Video Article

Germ Cell Transplantation and Testis Tissue Xenografting in Mice

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Abstract

Germ cell transplantation was developed by Dr. Ralph Brinster and colleagues at the University of Pennsylvania in 1994[1,2]. These ground-breaking studies showed that microinjection of germ cells from fertile donor mice into the seminiferous tubules of infertile recipient mice results in donor-derived spermatogenesis and sperm production by the recipient animal[2]. The use of donor males carrying the bacterial β-galactosidase gene allowed identification of donor-derived spermatogenesis and transmission of the donor haplotype to the offspring by recipient animals[1]. Surprisingly, after transplantation into the lumen of the seminiferous tubules, transplanted germ cells were able to move from the luminal compartment to the basement membrane where spermatogonia are located[3]. It is generally accepted that only SSCs are able to colonize the niche and re-establish spermatogenesis in the recipient testis. Therefore, germ cell transplantation provides a functional approach to study the stem cell niche in the testis and to characterize putative spermatogonial stem cells. To date, germ cell transplantation is used to elucidate basic stem cell biology, to produce transgenic animals through genetic manipulation of germ cells prior to transplantation[4,5], to study Sertoli cell-germ cell interaction[6,7], SSC homing and colonization[3,8], as well as SSC self-renewal and differentiation[9,10].

Germ cell transplantation is also feasible in large species[11]. In these, the main applications are preservation of fertility, dissemination of elite genetics in animal populations, and generation of transgenic animals as the study of spermatogenesis and SSC biology with this technique is logistically more difficult and expensive than in rodents. Transplantation of germ cells from large species into the seminiferous tubules of mice results in colonization of donor cells and spermatogonial expansion, but not in their full differentiation presumably due to incompatibility of the recipient somatic cell compartment with the germ cells from phylogenetically distant species[12]. An alternative approach is transplantation of germ cells from large species together with their surrounding somatic compartment. We first reported in 2002, that small fragments of testis tissue from immature males transplanted under the dorsal skin of immunodeficient mice are able to survive and undergo full development with the production of fertilization competent sperm[13]. Since then testis tissue xenografting has been shown to be successful in many species and emerged as a valuable alternative to study testis development and spermatogenesis of large animals in mice[14].

Video Link

The video component of this article can be found at http://www.jove.com/video/3545/

Protocol

PART A. Germ cell transplantation in mice

1. Preparation of recipient mice

1. Recipients should be immunologically tolerant (either genetically matched to donors or immune-deficient) to the donor testis cells.
2. Recipients should be either naturally devoid of spermatogenesis (e.g. *W/ W^v* mice) or depleted of endogenous germ cells. Germ cell depletion can be achieved by irradiation or chemotherapeutic drugs such as Busulfan. In this protocol, we describe the method of preparing recipient mice with Busulfan.
3. Treat recipients at 4-6 week of age through i.p injection of Busulfan. The optimal dose of Busulfan is strain dependent. In commonly used recipient strains, a dose of 40-50 mg/kg is sufficient to deplete endogenous germ cells (e.g. 44 mg/kg for nude mice, 50 mg/kg for B6/129 F1 recipients).
4. Dissolve Busulfan powder in DMSO and then add an equal volume of sterile distilled water to make a final concentration of 4 mg/ml. Keep solution warm at 35-40°C before use to avoid precipitation of Busulfan. Discard solution if precipitation is observed.
5. After Busulfan treatment, allow at least one month before using recipients. Recipients can be used between 1 month and 3 months post-Busulfan treatment.

2. Preparation of microinjection pipettes

1. Choose borosilicate glass pipettes (capillary tubes) with a 1.0 mm outer diameter, a 0.75 mm inner diameter, and a length of 3 inches.
2. Siliconize glass pipettes with Sigmacote, rinse pipettes with methanol and blow dry.
3. Pull the pipettes using a pipette puller. Different pipette puller machines have different settings, therefore, test a few settings. Generally, a setting similar to that used for making enucleation pipettes may work.
4. Break the pipette tips under dissecting microscope prior to use to achieve a diameter of approximately 50 µm at the tip.

