Cell. Mol. Life Sci. (2010) 67:1189–1202
DOI 10.1007/s00018-009-0247-4

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Accessibility of host cell lineages to medaka stem cells depends on genetic background and irradiation of recipient embryos

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Received: 24 September 2009 / Revised: 20 November 2009 / Accepted: 22 December 2009 / Published online: 10 January 2010

Abstract Chimera formation is a powerful tool for analyzing pluripotency in vivo. It has been widely accepted that host cell lineages are generally accessible to embryonic stem (ES) cells with the actual contribution depending solely on the intrinsic pluripotency of transplanted donor cells. Here, we show in the fish medaka (*Oryzias latipes*) that the host accessibility to ES cell contribution exhibits dramatic differences. Specifically, of three albino host strains tested (i^1, i^3 and af), only strain i^1 generated pigmented chimeras. Strikingly, this accessibility is completely lost in i^1 but acquired in i^3 after host γ-irradiation. Host irradiation also differentially affected ES cell contribution to somatic organs and gonad. Therefore, the accessibility of various host cell lineages can vary considerably depending on host strains and cell lineages as well as on irradiation. Our findings underscore the importance of host genotypes for interpreting donor cell pluripotency and for improving ES-derived chimera production.

Keywords Chimera · ES cell · Host accessibility · γ-Irradiation · Medaka

Abbreviations

dpf/hpf	Day(s)/hour(s) post-fertilization
ES	Embryonic stem
GFP/RFP	Green/red fluorescent protein
gfp/rfp	Gene coding for GFP/RFP
MBE	Midblastula embryo

Introduction

Embryonic stem (ES) cells derived from early developing embryos have the potency to differentiate into a wide variety of cell types of a developing embryo or the adult, and thus hold enormous potential in regenerative medicine [1]. Stem cells are also present in adult tissues for homeostasis. More and more stem cell lines have been established in recent decades, and induced pluripotent stem cells can be obtained via reprogramming of non-ES cells by forced expression of pluripotency transcription factors [2–4]. Criteria for characterizing putative stem cell lines include cell morphology, surface markers, and gene expression profile. The most powerful system to functionally define the developmental pluripotency is chimera formation, whereby stem cells are transplanted into early developing embryos, and their differentiation and contribution to multiple cell lineages are monitored. Early embryos undergo cell proliferation, lineage commitment, fate decision, and cell differentiation, thus providing an

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excellent host system for testing the potential of donor cells for differentiation into multiple lineages. In mice, ES cells can participate in organogenesis of chimeric embryos and, when introduced into tetraploid recipient embryos, can even generate an entire embryo proper that develops to a fertile adult [5]. These and other data have led to the hypothesis that all cell lineages are equally permissive for contribution by donor cells, and that the actual donor cell contribution depends completely on their intrinsic developmental potential.

There are several lines of evidence that chimera formation from ES cells in mice may also depend on the genetic background of the host. For example, host strain C57BL/6 produces germline chimeras efficiently from 129/Sv-derived ES cell donors, whereas CD1 or MF1 do not [6]. For C57BL/6 ES cells, BALB/c blastocysts are better than those of 129/Sv, C3H, and ICR [7, 8]. How these differences operate in mice and whether such strain differences are restricted to mice remains largely unknown.

The small laboratory fish, medaka (*Oryzias latipes*), is an excellent model system for analyzing the developmental potential of ES cells throughout external embryogenesis. Several ES cell lines comparable to mouse ES cells are available in this organism [9, 10]. One of these lines, MES1, has been characterized as being pluripotent in vitro and in vivo. In vitro, MES1 is capable of spontaneous differentiation into various functional cell types [9] and of directed differentiation into pigment cells [11]. In MES1 cells, the totipotency-specific mouse Oct4 promoter is active [12]. MES1 has a high efficiency for transient and stable gene transfer by physical/chemical procedures [13] and baculovirus transduction [14], and retains its developmental pluripotency even after long-term drug selection and stable gene transfer [13]. In vivo, MES1 after transplantation into host embryos can proliferate and differentiate during chimeric embryogenesis into many functional cell types that contribute to various organs [10]. The availability of ES cells and the possibility for robust genetic manipulations render medaka a promising model organism for cell culture-mediated germline transmission of defined genetic manipulations such as gene targeting.

Germline chimeras have been produced by blastula transplantation of non-cultivated midblastula embryonic (MBE) cells in a wide variety of non-mammalian vertebrates including zebrafish [15] and medaka [16]. Ma et al. [17] reported the production of zebrafish germline chimeras from short-term embryo cell cultures. However, attempts to produce germline chimeras from medaka ES cell cultures have so far been unsuccessful. In fact, the production of germline chimeras from cell cultures in non-mammalian species represents a critical challenge towards the development of gene targeting technology. The lack of ES cell germline transmission in fish has led to alternative approaches such as somatic cell nuclear transfer [15, 18], test-tube sperm production from testis-derived cell cultures [19], and semicloning by generating and utilizing haploid ES cells for direct nuclear transfer [20]. For germline chimera formation, an understanding of the accessibility and parameters for optimal contribution by ES cell cultures is required. Previously, we have shown that there are strain differences in ES cell derivation [21]. However, factors affecting ES donor contribution to various cell lineages of the host embryo have remained unclear.

In medaka, MBE-derived ES cell cultures including MES1 give rise to a high (up to 100%) frequency but a low (5% at best) degree of chimerism for somatic chimera formation [10]. This feature is common to ES cells after extended growth beyond 50 days of culture [21]. Such a low degree of chimerism has been thought to be responsible for the inability in egg-laying vertebrates to obtain contribution of donor ES cell cultures to particular cell lineages most important to the germline. Differences in several aspects between ES cell cultures and host embryos appear to account for this low degree of chimerism. For example, the recipient blastula embryos of medaka divide every 35 min [21, 22], whereas ES cell cultures such MES1 cells require more than 43 h to complete a cell cycle [9, 20]. Furthermore, there is a limited input number of ES cell cultures permissive to normal development [21]. Indeed, an increased input cell number by using differentiation-ablated ES cell cultures can enhance the degree of chimerism [13]. Another approach that has proven effective in enhancing the degree of chimerism is to use compromised recipients. For example, the use of γ-irradiated host embryos has enhanced chimera formation from non-cultivated blastodermal cells [23] and short-term ES cell cultures in chicken [24]. In medaka, Joly et al. [25] adopted this approach to boost chimera formation from non-cultivated MBEs. These experiments demonstrate the possibility to improve proportional donor contribution by adjusting both donor and host features.

One completely unsolved problem in chimera formation is whether the various host cell lineages are indeed equal or different in their capacity to allow for ES donor contribution. In zebrafish, the founder cells of the germline, the primordial germ cells (PGCs), are predetermined by maternal factors at cleavage stages [26, 27]. In medaka, PGCs are visible until stage 13 of pregastrulation by 12 h post-fertilization (hpf) [28, 29], and PGC formation also appears to be maternally determined [30]. It remains to be elucidated whether the germline and/or the somatic cells of the gonad primordium in these fishes is accessible to cultured ES cell donors.

