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Allogeneic Transplantation of Testicular Hyperplasia in *rag1* Mutant Zebrafish

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[Abstract] Allogeneic organ transplantation is a powerful tool for clinical and basic research studies. However, the graft is often rejected by the host organism. Here, we describe a protocol that uses immunodeficient *rag1* mutant zebrafish. These zebrafish escaped rejection, which made it possible to successfully transplant fragments of an allogeneic testis and testicular hyperplasia. This protocol can be used to amplify and maintain testicular hyperplasia grafts for several years (Kawasaki *et al.*, 2016). The amplified hyperplasias are likely to be a good source of somatic and germ cells such as Sertoli cells and spermatogonial stem cells.

[Background] Zebrafish have emerged as a tractable teleost genetic model for the study of vertebrate biology because several thousand mutants have been isolated by various genetic methods (Granato and Nüsslein-Volhard, 1996). Recently, this organism was used to study human diseases such as cancer (White *et al.*, 2013). Although the incidence of spontaneous cancers is low, with many zebrafish eventually surviving cancer, allogeneic organ transplantation is a powerful tool, because many of the cancers are not syngeneic. Unfortunately, this method is not well developed. A previous study reported that zebrafish embryos accept cell grafts prior to the development of a mature immune system (Nicoli *et al.*, 2007). However, it is difficult to successfully transplant grafts into embryos due to their minute size. For transplantation into adult zebrafish, sublethal γ-irradiation or immunosuppression with dexamethasone can block the rejection of the graft (Stoletov *et al.*, 2007; White *et al.*, 2008). However, it can be difficult to maintain cell grafts for long periods of time due to the short lifespans of recipients and the recovery of the immune response by 20 days after irradiation (Smith *et al.*, 2010; Eguiara *et al.*, 2011). Tissue grafts between identical clonal or inbred lines can survive without rejection (Kawasaki *et al.*, 2010; Mizgirev and Revskoy, 2010; Shinya and Sakai, 2011).

T lymphocytes are central to the allograft response (Ingulli, 2010). The *Recombination activating gene 1, 2* (*Rag1, Rag2*) are important for immune function, because it creates double-stranded DNA breaks and is essential for V(D)J recombination, as well as for T and B cell function. *Rag1* mutant mice lack mature T and B cells, and they maintain allogeneic heart grafts for long periods of time (Zhang *et al.*, 2006). By contrast, allogeneic transplantation has failed in *Rag1* mutant rats, probably due to the insufficient depletion of T and B cells (Ménoret *et al.*, 2013). Hypomorphic *rag2*^{E450fs} mutant zebrafish has been created, which have reduced V(D)J rearrangement and lymphocytes, and maintains various allogeneic cancer cells (Tang *et al.*, 2014). Although *rag1*^{t26683} mutant zebrafish (hereafter *rag1* mutant) have been isolated and they lack functional T and B cells (Wienholds *et al.*, 2002; Petrie-Hanson *et al.*,

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2009), they were not used for transplantation. Our recent study reported that *rag1* mutant zebrafish accept and maintain allogeneic testis organ and testicular hyperplasia grafts for long periods of time (Kawasaki *et al.*, 2016). Here, we describe a protocol that uses immunodeficient *rag1* mutant zebrafish for the subcutaneous transplantation of testis and testicular hyperplasia grafts.

Materials and Reagents

1. 100 mm dish (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 263991)
2. Paper towels
3. Surgical blades (FEATHER Safety Razor, catalog number: No. 25)
4. Aluminum foil
5. *rag1* mutant adult male zebrafish (Wienholds *et al.*, 2002)
6. L-15 medium (Sigma-Aldrich, catalog number: L5520-500ML)
7. 25x phosphate-buffered saline (PBS)
8. Ethyl *p*-aminobenzoate (Wako Pure Chemical Industries, catalog number: 057-03832)
9. Gentamicin (10 mg/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15710064)
10. Ethanol
11. 10% ethyl *p*-aminobenzoate stock solution (see Recipes)
12. 0.01% ethyl *p*-aminobenzoate working solution (see Recipes)
13. 0.4x PBS containing 10 µg/ml gentamicin (see Recipes)

Equipment

1. Stereoscopic microscope (OLYMPUS, model: SZ61)
2. Forceps (Style 5) (Dumont, catalog number: 0108-5-PO)

Procedure

A. Subcutaneous transplantation
   1. Maintain all adult male recipients of *rag1* mutant zebrafish without food 1 day prior to transplantation.
   2. Remove normal testes or spontaneous testicular hyperplasia from a donor fish as described in (Sakai, 2006), and cut to approximately 2-3 mm square containing the testis epithelium in L-15 medium.
   3. Place a paper towel in a 100 mm dish and pour a small amount of 0.4x PBS into the dish.
   4. Anesthetize the recipient zebrafish with 0.01% ethyl *p*-aminobenzoate.
   5. Place the anesthetized recipient fish into the 100 mm dish.
   6. Cover the head and tail of the recipient fish with wet paper towels (Figure 1A, Video 1).
   7. Descale the site of transplantation.