3. Preparation of donor cells for transplantation

1. Choise of donor strain is dependent on the experimental question studied. If quantification of spermatogenesis is used as endpoint, use of donors with an easily identifiable genetic marker such as Lac-Z (e.g. B6.129S7-Gt(ROSA)26Sor/J from Jackson Laboratory) or GFP (e.g C57BL/6-Tg(CAG-EGFP)1Osb/J from Jackson Laboratory).
2. Prepare 7 ml of collagenase in DMEM at 1 mg/ml, 4 ml of trypsin-EDTA (0.25% trypsin plus 1mM EDTA), and 2 ml of DNase in DMEM at 7 mg/ml. This amount is for digesting 2 donor mouse testes.
3. Collect testes aseptically and remove the tunica albuginea. Spread seminiferous tubules gently with fine forceps to facilitate enzymatic digestion.
4. Transfer tubules into collagenase, incubate at 37 °C for 5-10 min, and agitate frequently.
5. Wash tubules twice by spinning (200-300 g for 3 min) and re-suspending in PBS w/o Ca^{2++}.
6. Re-suspend tubules in trypsin-EDTA and shake until they become sticky and cloudy. Monitor the digestion of tubules at 37 °C as it should occur within 1-2 min.
7. Add DNase and shake well. Incubate for 1-2 min.
8. Add 3 ml of FBS to stop the action of enzymes.
9. Filter cell suspension using a nylon mesh with 40-70 µm pore size to remove cell/tissue chunks.
10. Collect cells at 600 g for 5 min and re-suspend cells in a small amount of DMEM (<100 µl).
11. Count cells and adjust the volume to a desired final concentration (usually 100 x 10^6). Keep cell suspension on ice prior to use.
12. Each Busulfan-treated mouse testis will be filled with only 10-15 µl of cell suspension. Due to potential waste and leakage, aim to have 30-50 µl per testis.

4. Transplantation procedure (Figure 1)

1. Anaesthetize recipients following approved protocols.
2. Place in dorsal recumbency and surgically prepare the abdominal area. Make a ~1cm midline abdominal incision to expose the abdominal wall. Lift abdominal wall by using small forceps at the point of the white line to avoid accidentally injuring abdominal organs, and proceed to make a ~0.5 cm incision at the midline of the abdominal wall to expose the peritoneal cavity.
3. Use one iris forceps to hold the abdominal wall, and use another pair of iris forceps to search for the fat pads attached to the epididymis and testis in the peritoneal cavity. Gently pull the fat pad out until the testis is exteriorized and the testicular artery and epididymis are clearly visible. Work on one testis at a time.
4. Place a thin sterile drape made from autoclaved index cards underneath the fat pad/testis for better visual identification (optional). The drape also works to absorb fluid.
5. Add a drop of Trypan Blue dye into the cell suspension and carefully load the cell suspension into the polyethylene tubing connected to a 1 ml syringe. Attach the pulled pipette into the tubing and gently force the cell suspension into the pipette by applying pressure to the syringe.
6. Identify the efferent ducts (that connect the testis to the epididymis) and gently remove fat tissue around the ducts. Work carefully as the ducts and the membrane around them are translucent.
7. Carefully insert the pipette into a duct in the bundle of efferent ducts, gently thread a few mm toward the testis.
8. While avoiding moving the injection pipette, reach for the syringe, gently depress the plunger of the syringe to ensure that the suspension flows into the rete testis and seminiferous tubules begin to fill.
9. The injection rate and flow of cell suspension is regulated by thumb pressure. Avoid sudden increase in pressure; monitor the movement of suspension in tubules.
10. Stop the injection when almost all surface tubules have been filled before the testis starts to become ischemic.
11. Return the testis to the abdominal cavity. Repeat procedure on the contralateral testis. Suture the abdominal wall with 6-0 silk suture and close the skin with metal wound clips. Monitor mice on a warming pad until full recovery.

