Allogeneic testes transplanted into castrated adult medaka (*Oryzias latipes*) are engrafted and can function over a long period for production of donor-derived offspring by natural mating

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Abstract

Germ cell transplantation protocols generally require induced immunosuppression in the recipient to avoid rejection of the transplanted cells; cytotoxic drugs may also be used to deprive the recipient germ cells and create a germ cell niche that allows donor-derived germ cells to thrive. In the present study, we demonstrate that in medaka, allogeneic adult testicular tissue will engraft in adult recipients immediately after the rough castration of the recipients and without use of immunosuppressive drugs. The allografted testes are retained in the recipient body for at least three months and are able to produce viable gametes that yield offspring after natural mating. Some recipients showed a high frequency (over 60%) of offspring derived from spermatozoa produced by the transplanted germ cell tissues. Histological analyses showed that allografted testicular tissues included both germ cells and somatic cells that had established within an immunocompetent recipient testis. The relative simplicity of this new approach will benefit investigations of the basic processes of reproductive immunology and will improve the technique of gonadal tissue transplantation.

Keywords

germ cell transplantation, reproductive immunology, surrogate broodstock, teleost, medaka

31 **Background**

32 Germline transplantation has been used in investigations of many aspects of reproductive biology
33 and reproductive immunology, and has also been successfully applied for selective breeding in
34 livestock and aquaculture, species conservation, and fertility treatment (1-7). A variety of allogeneic
35 or xenogeneic transplantation protocols for gonadal tissues have been developed and used to create
36 potentially superior broodstocks, as insurance against the accidental death of vital broodstocks, and
37 for maintenance of threatened breeds and species. The use of spermatogonial stem cells (SSCs) is
38 considered particularly valuable as these cells are present in large numbers in the testes of adult males
39 and are relatively easy to obtain (8, 9).

40 One of the potential drawbacks of allogeneic transplantation of tissues is the possibility of
41 immunorejection of donor cells and tissues. In SSC transplantation studies in mice and rats, it has been
42 found that the donors and recipients need to be closely related to avoid this potential problem;
43 alternatively, immunodeficient animals can be used as recipients, or the recipients can be treated with
44 drugs to suppress their immune responses (10, 11). Similar results have been reported in teleosts, in
45 which allografted tissues are rejected within a few days or weeks by the immune system of the
46 recipient (12-14). In teleosts, the immunorejection problem can be avoided by transplanting germ cells

47 into newly hatched recipient larvae whose immune system are immature (15-18). However, this
48 approach is technically demanding and requires the use of microinjection equipment.

49 A few sites in the body display "immune privilege" in which an antigenic response is not elicited
50 by the presence of transplanted cells. The testes are known to have immune privilege and are more
51 likely to accept transplanted tissues (immune privilege site) and also to be the source of donor cells
52 (immune privilege tissue) (19). In some domesticated species, such as pigs, goats, and dogs,
53 transplanted germ cells can be engrafted into recipient testes without the need to suppress the immune
54 response (4, 20, 21). It has been reported that in fish, transplants of body tissue (scales) are rejected
55 within a few days to about two weeks, while subcutaneous transplants of testicular tissue are accepted
56 for six to nine weeks (12-14). While the mechanism of this immune privilege is not completely
57 understood, it has nevertheless been of value for germ cell transplantation using adult fish as recipients
58 (22). When transplanting germ cells into an adult fish, it is important to create a germline niche in the
59 recipient testis. To achieve this, the number of recipient germ cells is reduced by administering
60 cytotoxic drugs such as busulfan (23-25). One of the problems that still needs to be solved is that the
61 frequency of offspring derived from donor sperm in the offspring rarely exceeds 40% (26).

62 The present study was initiated to develop a reliable method for allogeneic testis transplantation in

63 fish. We chose the model fish species medaka (*Oryzias latipes*) for our analyses as it spawns daily, is
64 amenable to gene editing, and a surgical method for gonadectomy has also been established (27, 28).
65 Medaka are immunocompetent as demonstrated by scale transplantation experiments. However, as
66 described above, the testis is immune privileged and histological analyses of recipient testes after
67 transplantation showed that they contained donor germ cells and somatic cells. These results indicate
68 the feasibility of developing a reliable method for creating male surrogate parents to efficiently obtain
69 donor-derived offspring.

70

71 ## Materials and methods

72 **Animals**

73 All medaka used in the study were maintained under a 14 hour light/ 10 hour dark photoperiod (light
74 on 09:00 to 23:00), with a water temperature of 28°C. The fish were fed 3-4 times per day with live
75 brine shrimp and commercial pellet food. We used d-rR/TOKYO (d-rR) strain medaka, along with
76 transgenic strains, and captive-bred wild type medaka. Transgenic medaka expressing GFP in the
77 neuropeptide B neuron (npb-GFP) were used in a previous study (29). Transgenic medaka consistently
78 expressing GFP (beta-actin-loxP-GFP, actb-GFP) were obtained from the National Institute for Basic

79 Biology (strain ID: TG862) via The National BioResouce Project-Medaka (NBRP-medaka). The
80 ancestor of the captive-bred wild strain medaka was caught in an irrigation channel of a rice field (GPS
81 coordinates: 32°58'21.9"N 132°58'12.6"E (32.972750, 132.970167); Isawa, Shimanto City, Kochi
82 Prefecture). This wild strain has been bred and maintained for a number of generations in our
83 laboratory.