This study was aimed at the use of externally developing embryos of medaka to analyze the accessibility of host cell lineages to contribution by ES cells. We show, by chimera

formation in three host strains, that host lineage accessibility exhibits considerable variability depending on several parameters including host strain genotypes and cell lineages. Furthermore, we show that this host accessibility can change dramatically upon sublethal irradiation, providing a so far neglected aspect to testing and interpreting donor pluripotency by chimera formation.

Materials and methods

Fish

Work with fish followed the guidelines on the care and use of animals for scientific purposes of the National Advisory Committee for laboratory animal research in Singapore and is in accordance with the regulations of the German Law for the protection of animals. Medaka was maintained under an artificial photoperiod of 14-h light to 10-h darkness at 26°C. Embryogenesis was staged according to [22].

Donor stains

HB32C is a wild-type pigmentation strain from which the ES cell line MES1 was derived [9]. To label liver cells, plasmid pLFABP-rfp was injected into 1-cell stage embryos and transgenic progeny were screened for four generations, resulting in a liver-red (Lr) transgenic line (Zeng and Hong, unpublished). This construct contains the 2.8-kb liver-specific promoter from the zebrafish liver fatty acid binding protein and drives RFP expression specifically in embryonic and adult liver [31]. Similarly, in Lr medaka, transgenic RFP expression occurs strongly and specifically in the developing and adult liver (Zeng and Hong, unpublished). Transgenic line Vg expresses specifically GFP from the medaka Vasa promoter and labels migratory PGCs [32]. Vg and Lr were crossed separately with HB32C for three generations to an essentially (87.5%) HB32C background. Embryos from crossing between heterozygous F3 males and females were used for transplantation. In some experiments, embryos from crossings between F3 Lr and Vg were used as donors.

Host strains

Albino strains i^1 and i^3 lack wild-type (black) pigmentation, due to mutations in the tyrosinase gene [33] and the *oca2* gene encoding a melanosome transporter protein [34], respectively. However, both strains have iridophores that produce yellow (positive for both green and red fluorescence) autofluorescence at 3 days post-fertilization (dpf), heavily interfering with the observation of fluorescence-labeled donor cells in advanced embryos [10]. By combining three recessive genetic loci including i^3, Wakamatsu et al. [35] developed an autofluorescence–free strain called see-through (ST2), which lacks melanophores and iridophores. The ST2 embryos are fragile for cell transplantation procedures. By crossing the ST2 with i^3 followed by interbreeding and selection for 15 generations, we obtained a robust autofluorescence-free (af) albino medaka.

Host irradiation

Embryos were collected shortly after fertilization and treated with proteinase K (10 mg/ml) for 1 h at room temperature. Attachment filaments were manually removed by pipetting and rolling. Embryos at the 4- to 8-cell stages were transferred into 2-ml tubes and irradiated at ambient temperature with a ^{60}Co source at doses of 5−10 gy during 2−4 min. Irradiated embryos were kept in darkness until use.

Cell culture

The medaka ES cell line MES1 was used as the transplantation donor throughout. It was maintained in ESM4 on gelatin-coated substrata as described [13]. MES1 cells expressing HygRGFP were described [13].

Chimera production

Irradiated and non-irradiated embryos were dechorionated and transplanted at the midblastula stage with approximately 100 MES1 cells [10, 21] or non-cultivated blastomeres [16, 21, 32]. In some experiments, embryos transplanted with blastula cells were reared in the presence of 1 mM of phenylthiourea to prevent pigmentation for easier fluorescence observations.

Microscopy and photography

Observation and photography on a Leica MZFIII stereo microscope, a Zeiss Axiovertinvert 2 invert microscope, and an Axiovert 200 upright microscope with a Zeiss AxioCam M5Rc digital camera (Zeiss, Germany) were as described previously [36].

Statistics

Statistical analyses were calculated by using GraphPad Prism v4.0. Consolidated data were presented as mean ± SD, and P values were calculated by using non-parametric Student's t test.

Results

Accessibility of multiple host cell lineages for contribution by blastula cells

We have previously revealed that MBE cells from nine genetically different pigmented strains of medaka can efficiently contribute to the pigment cell lineage of albino host strain i^1 [10]. However, the genotype of the donor turned out to be crucial for cultured MBE cells to produce pigmented chimeras, as the ability upon cultivation beyond 7 days is lost in some donor strains but maintained in others [21]. This implies that the accessibility of pigment cell lineage in i^1 is dependent on the donor genetic background. To assess a host effect on donor contribution, this study mainly used HB32C (Fig. 1a) as the donor strain and three different albino strains as hosts.

We first examined the host effect on accessibility of the pigment cell lineage by using three inbred albino strains as host, namely i^1, i^3, and *af* (Fig. 1b–d), the third being an autofluorescence-free albino strain on the i^3 genetic background. Transplantation of non-cultivated MBE cells produced a similarly high frequency (up to 80%; $n \geq 50$) for pigmented chimera formation in the three host strains (Fig. 1e–g). Furthermore, donor-derived melanocytes were similarly widely distributed to the eye, trunk, and yolk sac in all three host strains (Fig. 1e–h). In addition to melanocytes, fishes have a unique type of pigment cells called iridophores that are silvery white or greenish/bluish but appear brown in translucent light under a