5. Analysis of the recipient testes

1. Allow 2-4 months after transplantation before analysis.
2. Euthanize the recipient mouse according to animal care and use guidelines and collect the testis and epididymis into PBS. When a Lac-Z transgenic donor strain has been used, the epididymis can be used as a positive staining control as it has endogenous beta-galactosidase activity.
3. For detection of donor cells by Lac-Z staining, fix the testis for 1-2 hour at 4 °C in 4% paraformaldehyde (PFA) in PBS. Rinse 3 x 30 min at room temperature in lacZ rinse buffer.
4. Incubate overnight at 37 °C in lacZ staining solution. Testis can be stained as a whole or after dispersing the tubules.
5. Fix and store in 10% neutral buffered formalin. If sections are needed use neutral fast red as counter-stain.

PART B. Ectopic xenografting in mice

(From Dobrinski and Rathi 2008[15], and Rodriguez-Sosa *et al*. 2011[16]).

1. Collection of donor tissue

1. Obtain testis tissue by castration or biopsy from a donor male.
2. Place testis in PBS or biopsies into culture medium, maintaining sterile conditions.
3. Keep the collected tissue on ice and transport to the laboratory.

2. Preparation of donor tissue

1. Perform in the tissue culture hood to maintain sterility.
2. Wash each testis in ice-cold PBS containing antibiotics two to three times before transferring into a culture-dish with PBS. In the case of biopsies, wash testis fragments two to three times with ice-cold culture medium containing antibiotics by spinning at 150g for 2 min and resuspending in fresh ice-cold culture medium.
3. For intact testes, remove tunica vaginalis by making an incision along the surface and extrude the testis. Remove from testis all annex structures (spermatic cord, epididymis, connective tissue). Wash testes once in cold PBS and transfer into a culture-dish with PBS.
4. Carefully remove the tunica albuginea of the testis by using a scalpel blade and a pair of scissors. If the testis is very small, the tunica can be removed by squeezing the testicular tissue out of the tunica through a small incision made on one end while holding the tunica with a pair of small forceps on the other end.
5. Depending on the size of the testis either the whole testis tissue can be cut into small pieces of around 1 - 2 mm^3 in size using curved forceps and a scalpel blade, or large pieces of testis tissue can first be removed from the testis and then cut into smaller pieces. All this should be done in ice-cold culture medium and under sterile conditions in a small culture dish (60 x15 mm).
6. Transfer the prepared tissue fragments to ice-cold culture medium in small culture-dishes on ice until grafting.

3. Castration of recipient mouse

1. Anaesthetize mouse as above and prepare sterile surgical field by clipping the hair (not necessary in nude mice), wiping with 70% Ethanol and Betadine solution.
2. Make a 0.5 -1 cm ventral midline skin incision to expose the abdominal wall.
3. Carefully expose the testis, the testicular artery and epididymis as described in PartA, 4.2-4.3.
4. Detach the tail of the epidydimis from the gubernaculum by blunt dissection.
5. Ligate the testicular artery, and the vas deferens together with the blood vessel with silk, and section the ligated structures by cutting between the testis and the ligature.
6. Repeat the procedure for the second testis.
7. Suture the abdominal wall with one or two surgical stitches.
8. Close the skin incision with one or two Michel clips.

4. Ectopic xenografting

1. Position the mouse in ventral recumbency and prepare a sterile surgical field on its back as above.
2. Depending on how many grafts are to be inserted (generally 4-8/mouse), make ~0.5 cm long skin incisions on each side of the mid line of the back of the mouse.
3. Use forceps to hold a border of the skin incision, and make a subcutaneous cavity by teasing apart the connective tissue using scissors.
4. Using an iris forceps place a piece of testis tissue deep into the subcutaneous cavity, holding the border of the skin incision with another iris forceps.
5. Close the skin incision with one Michel clip and keep mouse on heating pad until it starts to recover from anaesthesia.
6. Transfer the mouse to a cage with additional insulation and cover and monitor until mice are fully recovered.