84 **Testis transplantation into recipient males**

85 A few medaka aged 3-5 months for each strain were used as donors; they were deeply anesthetized,
86 decapitated, and the testes were dissected. Isolated testes were kept in PBS until transplantation.
87 Twenty-two recipient medaka (aged 2-4 months) were anesthetized using 0.02% MS-222 and their
88 abdomens were incised using a razor blade. In male medaka, the testis is essentially a single organ
89 following fusion of bilateral testes during ontogeny (30). The rostral side of the recipient testis was
90 pinched using forceps, and most of the testicular tissue was removed using another set of forceps. The
91 isolated donor testis was cut into 1-2 mm pieces which were placed adjacent to the remaining part of
92 the recipient testis. After implantation, the abdominal incision was sutured with nylon thread. Post-
93 surgical recovery was carried out by placing the recipient medaka in the 0.8% saline for a few days;
94 the fish were transferred to a freshwater environment after recovery. The abdomens of the recipient

95 medaka and of their offspring were photographed using a stereo microscope (M165FC or M205FA,
96 Leica Microsystems, Wetzlar, Germany) equipped with a DFC7000T digital camera (Leica
97 Microsystems). GFP fluorescence was detected using excitation at 450-490 nm and images captured
98 at 500-550 nm.

99 **Scale transplantation experiments**

100 The immune responses of the fish strains used here were confirmed by scale transplantation
101 experiments; actb-GFP strain and captive-bred wild strain medaka (6-7 months old) were used as the
102 donor strains, and d-rR strain medaka (6-7 months old) were used as the recipients. As a control, we
103 transplanted scales between siblings of the d-rR strain (4- to 5-months old) that had been maintained
104 for a number of generations in our laboratory to confirm that body tissue transplants were not rejected
105 by the immune system of these fish.

106 Four recipient medaka were anesthetized using 0.02% MS-222. A few donor medaka were deeply
107 anesthetized and immediately decapitated; 20-23 scales were removed from the donor body and
108 transplanted into the caudal region around the lateral line of the four recipients (Day 0). The recipients
109 were kept in a tank throughout the experimental period. The number of engrafted scales on the
110 recipients was counted each day and the fish were photographed on Days 1, 7, and 10 under an

111 M205FA stereo microscope equipped with a DFC7000T digital camera. Fluorescent staining was
112 viewed after 450-490 nm excitation and 500-550 nm emission for GFP, and 540-580 nm and 593-667
113 nm, respectively for Alizarin red S (ARS).

114 **Vital staining of scales**

115 In the control analysis using d-rR siblings, we stained the scales of donor fish with Alizarin red S
116 (ARS) (Wako, Osaka, Japan), a vital stain for fishbone (31), to distinguish them from the scales of the
117 recipient. A few medaka were anesthetized using 0.02% MS-222 and dried with tissue paper. A
118 saturated solution of ARS (0.1% ARS in PBS) was dropped onto the fish body with a micropipette and
119 left for ten seconds up to a minute. Medaka with red scales were released into the tank and used as
120 donors on the following day. Scale transplantation was performed as described above. The stained
121 scales transplanted into recipients could generally be distinguished from the unstained scales of the
122 recipient by eye for up to 5 days; after 6 days, it was necessary to use fluorescence to identify donor
123 scales. The excitation and emission wavelengths for the detection of stained scales were as described
124 above.

125 **Immunohistochemistry (IHC)**

126 The testis of a recipient that had been transplanted with a testis from an actb-GFP strain fish was

127 excised (n = 1, recipient #2, 2 months after surgery) and fixed in 4% paraformaldehyde (PFA)/PBS
128 for approximately one week; the fixed testis was dehydrated through an ethanol series, cleared with
129 xylene, and embedded in paraffin. Sections (10 μm thick) were cut and treated with 0.3% H_2O_2 for 30
130 min, 2% normal goat serum (NGS) for 30 min, and incubated with anti-GFP rabbit polyclonal antibody
131 (#598, Medical and Biological Laboratories, Tokyo, Japan) diluted at 1:500-1:2000 in PBS containing
132 2% NGS overnight at 4°C. After two washes in PBS, the sections were incubated with biotinylated
133 goat anti-rabbit IgG (diluted according to the manufacturer's protocol) for 1 hour, and stained using
134 the VECTASTAIN Elite ABC reagent (VECTASTAIN(R) Elite ABC-HRP Kit, Peroxidase, PK-6101,
135 Vector Laboratories, Burlingame, CA) for 1 hour. The horseradish peroxidase-conjugated Avidin-
136 Biotin Complex was visualized using 3,3-diaminobenzidine (DAB) and 0.003% H_2O_2. After the DAB
137 reaction, some sections were counterstained with hematoxylin.