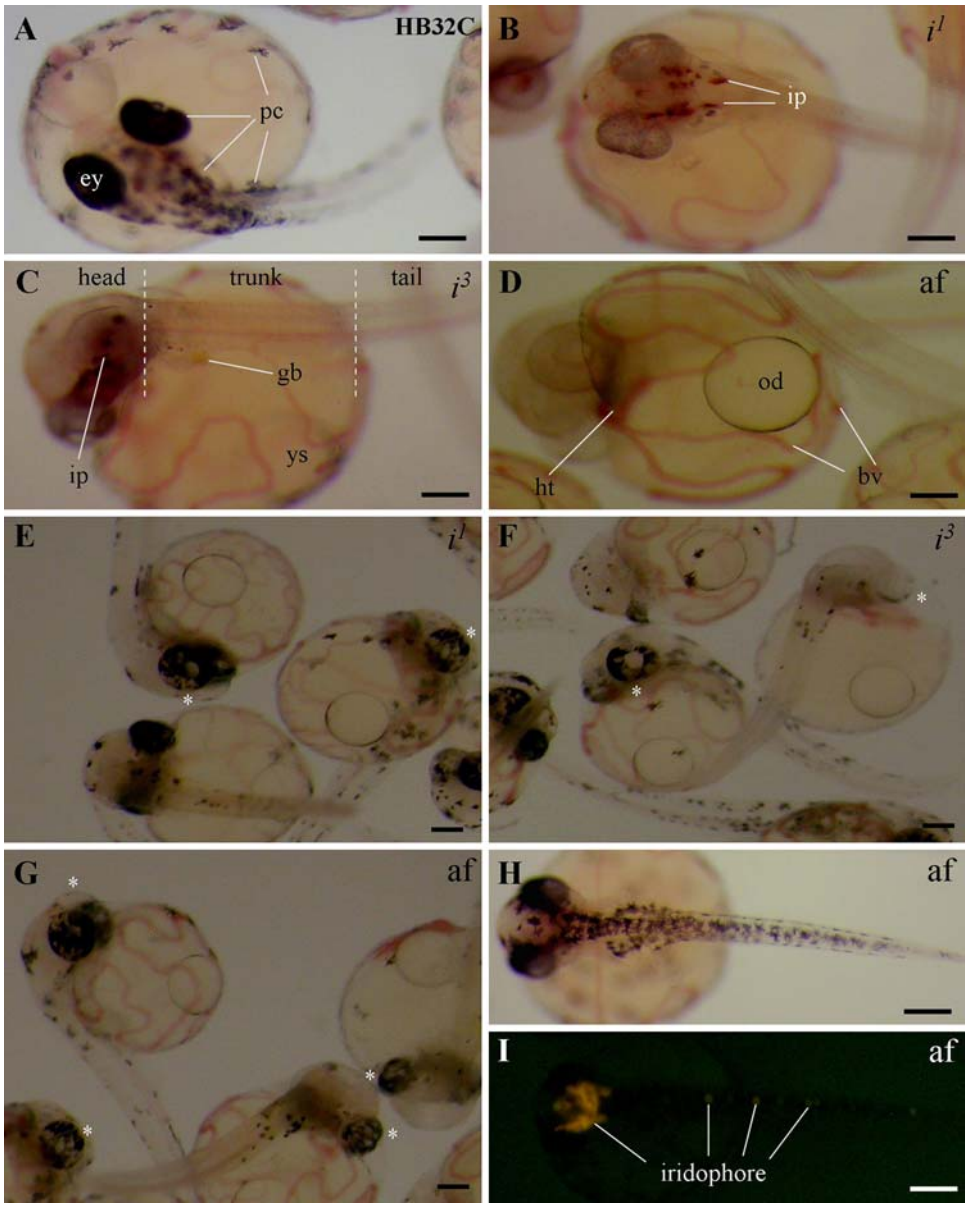

Fig. 1 Accessibility of the host pigment cell lineage to non-cultivated blastomeres. **a** Donor strain embryo (strain HB32C) with wild-type melanocyte pigmentation in the eye, trunk and yolk sac. **b–d** Control embryos of three albino host strains showing the absence of black pigmentation. Major embryonic compartments and organs are indicated. *bv* Blood vessel, *ey* eye, *gb* gall bladder, *ht* heart, *ip* iridophore, *od* oil droplet, *pc* pigmented cell, *ys* yolk sac. Notably, the autofluorescence-free (*af*) strain lacks visible pigmented cells. **e–i** Pigmented chimeras obtained by transplanting HB32C blastomeres into blastula embryos of the host strains indicated, showing wide distribution of pigmented cells in the embryo proper and extraembryonic yolk sac. Mosaic pigmentation is evident in the eyes (*asterisks*). In the *af* host, HB32C-derived donor iridophores are also clearly visible. **h**,**i** Embryos at 5–7 days post-fertilization. *Bars* 200 μm

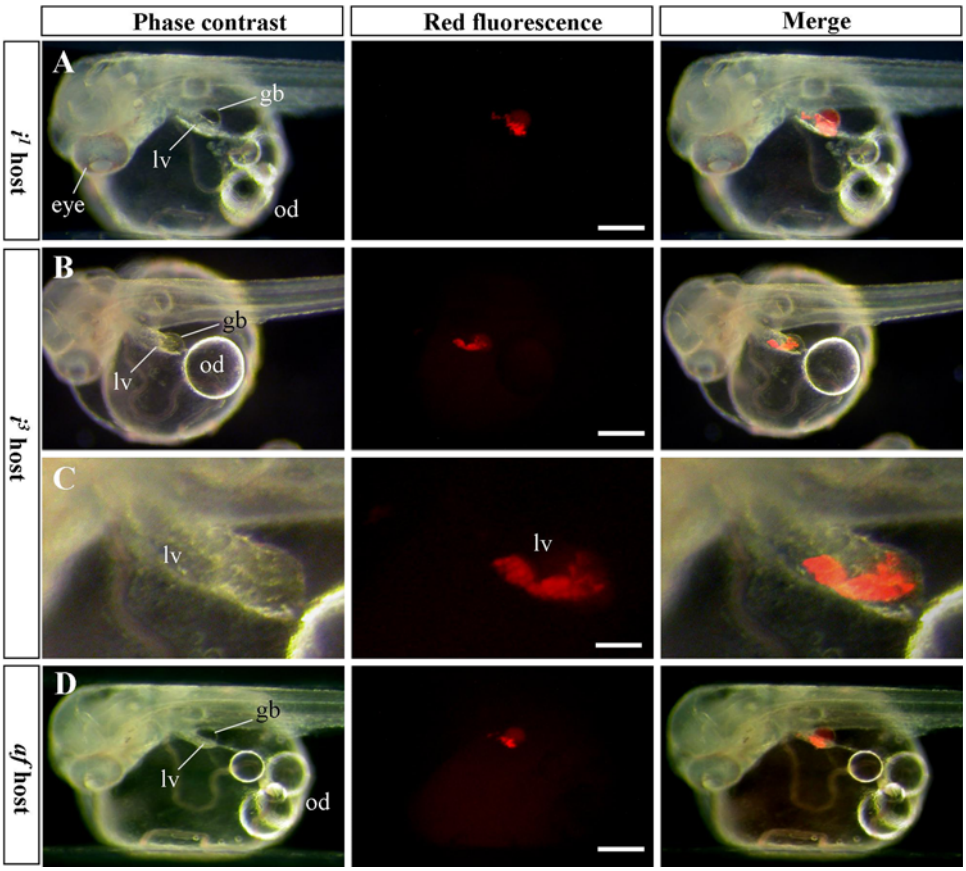

Fig. 2 Accessibility of the host liver to non-cultivated blastomeres. Following transplantation with Lr transgenic blastomeres expressing RFP in liver cells from the transferrin promoter, the embryos were incubated with 1 mM phenylthiourea to prevent pigmentation and documented at 7 dpf. **a** Donor-derived liver cells in i^1 host. **b**,**c** Donor-derived liver cells in i^3 host. **d** Donor-derived liver cells in *af* host. The chimeric liver (*lv*) consists of host cells and donor derivatives (*red*). The gall bladder (*gb*) exhibits cloud-like autofluorescence. *od* Oil droplet. *Bars* 200 µm (**a**,**b**,**d**) and 100 µm (**c**)

stereomicroscope. We observed that non-cultivated MBE cells also contributed to iridophores (Fig. 1i). This demonstrates that the pigment cell lineage of the three host strains is fully accessible to non-cultivated MBE donor cells.