5. Collection of testis xenografts for analysis and sperm harvesting

1. Euthanize the host mouse according to animal care and use guidelines, and make a midline skin incision on the back skin running from the tail to the neck and open skin. This exposes the grafts which can be located either on the subcutaneous tissue or attached to the skin.
2. Carefully remove the grafts using a pair forceps and a pair of scissors.
3. Record number of grafts recovered, size and weight of individual grafts.
4. Retrieve the seminal vesicles from the abdomen of the mouse and record their weight as an indication of testosterone production by the grafted tissue.
5. For histological analysis
    a. Suspend xenografts into sample vial containing Bouin's solution (or other fixative) in a volume ~10x that of the xenograft, and label vial appropriately.
    b. Incubate overnight in the refrigerator followed by washing at least 3 times in 70% ethanol at intervals of 24 hours preferably.
    c. Proceed for processing and embedding in paraffin.
6. For sperm harvesting
    a. Wash xenografts by spinning them down at 300g for 1 min and resuspending them in culture medium containing antibiotics.

b. Cut grafts into small pieces and mince carefully with the forceps in a tissue culture dish containing 3 - 5 ml of culture medium.
c. Filter minced tissue through the 40 µm cell strainer.

PART C. Representative Results

1. Germ cell transplantation

If the transplanted donor cell suspension contains spermatogonial stem cells carrying a genetic marker such as the LacZ transgene, colonization of donor SSCs in the recipient testis can be visualized by X-gal staining as distinctive blue segments of the seminiferous tubules after 2-3 month post-transplantation[3] (Figure 2A). A well- established colony should have a long dark blue stretch of completely filled segments with two or more layers of blue cells closer to the center, and relatively weaker stained regions at both ends where a network of single, paired or small groups of blue cells is apparent[3] (Figure 2B). A cross section of the dark blue seminiferous tubule region should reveal well-established and well-organized spermatogenesis with blue germ cells at various differentiation stages (Figure 2C).

2. Testis tissue xenografting

The viability of testis tissue after transplantation is inversely correlated with the developmental stage of the donor; the best outcome is obtained when tissue from newborn males is used, while adult tissue shows a high tendency to degenerate and die[17-21]. Generally, xenograft success decreases as donor tissue undergoes meiosis of the first wave of spermatogenesis. Time to full development of immature donor tissue and complete spermatogenesis is species specific, and often somewhat shorter in comparison with testes in situ. The number of seminiferous tubules with complete spermatogenesis is variable according to the species. While in sheep, goats and pigs that number is greater than 50%, in cattle and cats it is less than 15% [14](Figure 3).