138

139 ## Results

140 **Adult donor testis transplanted into an adult recipient male is functionally engrafted without**
141 **immunosuppression**

142 Four of the ten d-rR males whose testis was replaced with an actb-GFP testis showed strong green

143 fluorescence in their abdomens at 2 months after surgery (Figure 1a-c). Thus, successful allografts
144 were present in four of the fish. To determine whether the engrafted testis was functional, we mated
145 the GFP-positive recipients with d-rR females and assessed the frequency of GFP-positive eggs 2-7
146 weeks after surgery (Figure 1 d, e, and Table 1). The frequency of GFP-positive eggs was
147 approximately 9, 18, and 66% for three fish; the fourth fish produced no GFP positive eggs (Table 1).
148 We also performed testis transplantation using donor npb-GFP medaka that were generated in our
149 laboratory and had the same genetic background as the recipient fish (Table 1, #5, #6). Two of four
150 recipients had high frequencies (95% and100%, respectively) of GFP-positive eggs (Table 1).

151 **Functional allografts produced by transplanting testis from wild-derived medaka into d-rR**
152 **recipients**

153 To determine whether testis transplantation can be applied to genetically distant strains, we
154 transplanted testes from wild-derived medaka into d-rR strain medaka males; this wild-derived strain
155 and the d-rR strain belong to different subclades (32). We also allografted testes from wild strain
156 donors to d-rR male recipients (Figure 2 a). Testicular tissues from wild-derived males were
157 transplanted into eight d-rR males; the recipients were subsequently mated with d-rR females (Figure
158 2 a,b). Interestingly, black pigmented eggs, which indicates fertilization by sperm from the wild-

159 derived donor testis, were produced in matings of two of the eight recipients (Figure 2c); all of the
160 fertilized eggs of one of these recipients (#7) were pigmented, the other produced 9% pigmented eggs
161 (Table 2).

162 **Transplanted scales are rejected by the immune system of the recipient**

163 The wild-derived medaka strain belongs to a different subclade to the d-rR strain due to geographical
164 isolation (32). We performed a scale transplantation experiment to confirm that d-rR recipients would
165 reject somatic tissues from other strains (Figure 3 a-f, and Table 3). Loss of transplanted scales may
166 be caused by immunorejection or mechanical injury; these two causes can be distinguished by the fact
167 that mechanical injury during the transplantation process results in the loss of the scales on the day
168 after transplantation (12). Our analysis of the recipient fish on successive days after scale
169 transplantation indicated that 10-15 scales derived from wild-derived and actb-GFP strain fish had
170 been engrafted in recipients. Almost all of the transplanted scales were rejected by days 7 to 9, and all
171 scales were lost within 12 days. To confirm that the scale transplantation was successful, we performed
172 vital staining of the scales with ARS in d-rR donors and transplanted these stained scales into d-rR
173 recipients (Figure 3 g, h). After the loss of some scales on Day 1 due to mechanical injury, most of the
174 allografted d-rR scales had been accepted at 12 days by the d-rR recipient (Table 3). Donor medaka of

175 actb-GFP strain were generated from the d-rR strain, and thus the genetic backgrounds of the d-rR
176 recipients and actb-GFP donors appear to be the same. However, it should be noted that the d-rR strain
177 is not an inbred strain. Based on the fact that transplanted actb-GFP scales were rejected by the
178 recipient immune system, we conclude that the genetic backgrounds are sufficiently distant to cause
179 immunorejection. Our results demonstrate that recipient d-rR strain medaka reject allografted tissues
180 from donor medaka (actb-GFP strain and wild-derived strain).

181 **Allografted testis is functionally retained in recipients for more than 3 months**

182 To determine the functional longevity of donor-derived testis in recipient medaka, we mated
183 recipients for up to 13 weeks after surgery (Table 4). One recipient (#2) was sacrificed for abdominal
184 analysis, and a second (#5) died accidentally; the other recipients were included in this analysis. As
185 described in Table 4, four individuals (#3, #6, #7, and #8) showed almost equal frequencies of donor-
186 derived eggs; two males did not produce any donor-derived offspring (Table 1); they have been re-
187 numbered #1' and #2' in Table 4. This analysis demonstrated that allografted testis remained functional
188 over an extended period, at least 13 weeks, except for one individual.

189 **Somatic cells derived from the donor testis also engraft into recipient testis**

190 We performed an IHC analysis to detect GFP-expressing cells derived from the recipient testis.

191 GFP-positive cells (donor-derived cells) were distinguished as DAB positive cells in histological
192 sections while GFP negative cells (recipient cells) were only stained with hematoxylin (Figure 4a, b).
193 For the classification of each developmental stage of spermatogenesis, we used the descriptions
194 provided in previous studies (33, 34). GFP signals were detected in the allografted testis of the
195 recipient male (Figure 4a, c, d). The recipient testis contained spermatogonia with GFP signals,
196 indicating that these spermatogonial cells proliferated and supplied the donor-derived germ cells
197 (Figure 4c). These observations also indicate the reason why some recipients produced donor-derived
198 offspring over a long period. Interestingly, testicular somatic cells, such as basement membrane (bm)
199 cells and lobule boundary cells (lbc), had allografted into the recipient testis (Figure 4d). These
200 observations suggest that the donor-derived testicular tissue probably included Sertoli cells and Leydig
201 cells that were not immunorejected but integrated into the recipient testis and supported functional
202 spermatogenesis.