We then examined the accessibility of host liver to non-cultivated blastomeres. For this, we generated a transgenic line called Lr (Liver red), which specifically expresses red fluorescent protein (*rfp*) from a hepatocyte-specific promoter in the developing liver. Upon transplantation, Lr-blastomeres contributed at a 4% frequency to the host liver in all three host strains, as evidenced by RFP expression (Fig. 2a−d).

We finally examined the accessibility of the host germline. For this, the *olvas-gfp* transgene was introduced into the HB32C background by crossing, producing Vg (*Vasa-gfp*) that specifically expresses GFP in PGCs (Fig. 3a, b; [32]). Upon transplantation, Vg-blastomeres contributed to the germline of host strain *af*, as evidenced by GFP expression in the gonad (Fig. 3c). Observation on cryosections revealed that the GFP-positive cells were indeed PGCs (Fig. 3d). Similar results were also obtained with host strains i^1 and i^3 (data not shown). Furthermore, when blastomeres from Vg and Lr double transgenic embryos (Fig. 4a, b) were transplanted into host *af*, we observed simultaneous contribution to both germline and liver (Fig. 4c−f). Taken together, all three host strains show a similar accessibility of their pigment lineage, germline, and liver to contribution by non-cultivated blastomeres.

Host strain difference in pigment cell contribution of ES cell lines

Previously, serial cultivation has been found to reduce the frequency of pigmented chimera formation in the i^1 host by 15-fold from 75% for non-cultivated blastomeres cells to 5% for MBE cultures [10, 21]. This indicates a clear difference in pigmented chimera formation between MBE cells and MBE-derived serial cultures. To examine the effect of host strains on pigmented chimera formation from ES cell cultures, we used the HB32C-derived ES cell line MES1, because it has demonstrated the ability to produce pigmented chimeras in strain i^1 [10]. MES1 transplantation into 1,142 blastulae of host strain i^1 generated a total of 783 fry, of which 41 were pigmented chimeras at day 5 of development, amounting to 5.3% pigmented chimera formation (Table 1). These MES1-derived chimeras had an average of 1.5 melanocytes. Compared to approximately 100 melanocytes per HB32C control embryo at the same

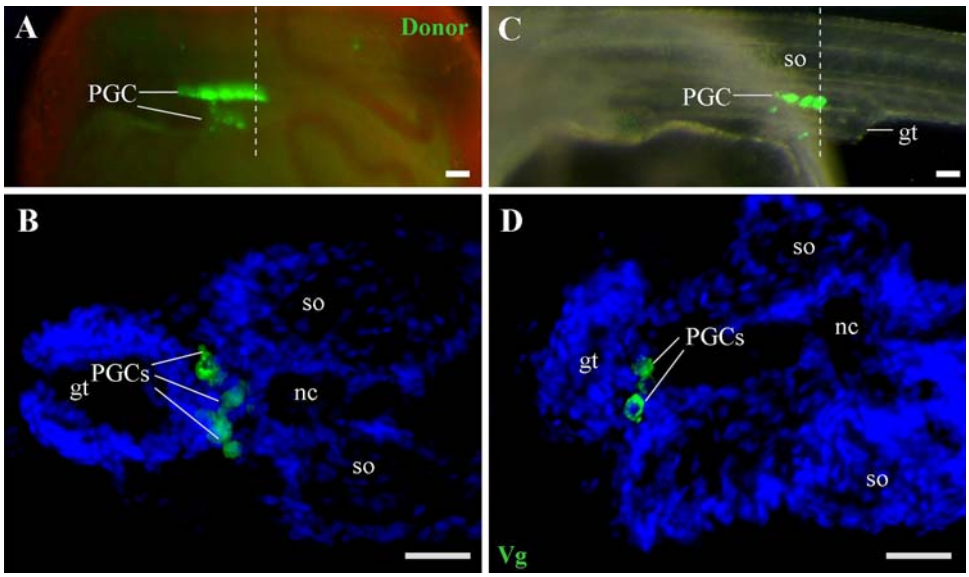

Fig. 3 Accessibility of the host germline to non-cultivated blastomeres. **a** Control donor embryos showing *green* fluorescing PGCs in the lateral plate mesoderm (site of the gonad primordium. **b** Section through the *broken line* in (**a**) showing four PGCs. Vg transgenic blastomeres of strain HB32C with GFP-labeled PGCs were transplanted into *af* host blastulae. **c** Chimera showing a few donor-derived PGCs (*green*) in the genital ridge. **d** Section through the *broken line* in (**c**) showing two PGCs. *br* Brain, *ey* eye, *gb* gall bladder, *gt* gut, *lv* liver, *nc* notochord, *od* oil droplet, PGCs primordial germ cells, *pf* pectoral fin, *so* somite, *ys* yolk sac. *Bars* 50 μm

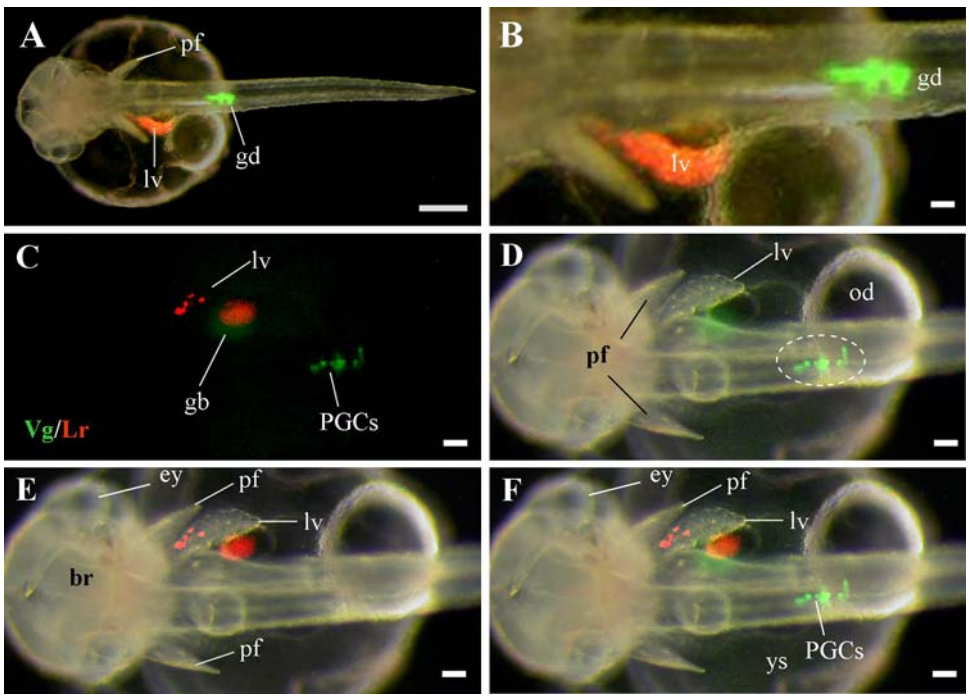

Fig. 4 Co-colonization of the host germline and liver by non-cultivated blastomeres. Heterozygous Vg and Lr double transgenic blastomeres were transplanted into *af* blastulae and chimeras were documented at 7 dpf. **a**,**b** Control donor embryos showing *green* PGCs and *red* liver. Vg- and Lr-blastomeres were transplanted at 1:1 ratio to blastulae of host *af*. Chimeras were documented at 7 dpf. **c** Merged fluorescent micrograph showing PGCs (*green*) and liver cells (*red*). **d** Donor-derived PGCs in the genital ridge (*circle*). **e** Donor-derived liver cells. **f** Merged bright-field and fluorescent photographs showing PGCs and liver cells relative to other major organs. Mosaic composition is evident in the gonadal PGCs and liver. The signal in gall bladder is due to autofluorescence (*green* and *red*). *br* Brain, *ey* eye, *gb* gall bladder, *gt* gut, *lv* liver, *nc* notochord, *od* oil droplet, *pf* pectoral fin, *PGCs* primordial germ cells, *so* somite, *ys* yolk sac. *Bars* 100 μm