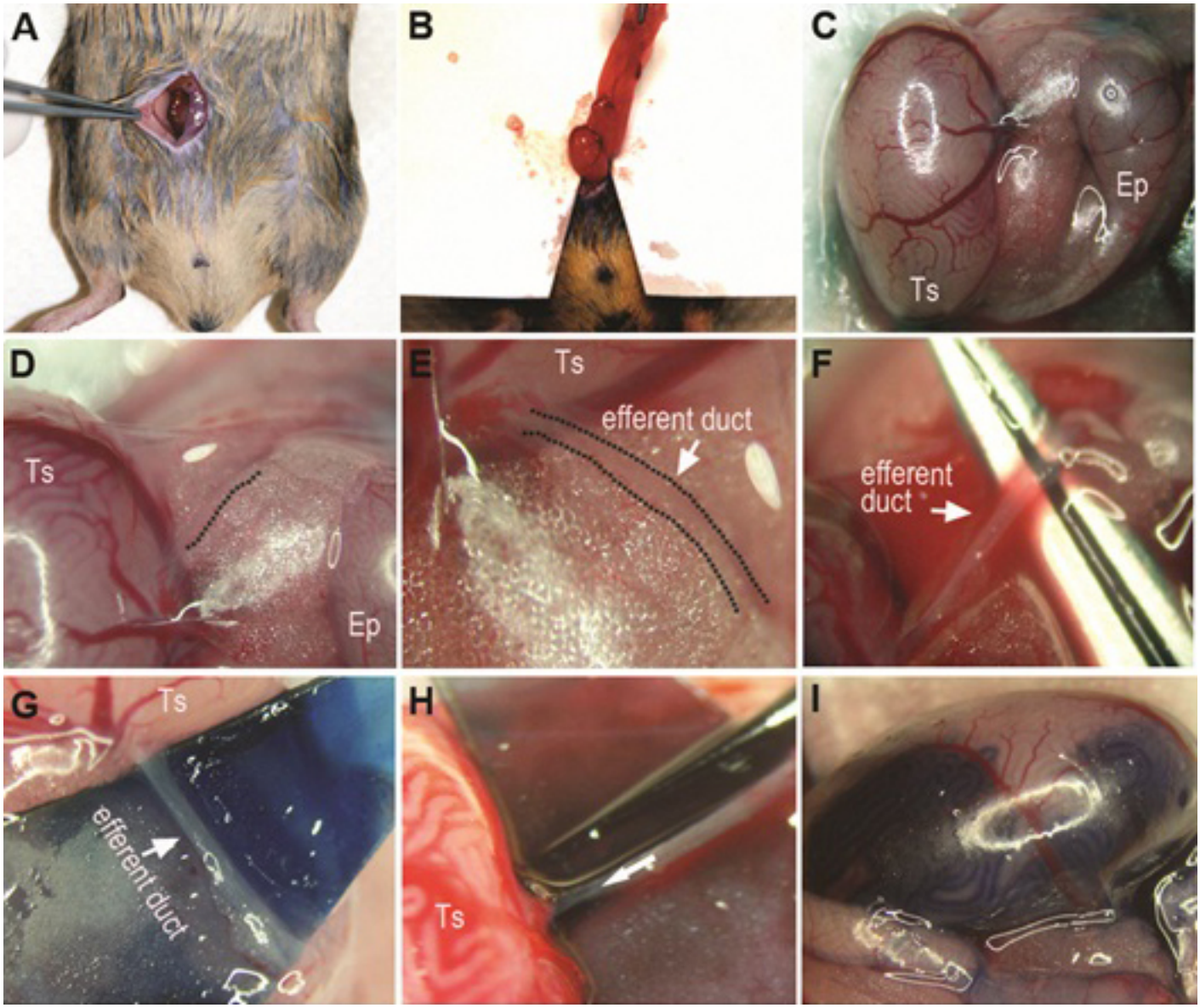

Figure 1. Germ cell transplantation procedure. Place recipients in dorsal recumbency after anaesthesia and make a ~0.4-inch midline abdominal incision (A). Expose testis by withdrawing the fat pad attached to the epididymis and testis and place a thin sterile drape underneath the fat pad/testis for better visual identification (B). Position the testis and epididymis so that the efferent ducts buried in the fat pad are discernible (C, D, E). Identity the efferent ducts and gently remove fat tissue around the ducts. A piece of colored paper or plastic can be placed underneath the ducts for better visualization (G). Break or grind the pipette tip according to the size of the ducts and load cell suspension into the pipette (H). Carefully insert the pipette into a duct in the bundle of efferent ducts, gently thread a few mm toward the testis (the arrow in H shows the direction of pipette injection and threading). A testis with successful injection into the seminiferous tubules is shown (I). Ts: Testis; Ep: epididymis.