203 Our testis transplantation analyses demonstrated that transplanted adult donor testis can functionally
204 engraft into an adult recipient body without any immunosuppression treatment for 13 weeks even
205 when their genetic backgrounds are different; this behavior contrasts sharply with that of transplanted
206 scales, which were rejected from the recipient body within 12 days.

207

208 **Discussion**

209 In the present study, we demonstrated that transplanted allogeneic testicular tissue could engraft in
210 the body of recipient adult medaka without use of an immunosuppressive treatment. Additionally, we
211 showed that allografted testicular tissue derived from medaka with a different genetic background was
212 functional and produced sperm that resulted in fertilized eggs after natural mating. An IHC analysis
213 also showed that both germ cells and testicular somatic cells were engrafted in allogeneic adult
214 recipients.

215 Methods for allogeneic or xenogeneic transplantation of SSCs, which are abundant in the testis,
216 have been developed in many species. In mammals, SSCs allografted to Sertoli cells of the recipient
217 migrated into the basement membrane through the blood-testis barrier, a physical barrier between the
218 blood vessels and the seminiferous tubules (35). Thus, transplanted SSCs can evade immunorejection
219 and become established in the recipient testis. In the present study, IHC was used to analyze the cellular
220 structure of the testicular allograft (Figure 4); GFP signals were observed not only in the germ line
221 cells but also in the testicular somatic cells such as the tunica serosa, tunica albuginea, and interstitial
222 cells that probably included Sertoli and Leydig cells. These observations suggest that the transplanted

223 testicular tissue became established and formed blood vessels within the recipient testis. As some
224 recipients fertilized eggs with donor-derived sperm by natural mating (Tables 1, 2, and 4), then the
225 sperm derived from the donor testicular tissue must have been released to the efferent duct, which
226 would have been re-established after the transplantation surgery. It is interesting that the allografted
227 testicular tissue, which included somatic cells, was accepted by the immunocompetent recipient whose
228 genetic background was distant to that of the donor (Figure 2; Tables 2 and 4). In domesticated
229 mammals, such as the pig and goat, it has been reported that allografted testicular cells including
230 Sertoli cells successfully engraft in a recipient testis without use of immunosuppressive treatment (4,
231 6). Molecular analyses suggest that the domestication of these species started 9,000-10,000 years ago
232 (36, 37). Examination of the geographic distribution of mitotypes of Japanese medaka (32) showed
233 that the wild-derived medaka strain used as a donor in the present study belongs to subclade B-V,
234 while the d-rR strain belongs to the subclade B-II; the divergence time among the B subclades is
235 estimated as 0.5-2.3 mya. Our transplantation experiments here demonstrate that allogeneic
236 transplantation of testicular tissue can succeed even in medaka with divergent genetic backgrounds.

237 Generally, allografted tissue is rejected by the immune system of the recipient. A previous study of
238 allogeneic scale transplantation in medaka conformed to this expectation as the allografted scales were

239 rejected within 7 days (12). We confirmed that the recipient strain used here was immunocompetent
240 by allografting scales from a wild-derived strain (black scales) and the actb-GFP strain into recipient
241 d-rR strain fish; scales derived from the genetically distant donor were rejected within 12 days (Figure
242 3 and Table 3). Although the genetic backgrounds of the recipients (d-rR) and actb-GFP (generated
243 from d-rR strain) might be expected to be similar, these strains are not inbred and have different genetic
244 backgrounds. These results of our experiments show that testicular tissue can engraft in allogeneic
245 individuals whereas somatic tissue, such as scales, are rejected by the immune system. This finding is
246 consistent with the general consensus that the testis has immune privilege (19). In a previous study on
247 rainbow trout, testis allografted into subcutaneous tissue was retained for 6-9 weeks but rejected after
248 9 weeks (13, 14). In the present study, testicular allografts inserted into the abdomen of the recipient
249 were retained for 13 weeks (Table 4). These results indicate that allografted testicular tissue is more
250 readily accepted by the recipient than somatic tissues. Teleost species have developed a variety of
251 reproductive modes such as ovo-viviparity and viviparity (38). These species must have developed
252 immunological tolerance to accept allogeneic spermatozoa during reproduction. Thus, our results
253 suggest that modern non-viviparous species may have retained this immunological tolerance of
254 allogeneic spermatozoa.

255 The methods for germ cell transplantation in teleosts can be classified into three approaches (22):
256 primordial germ cell transplantation in fish embryos (39); germ cell transplantation in hatched fish
257 larvae (16, 18, 40-42); and germ cell transplantation in adult fish (23-26, 43, 44). The latter method,
258 germ cell transplantation in adult fish, has advantages over the other two approaches for aquaculture
259 and species preservation. For example, it avoids the time lag between transplantation and sexual
260 maturity of the recipient. Moreover, it does not require sophisticated techniques and equipment for
261 microinjection into eggs or larvae. Adult tissue transplantation is relatively easy as it involves a simple
262 transplantation procedure through the genital duct of the recipient after germ cell extraction from the
263 donor testis (22, 45, 46). To improve the success rate of germ cell transplantation to allogeneic
264 individuals, it is considered crucial that the germ cells of the recipient are depleted but that the ability
265 of the recipient to nurse donor-derived germ cells is maintained, e.g. through use of triploid individuals
266 (1, 47-49) or *dead end* gene knockdown fish (50). Cytotoxic drugs such as busulfan may be used for
267 germ cell depletion; use of these drugs adds a relatively short time to recipient preparation (2-4 weeks)
268 (23-25). However, the study using cytotoxic drug reported that the frequency of offspring derived from
269 donor sperm generally does not exceed 40% (26). In the present study, some of the recipients that had
270 received donor testicular tissue immediately after castration showed a high rate (60-100%) of offspring
271 derived from donor spermatozoa (Tables 1 and 4). This may be due to co-engraftment of germ cells