Table 1 MES1-derived pigmented chimeras in control and irradiated albino hosts

Host	Irradiation dose (gy)	Number of experiments	Total blastulae transplanted	Number of embryos survived[a]	Pigmented chimeras[b]	
					Number	(%)[c]
Strain i^1	0	12	1,142	783	41	5.3
	6	13	1,004	247	0	0
Strain i^3	0	15	1,336	682	0	0
	6	12	1,015	715	270	38

[a] Embryos survived until day 5 of development. At this time, irradiated and non-irradiated embryos appeared to be at the same or similar morphological stage, regardless of whether they were transplanted or not

[b] Host embryos that displayed varying numbers of MES1-derived, black pigmented cells were scored as pigmented chimeras

[c] Comparison between pigmented chimeras and total survivors at the same stages. They are used for calculating the degree of pigmented chimerism as shown in Table 2

stage, this gives rise to 1.5% for the degree of pigment cell chimerism. Taking together, the 5.3% frequency and 1.5% degree leads to 0.08% for the overall MES1 contribution in all transplanted embryos. This frequency and degree of pigmented chimera formation (Table 2) conforms to our previous data [10]. The MES1-derived melanocytes distributed in the embryo proper as well as the extraembryonic yolk sac (see below). Therefore, MES1 has retained the competence for pigmented chimera formation, and the pigment lineage of host strain i^1 is accessible to colonization by ES cell cultures.

In contrast, when i^3 was similarly used as host, MES1 transplantation into 1,336 blastulae produced 682 embryos that survived until 5 dpf, but none of them had donor-derived melanocytes (Table 1). Clearly, the host strain i^3 is refractory for MES1 cells to contribute to the pigment cell lineage.

Host irradiation changes the pigment lineage accessibility

Since the use of compromised hosts can improve the efficiency of chimera formation [23, 25], we asked whether irradiation could enhance pigment chimera formation in i^1 and i^3 hosts. First, we established parameters of host irradiation in medaka. We observed that irradiation had three dose-dependent effects on medaka embryo development. First, irradiation at doses of 5−9 gy severely affected the survival rate. The hatching rate declined from 90% for non-irradiated embryos to 60, 52, 44, 35, 25, and 7% after irradiation at 5, 6, 7, 8, 9, and 10 gy, respectively (Fig. 5). The mechanical damage caused by dechorionation and cell transplantation procedure further reduced the normal hatching rate by approximately 30%. These observations indicate that γ-irradiation compromised embryonic development in a dose-dependent manner, and that irradiation at 5−7 gy still maintained the survival rate at a practical level. Therefore, we used a 6-gy dose in subsequent experiments.

Table 2 Numbers of MES1-derived melanocytes in control and irradiated albino hosts

Chimera	Non-irradiated i^1 host	Irradiated i^3 host (6 gy)	
		Normal development[a]	Abnormal development[b]
1	1[c]	5	40[d]
2	2	7	17
3	1	4	4
4	1	8	27
5	4	3	11
6	1	2	18
7	1	11	6
8	1	3	9
9	1	5	12
10	1	6	21
Number of melanocytes[e]	1.5	5.4	16.5
Chimera frequency (%)[f]	5.3	38	38
Chimerism degree (%)[g]	0.08	2.00	6.27
Fold enhancement by irradiation[h]		25	78

Transplanted embryos were scored at day 5 of development

[a] Pigmented embryos displaying seemingly normal development (Fig. 6d)

[b] Pigmented embryos displaying evident disorganization (Fig. 6b, c)

[c] This chimera is shown in Fig. 6e

[d] This chimera is shown in Fig. 6b

[e] The average number of melanocytes is the same as percentage pigmented degree, as the embryo of the MES1 donor strain HB32C has approximately 100 melanocytes at the same stage

[f] See Table 1

[g] Degree of pigmented chimerism is derived by multiplying the pigmented chimera frequency and the average number of melanocytes per host

[h] Comparison in chimerism degree between non-irradiated i^1 host and irradiated i^3 host

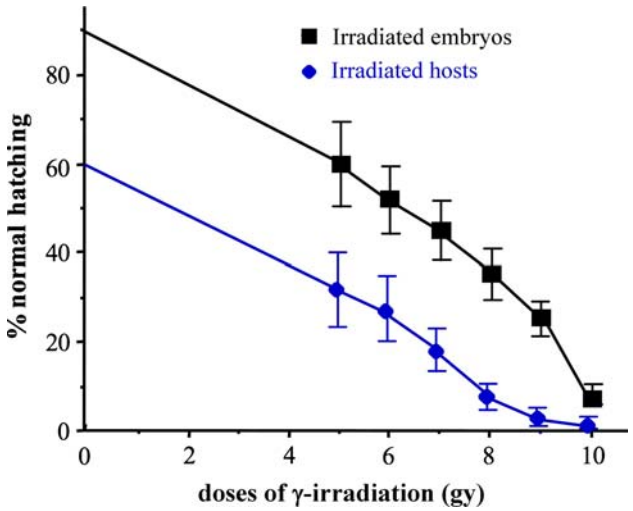

Fig. 5 Effect of γ-irradiation on survival at hatch. Irradiated embryos, embryos irradiated but not dechorionated and not transplanted; irradiated host, embryos irradiated with indicated doses (gy) followed by dechorionation and transplantation with approximately 100 MES1 cells at the midblastula stage. The survival rate is 90% for non-irradiated embryos (chorion-bearing controls) and 60% for non-irradiated hosts (dechorionated hosts), respectively. Values are means ± SD (*error bars*) of three independent experiments

Second, irradiation delayed early embryonic development, as has been described for chicken and for medaka in previous reports. In chicken, retardation persists throughout development [23]. In medaka, the delay is at early stages but becomes compensated at later stages [25]. In our experiments, non-irradiated embryos by 17 hpf had reached stage 23 with 12 somites, whereas irradiated embryos had not a single somite at the same time point. Surprisingly, both irradiated and non-irradiated embryos appeared to be at comparable developmental stages from 2 days post-fertilization (dpf) onwards (data not shown). Finally, irradiation affected embryonic development at later stages, regardless whether they were transplanted with cultivated ES cells or not. Approximately 30% of irradiated embryos displayed severe disorganization. These embryos sometimes developed the anterior–posterior axis and incomplete eyes, but did not develop clearly visible internal organs (Fig. 6a). Such embryos usually died before 5 dpf and were not used for further analyses. The remainder fell into two major classes with a similar frequency. Class I appeared normal or nearly so (Fig. 6b) and contributed substantially to the hatching rate of irradiated hosts (Fig. 5). Class II exhibited clear defects but developed macroscopically visible organs (Fig. 6c, d). They usually survived to 7 dpf but died before hatching. The embryos were pooled from several females that were laid within 30 min. Although these embryos at irradiation were at the 4−8 cell stages, they might be asynchronous at different cleavage phases (e.g., S and M phases) and thus different in sensitivity to irradiation.