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8. Make an abdominal incision of approximately 5 mm in length with a razor blade.
9. Insert the tips of a pair of forceps between the muscle and skin to prepare for the transplantation (Figure 1B, Video 1).
10. Insert a fragment of the testis or testicular hyperplasia containing a small portion of adjacent tissue (Figure 1C, Video 1).
11. Transfer the recipient zebrafish to a tank containing 0.4x PBS supplemented with 10 μg/ml gentamicin.
12. Wrap the tank with aluminum foil to prevent the entry of light and incubate the tank at 26 °C for 4 days to promote wound healing.
13. Rear the transplanted fish 4 days after surgery.

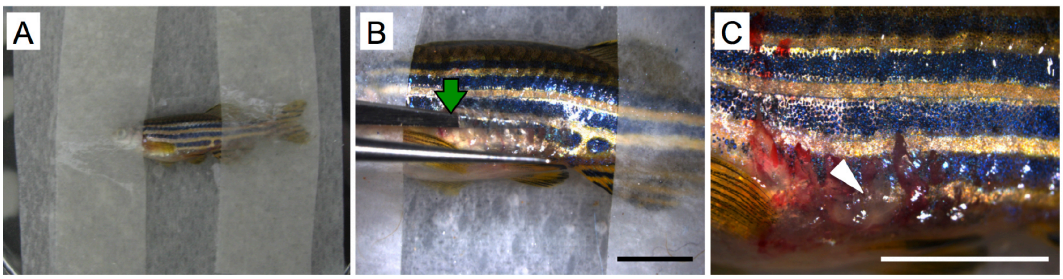

Figure 1. View of subcutaneous transplantation. A. Preparation of the recipient fish. The recipient fish is protected from drying by covering the head and tail with wet paper towels. B. Separation of the skin and muscle. The inserted forceps (arrow) separate the abdominal muscle from the skin of the recipient fish. C. View of the transplanted testis fragment. The testis fragment (arrowhead) is inserted into the treated area shown in (B). Scale bar = 5 mm.

Video 1. Procedures of subcutaneous transplantation

B. Serial transplantation of testicular hyperplasia

1. After growth of the testicular hyperplasia graft, anesthetize the recipient with 0.01% ethyl *p*-aminobenzoate.
2. Remove the testicular hyperplasia and transfer to a 60 mm dish containing L-15 medium.

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3. Cut the graft into approximately ten fragments of equal size.
4. To confirm the condition of the testicular hyperplasia graft, set aside several fragments for histology.
5. Re-transplant the remaining fragments, which contain epidermis of the hyperplasia, as outlined in Procedure A: Subcutaneous transplantation.

Data analysis

We have performed a total of 156 cases of serial transplantation using 4 testicular hyperplasias, and growth of the transplanted grafts was observed in 149 cases, including data previously reported (Data is presented in Kawasaki *et al.*, 2016). We have succeeded in maintaining a testicular hyperplasia more than 3 years at the longest (Data is presented in Kawasaki *et al.*, 2016). Figure 2 shows the tree diagram of an additional testicular hyperplasia by serial transplantation for more than one year. However, malignant transformation and testis-ova arose very occasionally in the serial transplantation of testicular hyperplasias (Data is presented in Kawasaki *et al.*, 2016). Therefore, histological observation of the grafted testicular hyperplasias should be performed in each generation to avoid undesired transformation.