272 and somatic cells in the transplanted testicular tissue, and the donor derived testicular tissue may be
273 able to nurse their own germ cells (Figure 4). Further study on immunological tolerance in the testis
274 will undoubtedly benefit aquaculture and species preservation.

275 Cryopreservation methods for the whole testis have been developed in medaka (51). The combined
276 use of testicular cryopreservation and the present approach for testicular tissue transplantation using
277 adult recipients and natural mating may make it possible to shorten the time for recovery of larger
278 numbers of offspring from cryopreserved testes compared to artificial insemination or injection into
279 larvae. In our IHC analysis, we observed GFP-positive spermatogonia (Figure 4c). In medaka, it takes
280 at least 5 days for spermatogonia to develop into spermatids and approximately one week for the
281 spermatids to metamorphose into spermatozoa (52, 53). We mated each recipient used in the analysis
282 here with three d-rR females for 2-3 weeks. Therefore, spermatogenesis in the donor-derived testis
283 had sufficient time to complete at least one cycle of maturation before the mating analysis (Table 4).
284 Our results suggest that the allografted germ cells proliferated in the recipient testis, allowing the
285 recipient males to produce donor-derived offspring over a prolonged period (13 to 15 weeks). The rate
286 of success for functional engraftment was approximately 30% in the present study; it will be necessary
287 to improve this success rate to enable development of a simple, fast, and effective approach for

testicular transplantation into adult recipient fish.

Conclusions

We demonstrated the feasibility of allografting testicular tissue into immunocompetent recipients whose genetic background was distinctly different to those of the donors; functional engraftment was achieved after rough castration of the recipient without use of immunosuppressive treatments or chemical castration of the recipient. Further studies are required to improve our understanding of the immunological responses after testicular transplantation, and the results of these will be of value for aquaculture.

Abbreviations

ARS: Alizarin red S, bm: basement membrane, DAB: 3,3-diaminobenzidine, IHC: immunohistochemistry, lbc: lobule boundary cells, MS-222: Ethyl 3-aminobenzoate methanesulfonic acid salt, NGS: normal goat serum, PBS: Phosphate Buffered Saline, PFA:

paraformaldehyde, sc: spermatocyte, sg: spermatogonia, sp: spermatozoa, SSC: spermatogonial stem cells, st: spermatid

Declaration of competing interest

Ethics approval and consent to participate

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Tokyo. The committee requests the submission of an animal-use protocol only for use of mammals, birds, and reptiles, in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71; June 1, 2006). Accordingly, we did not submit an animal-use protocol for this study, which used only teleost fish and thus did not require approval by the committee.

Consent for publication

Not applicable

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Authors' contributions

D.K. carried out all experimental work, participated in the design of the study, and drafted the manuscript; S.K. and K.O. helped in the interpretation of the data and preparation the manuscript. All authors gave final approval for publication.

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Tables

Table 1. Results of the mating analysis: surrogate father of d-rR strain allografted with npb-GFP strain or actb-GFP strain testis.

After surgery (weeks)	2 to 7	2 to 7	2 to 7	2 to 7	2	2 to 7
Individual	#1	#2	#3	#4	#5	#6
GFP +	3	70	9	0	20	54
GFP -	30	36	40	78	1	0
%	9.09	66.04	18.37	0.00	95.24	100.00

Table 2. Results of the mating analysis: surrogate father of d-rR strain allografted with wild strain testis.

After surgery (weeks)	6 to 9	6 to 9
Individual	#7	#8
Pigmented	150	10

Non-pigmented	0	97
%	100.00	9.35

Table 3. Results of scale transplantation into a d-rR recipient

		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 12	Day 17
Pigmented scale	observed	23	10	10	9	9	8	8	6	6	0	-	-	-
	lost		13	0	1	0	1	0	2	0	6	-	-	-
GFP scale	observed	23	15	15	15	10	7	4	3	2	1	1	0	-
	lost		8	0	0	5	2	3	1	1	1	0	1	-
Alizarin red S positive scale	observed	20	13	13	13	13	13	13	12	12	12	12	12	12
	lost		7	0	0	0	0	0	1	0	0	0	0	0

Table 4. Results of the mating analysis at 13 weeks or more after surgery: surrogate father of d-rR strain allografted with actb-GFP strain or wild strain testis.

After surgery (weeks)	13	13	13	15	13	13
Individual	#1' *	#2' *	#3	#6	#7	#8
GFP + or pigmented eggs	0	0	15	97	86	20
GFP - or non-pigmented eggs	94	17	69	0	0	106
%	0.00	0.00	17.86	100.00	100.00	15.87

*, #1' and #2' were re-numbered because they could not be distinguished as #1 or #4.