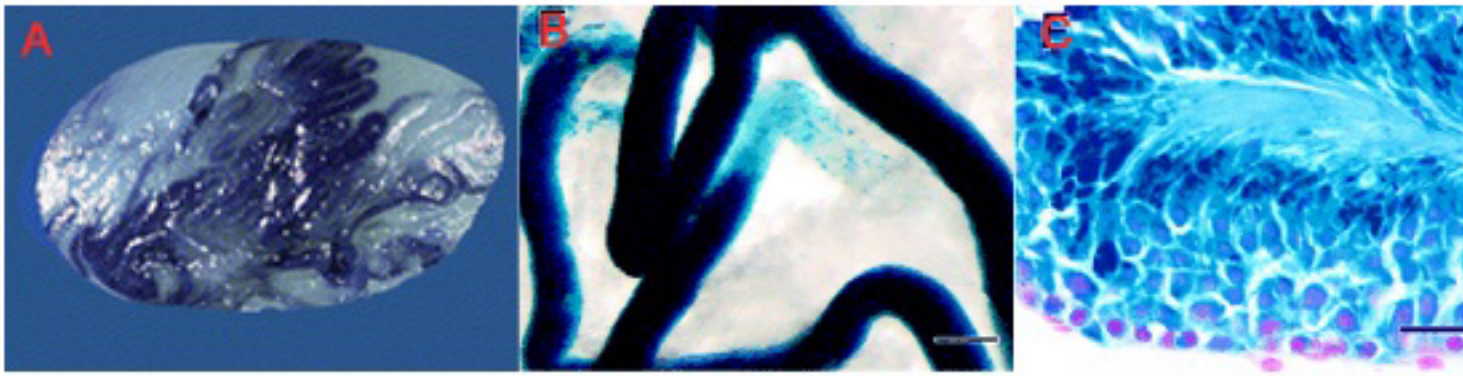

Figure 2. Representative results for germ cell transplantation A) An injected testis stained with X-gal. The blue segments of the seminiferous tubules represent established colonies from transgenic donor SSCs (LacZ transgene). B) Higher magnification of a SSC colony at 3 month post-transplantation. The dark blue region in the center represents complete spermatogenesis and the pale blue regions at the ends represent growing extension of the colony. C) A histological section of the dark blue seminiferous tubule (3 month post-transplantation) shows well organized spermatogenesis. Scale bars in B and C are 200 μm and 30 μm, respectively. (Figures adapted from Annu Rev Cell Dev Biol. 2008;24:263-86 and Biol Reprod. 1999 Jun;60(6):1429-36).