MES1 cells were then transplanted into irradiated recipients. Transplantation of 1,015 irradiated i^3 recipients led to 715 embryos that survived to the pigmentation stages. Amazingly, 270 of the 715 embryos possessed pigmented melanocytes, giving rise to a 38% frequency for pigmented chimeras (Table 1). The number of MES1-derived melanocytes per chimera varied considerably, ranging from a few up to 40 melanocytes (Fig. 6b−d).

Donor-derived pigmentation in irradiated i^3 hosts showed two intriguing features. First, pigmentation began at 3 dpf, as is the case in normal embryonic development and in chimeras from non-cultivated (Fig. 1) or short-term cultivated MBE cells [21], but in contrast to delayed pigmentation at 5 dpf from transplantation of serial MBE cultures and MES1 in non-irradiated i^1 hosts (see above) [10, 21]. Second, melanocytes sometimes showed a wide distribution to various embryonic compartments (Fig. 6b−d). A MES1-derived pigmented chimera in a non-irradiated i^1 host usually had only one or two melanocytes (Fig. 6e; Table 2) consistent with our previous experiments [10].

Transplantation of 1,004 irradiated i^1 recipients resulted in 247 embryos that survived for observation until pigmentation stages. Surprisingly, none of them displayed any pigmented cells (Table 1). On the other hand, when non-cultivated HB32C blastomeres were used as the donor, all transplanted i^1 embryos ($n = 24$) were pigmented chimeras containing donor-derived pigment cells in a wide variety of areas, regardless of normal or abnormal development (Fig. 6f). Similarly, all irradiated i^3 hosts ($n = 28$) transplanted with HB32C blastomeres also produced pigmented chimeras (Fig. 6g). This indicates that irradiation in the i^1 genetic background abolishes the accessibility of the melanocyte lineage for contribution by MES1 but not by non-cultivated blastomeres. Collectively, the accessibility of pigment lineage in the two host strains has dramatically different responses to γ-irradiation: i^1 becomes inaccessible, whereas i^3 acquires accessibility to MES1 contribution.

Host irradiation quantitatively enhances the degree of pigmented chimerism

The number of melanocytes per chimera at 5 dpf was scored as the degree of pigment cell chimerism in irradiated i^3 hosts. Interestingly, the number of melanocytes appeared to be correlated with differential compromising effects, as more melanocytes were usually seen in class-II embryos with severe developmental defects than in class-I specimen (Table 2). A closer inspection of 10 chimeras from class I and II each revealed an average of 5.4 and 16.5 melanocytes per embryo (Table 2). Considering the 38% chimera frequency, this is a degree of pigmented chimerism of 2 and 6.27% for class I and II chimeras, respectively (Table 2). Comparisons of these elevated