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Figure legends

Figure 1.

The appearance and functionality of testicular tissue allografted into the abdomen of a recipient.

(a-c) Representative images of a recipient male that received testicular tissue derived from an actb-GFP strain male. Bright field image of the intact abdomen (a), bright field image of the incised abdomen (b), and fluorescence image of the incised abdomen (c); scale bar, 1 mm. (d-e) Representative images of the eggs fertilized by the recipient male that had been transplanted with testicular tissue of an actb-GFP strain male. Bright field image (d) and fluorescence image (e); scale bar, 1 mm

Figure 2.

Functional allografts of testicular tissue from donor medaka with a different genetic background to the recipients.

498 (a) An outline of the surgical procedure used here. (b) An outline of the mating scheme used here.
499 Pigmented eggs are produced following fertilization by spermatozoa of wild-derived strain germ cells.
500 Non-pigmented eggs result from fertilization with d-rR strain sperm. (c) Representative image of eggs
501 fertilized by a recipient that had been transplanted with testicular tissue from a wild-derived medaka
502 strain; arrowhead, pigmented egg resulting from fertilization with a wild-derived spermatozoon; scale
503 bar, 1 mm

504 **Figure 3.**

505 **Allografted scales were immunologically rejected by the recipient.**

506 (a, c, e) Representative bright field images of scales from a donor (actb-GFP strain and wild strain)
507 transplanted into a recipient (d-rR strain). Day 1 (a), Day 7 (c), Day 10 (e); arrowhead indicates
508 transplanted scale. (b, d, f) Representative fluorescence images of scales transplanted into a recipient.
509 Day 1 (b), Day 7 (d), Day 10 (f); arrowhead indicates transplanted scale. asterisk, autofluorescence
510 originating from a scale on the recipient. (g, h) Representative images of a donor (d-rR strain) whose
511 scales were vital stained with ARS and transplanted into a d-rR strain recipient: bright field image (g),
512 and fluorescence image (h) on Day 10. Arrowhead, transplanted scale. Scale bar, 1 mm

513 **Figure 4.**

514 **Allografted testicular tissue including somatic cells became established within the recipient testis.**

515 (a-c) Representative images from the IHC analysis after DAB and hematoxylin staining. DAB positive
516 cells are donor-derived (actb-GFP strain) cells. (a) Testis of individual #2 (GFP positive recipient).
517 The boxed area is magnified in panel (c). Scale bar, 100 μm. (b) GFP negative recipient. No DAB
518 positive signals are seen in any section. Scale bar, 100 μm. (c) Magnified image of boxed area in panel
519 (a). sg, spermatogonia; sc, spermatocyte; st, spermatid; sp, spermatozoa. Scale bar, 50 μm. (d)
520 Representative image of the IHC analysis after DAB staining but without hematoxylin staining. The
521 upper panel shows a section of the testis of individual #2. bm, basement membrane; lbc, lobule
522 boundary cells. The lower panel shows a section of a GFP negative recipient testis. Scale bar, 50 μm.