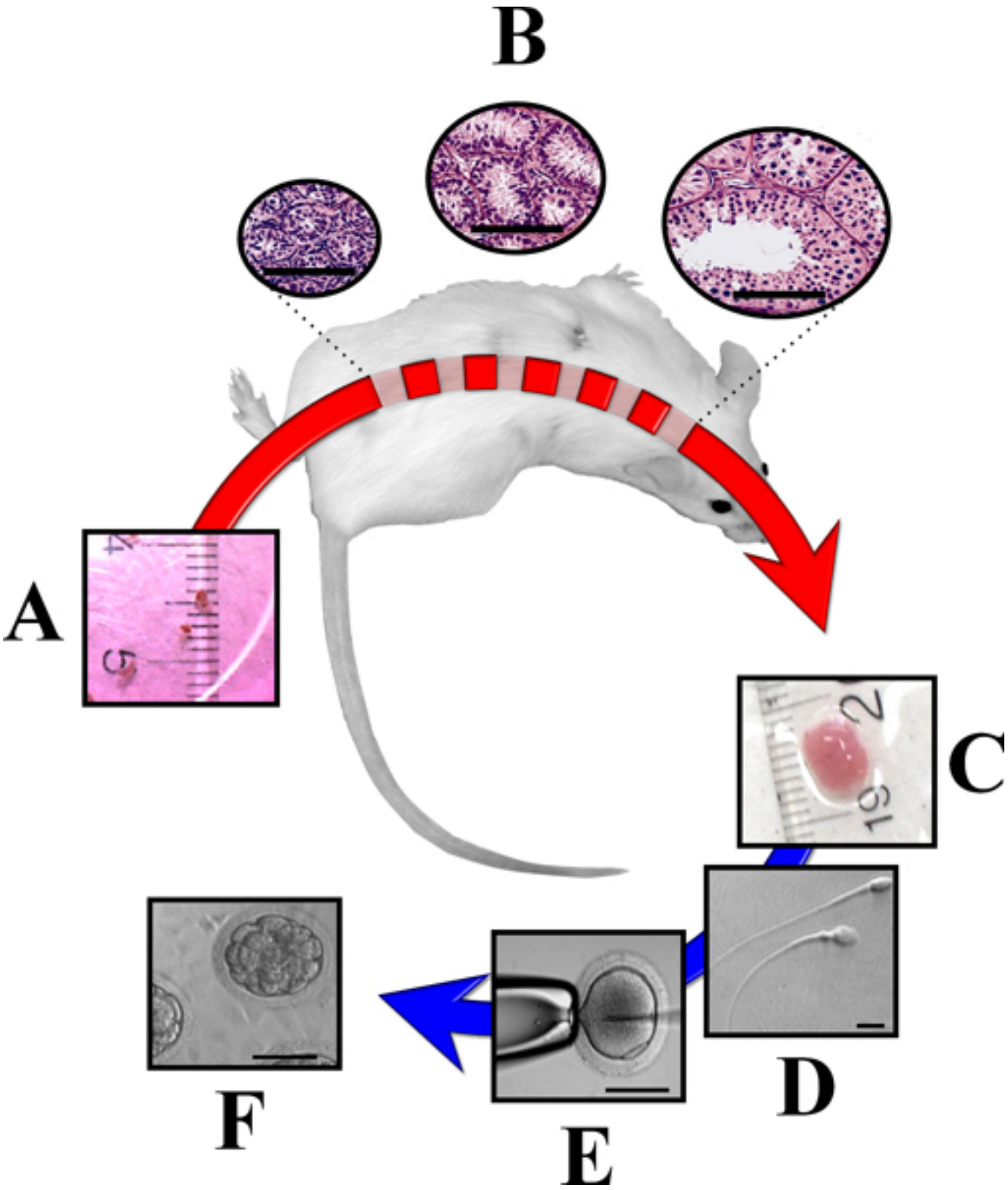

Figure 3. Ectopic xenografting of immature testis tissue from large animals into immunodeficient mice. Fragments of immature donor testis (~1 mm^3) transplanted under the dorsal skin of immunodeficient mice (A) are able to survive and respond to mouse gonadotropins. As a result, testis tissue undergoes complete development, including formation of fertilization competent sperm (B). Once testis xenografts are collected (C) they can be used for analysis or to obtain sperm (D) for ICSI (E) and embryo production (F). Bars equal 50 µm (B, E and F) or 10 µm (D). (Modified from Rodriguez-Sosa and Dobrinksi 2009[14]).

Discussion

1. Germ cell transplantation

Germ cell transplantation provides the only functional assay for unequivocal confirmation of the presence of spermatogonial stem cells (SSCs) in a cell population. Only SSCs can home to and colonize the SSC niche at the basement membrane and initiate donor-derived spermatogenesis. Germ cell transplantation made it possible to study and manipulate SSCs in an unprecedented manner. The technique has been used to produce transgenic animals through genetic manipulation of SSCs[4]; to elucidate the pattern, efficiency and kinetics of SSC colonization[3,8]; to study the signalling pathways that regulate SSC self-renewal and differentiation[9,10]; to characterize surface markers on SSCs for their identification[22,23]; to study the niche environment for SSCs[6,7,24]. Furthermore, reciprocal transplantation has been employed to investigate whether a phenotype of infertility originated from a defect in Sertoli cells or in germ cells[25,26].

For transplantation to work efficiently, choice and treatment of recipient animals is important. Recipients should be either genetically matched to donors or immune-suppressed. Recipients should also lack endogenous spermatogenesis: either due to a mutation as in *W/ W^{v}* mice, or rendered infertile as a result of germ cell depletion by irradiation or chemotherapeutic drugs such as Busulfan. Moreover, a good preparation of donor cell suspensions and proficiency in transplantation procedure are important for the success of the technique as well.