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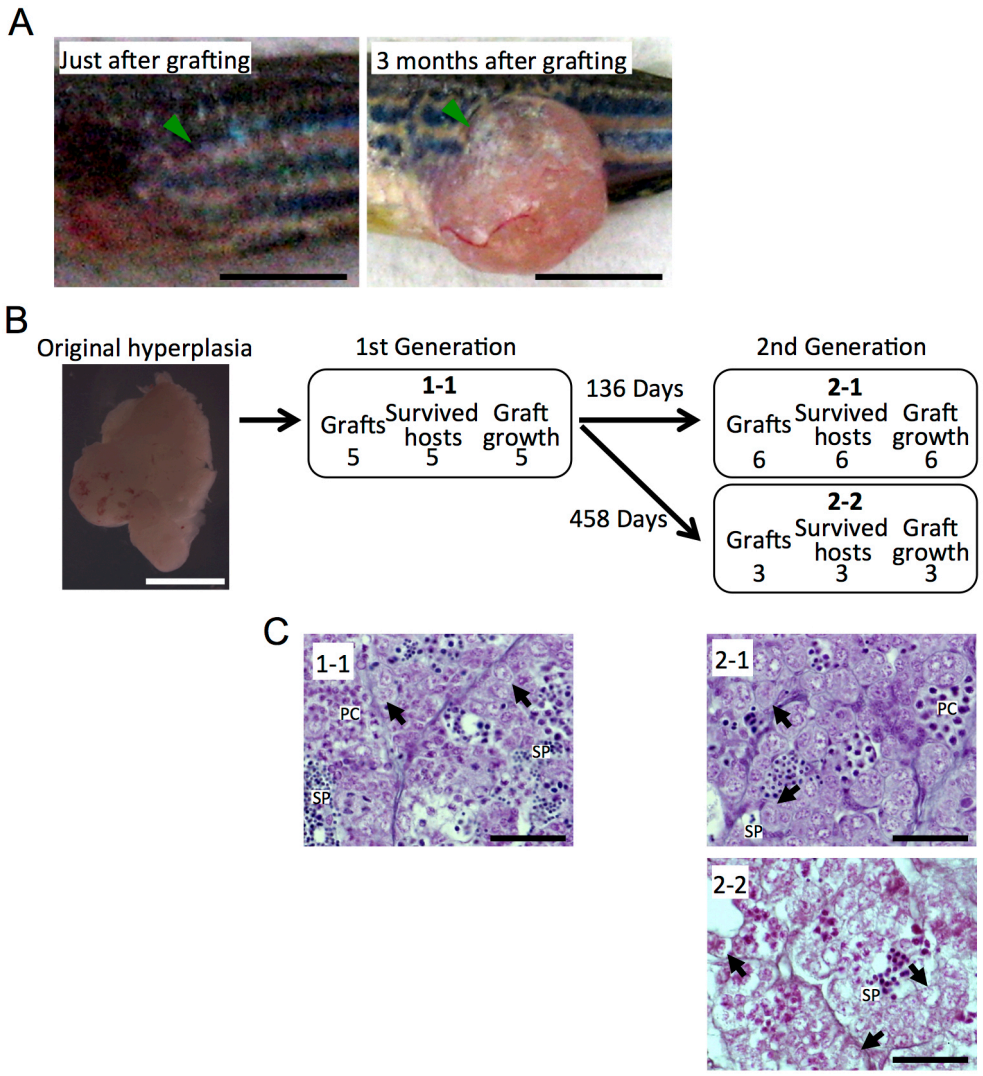

Figure 2. Maintenance of testicular hyperplasias by serial transplantation. A. Growth of the grafted fragment of a testicular hyperplasia. Arrowheads indicate the transplanted grafts. Scale bar = 5 mm. B. The tree diagram of serial transplantation of a testicular hyperplasia. Each box shows the number of the grafted fragments, the number of recipients survived for more than 1 month, and the number of the grown graft in each transplantation steps. Note that the fragment of the original testicular hyperplasia (the left photograph) grafted in the case of 1-1 was maintained for 458 days after the 1st transplantation, and that all grafts regrown in the 2nd transplantation. Scale bar = 5 mm. C. Histological observation of the grafted hyperplasia. Sections of each testicular hyperplasia correspond to the grafts shown in (B). Although the proportion of each stage of spermatogenic cells seemed to be changed, spermatogenesis was maintained through the serial transplantation. Arrows, spermatogonia; PC, spermatocytes; SP, sperm. Scale bar = 20 µm.

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Recipes

1. 10% ethyl *p*-aminobenzoate stock solution
   Dissolve 5 g of ethyl *p*-aminobenzoate in 50 ml of ethanol
2. 0.01% ethyl *p*-aminobenzoate working solution
   Dissolve 50 µl of 10% ethyl *p*-aminobenzoate stock solution in 50 ml of rearing water
3. 0.4x PBS containing 10 µg/ml gentamicin
   Combine 16 ml of 25x PBS and 1 ml of gentamicin stock solution (10 mg/ml) in 983 ml of autoclaved rearing water

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