Figure 1

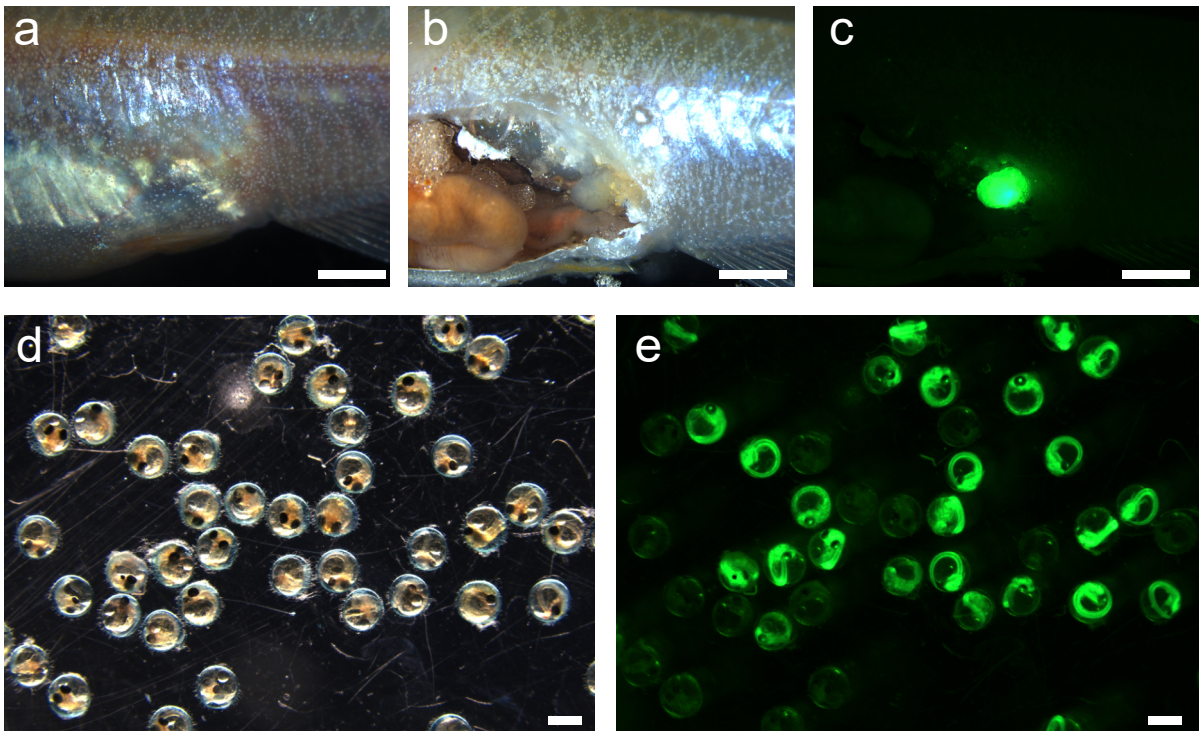


Figure 2

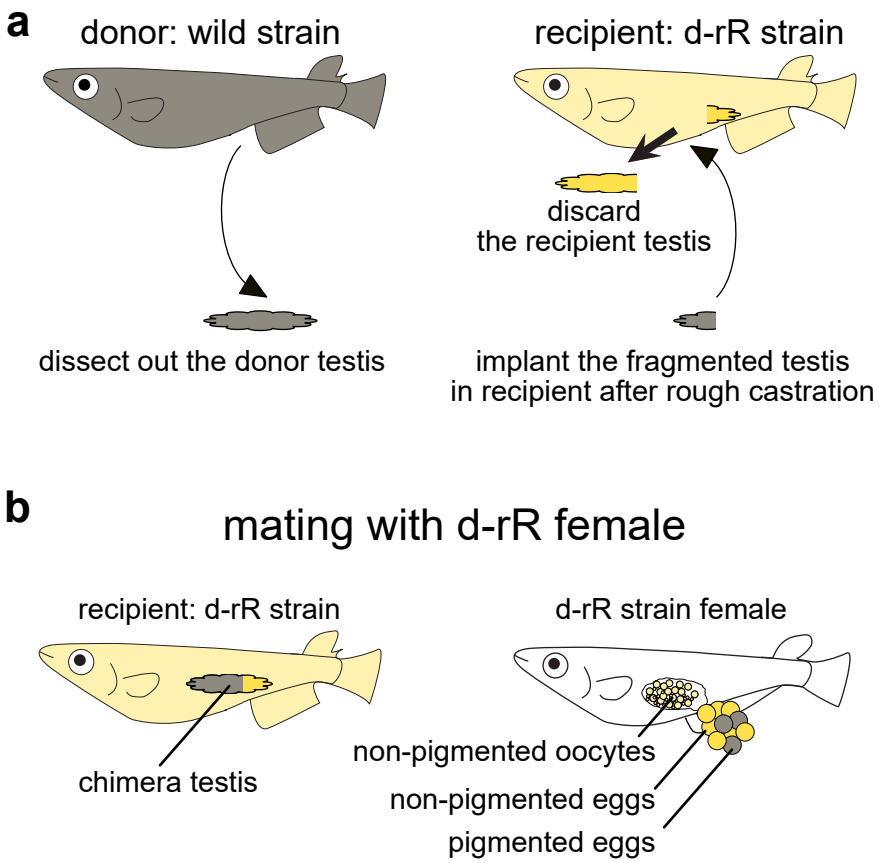

a
donor: wild strain
recipient: d-rR strain
discard
the recipient testis
dissect out the donor testis
implant the fragmented testis
in recipient after rough castration
b
mating with d-rR female
recipient: d-rR strain
d-rR strain female
chimera testis
non-pigmented oocytes
non-pigmented eggs
pigmented eggs
c