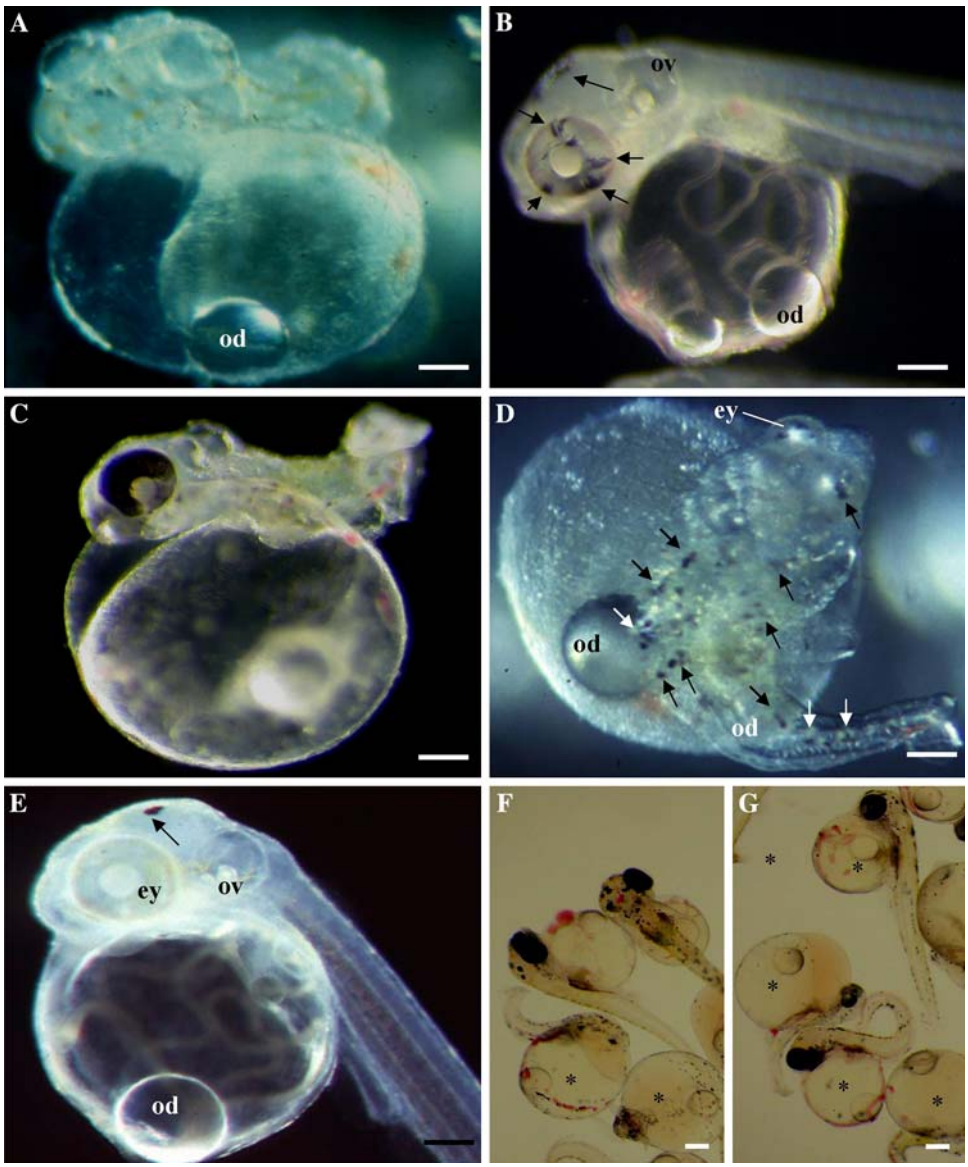

Fig. 6 Changes in accessibility of host pigment cell lineage after γ-irradiation. **a** Representative irradiated i^3 embryo, showing disorganized cell mass (3 dpf; *lateral view*). The anterior is to the *left*. MES1-derived pigmented chimeras in irradiated i^3 host at 7 dpf, showing wide distribution of numerous pigmented melanocytes. **b** Class I showing seemingly normal development and several pigmented melanocytes (*arrows*) in the eye and head. **c** Class II showing development defects and pigmented cells in the eye. **d** Class II showing a visible axis with organs and approximately 40 donor-derived melanocytes (*arrows*) on the surface of the trunk. **e** MES1-derived pigment chimera in non-irradiated i^1 host for comparison, showing a single melanocyte (*arrow*) and normal development (6 dpf). Non-cultivated MBE-derived pigment chimeras in irradiated i^1 hosts (**f**) and irradiated i^3 hosts (**g**) at 7 dpf, showing wide distribution of numerous pigmented melanocytes and irradiation-caused abnormal development (*asterisks*). *ey* Eye, *od* oil droplet, *ov* otic vesicle. *Bars* 200 μm

values to the basal level of 0.08% in non-irradiated i^1 hosts (Table 1) mean a 25- and 78-fold increase in the degree of pigmented chimerism. Therefore, irradiation in i^3 hosts not only abolishes the inaccessibility of the pigment lineage to contribution by MES1 cells but also quantitatively increases the degree of pigmented chimerism.

Host irradiation quantitatively modulates non-pigment cell lineages for ES cell contribution

The experiments described so far have focused on melanocytes because of the ease with which distribution and functional differentiation are discernible due to easily visible melanin. The pigment lineage represents just one of many cell lineages in a developing embryo. Hence, we asked whether irradiation also differentially affected MES1 contribution to other cell lineages. For this, we labeled MES1 cells with pHygEGFP [13] to visualize their fate in non-irradiated and irradiated i^3 hosts. Irradiation did not prevent wide contribution of MES1 as they were found in many organs of irradiated hosts. These included the ectoderm-derived organs brain, eye, neural tube, otic vesicle, and operculum, the mesoderm-derived organs heart, somatic muscles, spleen, and blood cells of hematopoietic islands or from circulation, and the endoderm-derived organs gut and liver (Figs. 7a–h and 8a, b).

Our observations so far have been made on donor distribution in living embryos. In cases of melanocytes and blood cells, terminal differentiation was apparent at the single cell level by their vital staining, namely dendrites with pigmentation for melanocytes (Fig. 6) and red cells in the islands and circulation for blood cells (Fig. 7d, e). To

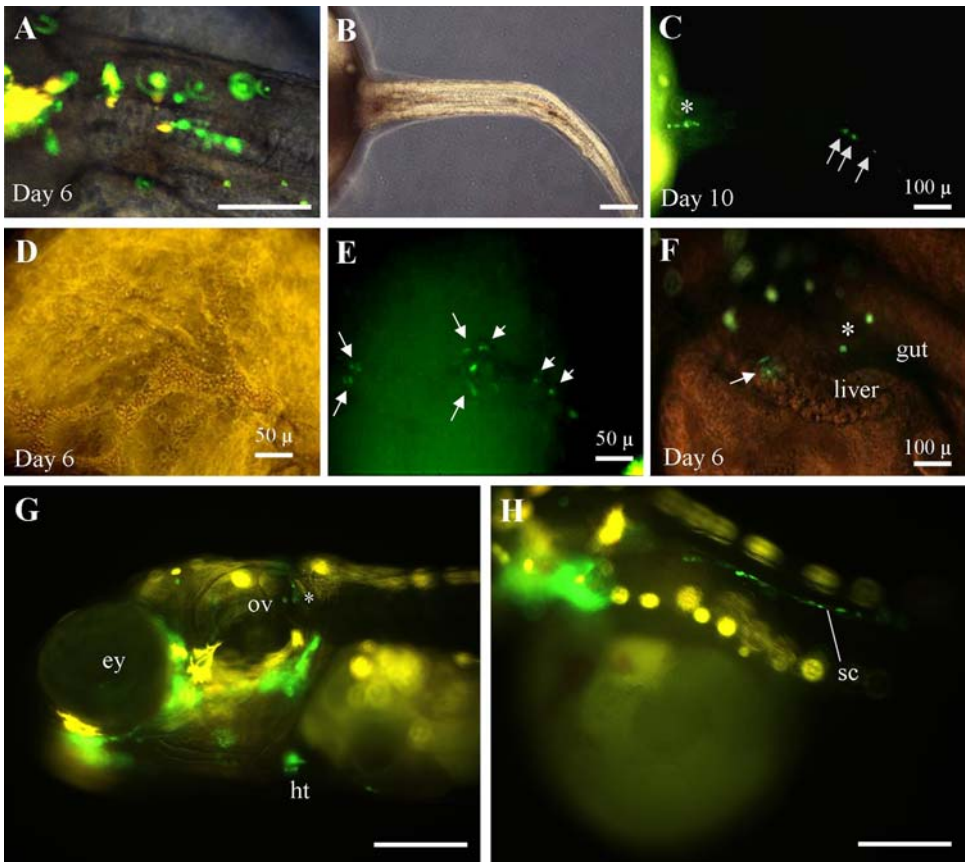

Fig. 7 Chimera formation in irradiated hosts. Embryos of host strain i^3 were subjected to 6 gy at the 4−8-cell stage and transplanted with approximately 100 GFP transgenic MES1 cells. Chimeras were photographed at indicated days of development. GFP-labeled MES1 derivatives are easily distinguished from large, *yellow* autofluorescent pigment cells. **a** MES1 derivatives in the trunk. **b**,**c** Chimeric fry showing MES1 cells in the gonad (*asterisk*) and tail (*arrows*) (ventral view). The *insert* shows the posterior part of the fry. **d**,**e** MES1-derived red blood cells (*arrows*). **f** MES1 derivatives in the gut (*asterisk*) and liver (*arrow*). **g** MES1 derivatives in the otic vesicle (*ov*) and heart (*ht*). **h** MES1 derivatives in the spinal cord (*sc*). *Bars* 100 µm

determine whether MES1 cells in other organs/lineages also terminally differentiated into other functional cell types, we combined live imaging and histological analysis. To this end, we focused on the eye and heart. In the eye, several cell types are arranged regularly into layered structures, allowing for unambiguous identification of particular cell types. For detailed observation, we chose a chimera that displayed eye pigmentation and GFP signals within the eye and heart by live imaging (Fig. 8d). On serial sections, MES1 derivatives were also found in the lens, ciliary marginal zone, neuroepithelium, pigment epithelium, optic nerve, and brain (Fig. 8e, f). More importantly, MES1 derivatives together with GFP-negative host cells formed a continuous neuroepithelium and pigment epithelium (Fig. 8f). It is noteworthy that black pigmentation of MES1 derivatives in the pigment epithelium masked the GFP observation (Fig. 8b, c, f).