Germ cell transplantation has its own limitations. There is no fast read-out for results. The analysis of recipient testes needs to wait at least two month as reestablishment of complete spermatogenesis in the otherwise infertile recipients happens two month after transplantation[3]. It is a qualitative or semi-quantitative assay due to large variations in cell number injected and degree of recipient germ cell suppression. Although the concept of germ cell transplantation has been adapted to other animal species, the procedure itself is technically different and somewhat more challenging in non-rodent species as a result of the anatomic differences across species[11].

2. Testis tissue xenografting

Testis tissue xenografting works across many mammalian donor species and is a relatively simple technique. As in other types of transplantations, the sooner after collection the tissue is transplanted the bigger chance of success. Therefore, preservation and handling of the tissue from collection to transplantation is important. However, in our experience testis tissue does not require special handling other than being kept refrigerated. Testis tissue can be maintained at refrigerator temperature up to 24 - 36 hr, and then fragments can be prepared for transplantation. Furthermore, fragments of fresh testis can be maintained in standard culture medium at 4 °C overnight prior transplantation without a noticeable effect on the grafting outcome[27]. Testis tissue can also be cryopreserved if long-term storage is desired. Studies performed in goat[13], pig[13,27], and monkey[28] have shown that freezing and subsequent thawing of testis tissue does not affect significantly its capability to develop and produce sperm after ectopic xenografting in mice. Successful cryopreservation of testis tissue can be achieved by automated-freezing[28] or conventional slow-freezing in an alcohol bath[13,27], using DMSO as cryoprotectant in standard tissue culture medium containing FBS. For transplantation, cryopreserved testis tissue is then thawed by standard methods and subsequently washed in culture medium before transplantation[27]. Once the tissue has been transplanted the recipient mouse serves as an in vivo incubator and no major interventions are required. However, in some cases supplementation with exogenous gonadotropins may be required; testis tissue from 6-month-old rhesus monkeys required injecting recipient mice subcutaneously with 10 IU of hCG twice a week to attain full spermatogenesis at 6-7 months[29].

As mentioned above, the best outcome is obtained when tissue from newborn males is used. Tissue from males in which postmeiotic germ cells are present shows a tendency to degenerate. However, with immature animals complete recapitulation of testis development is possible and has numerous clinical and research applications. In a clinical setting, testis tissue xenografting can be used for fertility preservation, particularly in immature males in which sperm recovery is not an option. Small pieces in the form of biopsies can be collected and frozen for long-term storage. When desired, the fragments can be thawed and grafted into mice[27,28]. Another alternative is the cryopreservation of the sperm that is harvested from xenografts. Microinjection with snap-frozen sperm from pig testis xenografts resulted in generation of morphologically normal embryos, although at a lower efficiency in comparison to testicular, epididymal, or ejaculated sperm[27]. After tissue has developed, it can be collected for harvesting sperm and sperm can be used to produce embryos *in vitro*[13,16,30]. A limitation of this, however, is the fact that resulting sperm do not undergo epididymal maturation and therefore require ICSI for fertilization. Therefore, use of xenograft-derived sperm for fertilization is limited to species where ICSI has been established.

In research, testis tissue xenografting is an attractive alternative to study testis development and spermatogenesis of large species in a rodent model. For example, a single donor testis can be transplanted to multiple mice. Recipient mice can then be exposed to different treatments, and/or sacrificed at different time points for xenograft collection. This not only eliminates donor effects, but also reduces the number of large males required for a particular experiment or study. This is particularly important in large animals where studies involving numerous males are logistically difficult and expensive, and may carry ethical limitations, particularly in primates. However, applications of testis tissue xenografting are limited as manipulation of specific cell types before transplantation is not easily possible and efficiency of spermatogenesis is low in certain donor species[14].

Disclosures

No conflicts of interest declared.

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