Figure 3

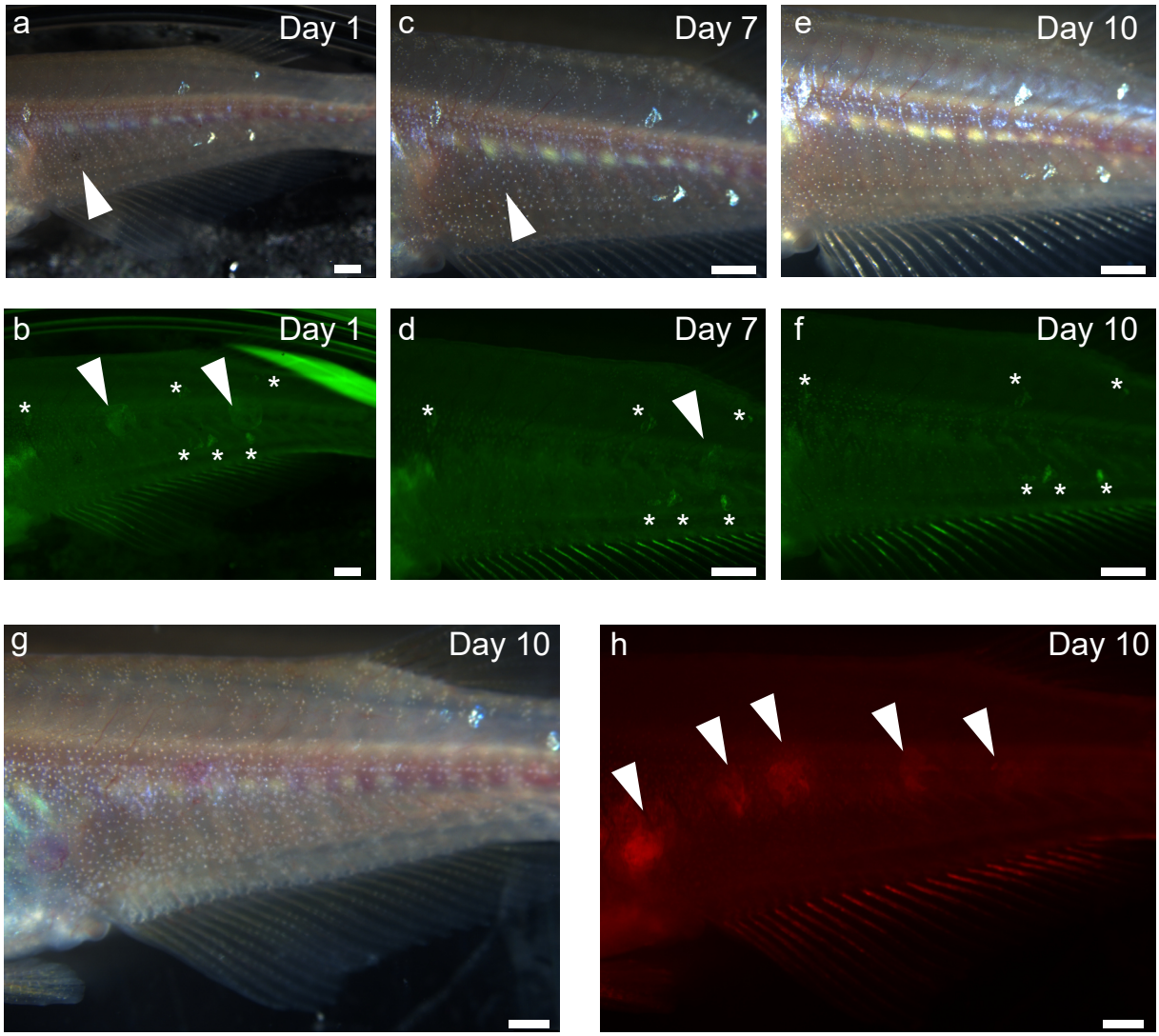
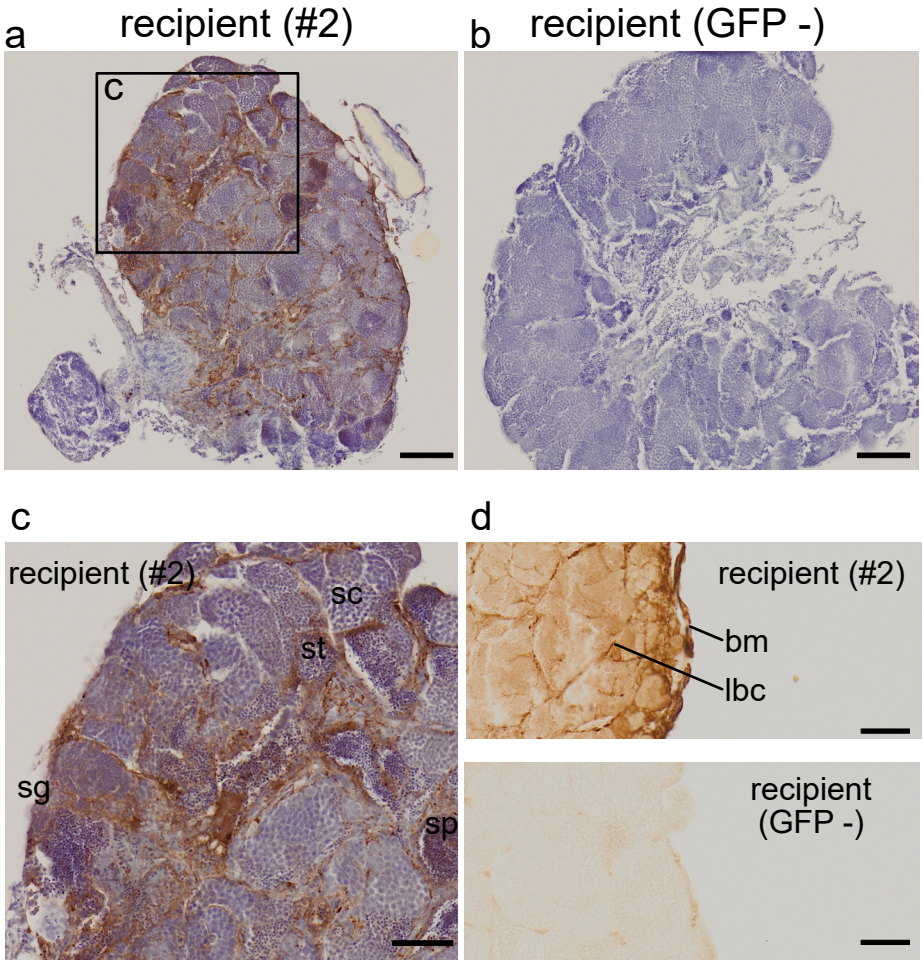


Figure 4


a
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