The heart visually manifests its functionality by rhythmic contraction in living embryos, and consists of large, contracting cardiomyocytes. On sections, at larger magnifications, MES1 derivatives in a contracting heart were recognized as functional cardiomyocytes, because they were morphologically indistinguishable from the host-derived GFP-negative muscles (Fig. 8e, f). Taken together, MES1 derivatives in both the heart and eye appear to have undergone morphologically terminal differentiation and to show an architectural organization, which is consistent with a functional contribution to the respective organs.

Together with a similar observation in non-irradiated i^1 host [10], these data suggest that host irradiation does not prevent ES cell distribution to the major organ systems while conferring accessibility on i^3 for pigment cells.

To evaluate the effect of host irradiation for MES1 contribution to various cell lineages besides pigment cells, we inspected GFP-MES1 cell distribution during chimeric embryogenesis until hatching, now focusing on MES1 contribution from 5 dpf onwards, when major organ systems are easily discernible. Upon MES1 transplantation into a total of 534 non-irradiated i^3 hosts, 361 survived until observation. MES1 derivatives were found in three major organs, namely the brain (38%), eye (22%), and heart (42%). A high frequency was also detected in blood (8%). The liver and gonad exhibited low frequencies of 3 and 0.8%, respectively (Fig. 9). This is similar to what we reported previously for MES1 in non-irradiated i^1 hosts [10]. Upon transplantation into 649 irradiated i^3 hosts, 237 exhibited many organs that had MES1-specific reporter gene expression (Fig. 9). The frequency of MES1 distribution decreased significantly in the three major organs about three- and fourfold (brain, 10%; eye, 8%; and heart,

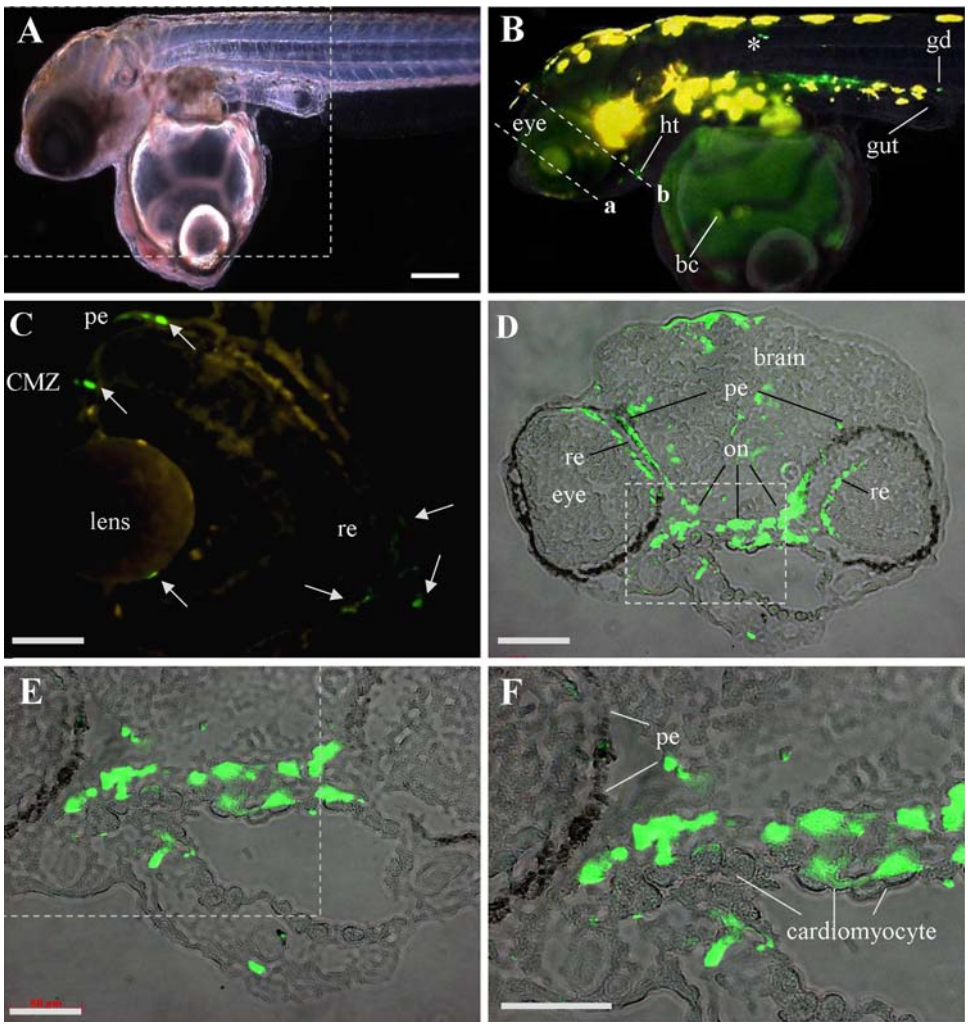

Fig. 8 Histological analyses of MES1 derivatives in irradiated hosts. **a**,**b** Live embryo showing MES1 derivatives the trunk (*asterisk*); ht heart, *gd* gonad, *bc* blood and gut. **c** Section at *broken line a* in (**b**) showing wide distribution of MES1 derivatives in the lens, ciliary marginal zone (CMZ), neural epithelium and pigment epithelium of the eye and cardiomyocytes. Section through the heart indicated by the *broken line b* in (**b**). **d** Contribution of MES1 derivatives to the brain, the pigment epithelium (*pe*) and neural epithelium (*re*) of the eye and optic nerve (*on*) between the eyes. **e**,**f** MES1-derived cardiomyocytes and host cardiomyocytes with striation at larger magnification (**f**). For chimera formation and observation, see caption to Fig. 7. *Bars* 100 μm

15%). In contrast, the frequency doubled to 16% for blood distribution and 7% for liver distribution, respectively. These results suggest that host irradiation can quantitatively modulate many somatic cell lineages besides melanocytes.

Host irradiation boosts gonadal contribution by MES1

In non-irradiated i^1 hosts, the gonad has been found to be one of the organs refractory to MES1 contribution [10]. Of 361 chimeras produced in non-irradiated i^3 hosts, only 0.8% displayed MES1 distribution ($n = 3$; Fig. 9). Interestingly, upon transplantation in irradiated i^3 hosts, 11 out of 237 chimeras had MES1 cells in the developing gonad (Fig. 7c), giving rise to a frequency of 4.6, an increase by nearly sixfold (Fig. 9). It appears that host irradiation boosts gonad contribution by MES1.

In summary, the accessibility of host cell lineages to donor cell colonization depends on donor cell source, host strain, and particular cell lineages, and can be dramatically modulated by experimental manipulations such as irradiation.

Discussion

This study reports the usefulness of externally developing embryos to assess the accessibility of host cell lineages to contribution by transplanted ES cells. We have provided four lines of evidence that the host cell lineages have dramatic differences in the accessibility to qualitative and quantitative contribution by donor cells, depending on the host strain and cell lineage. Amazingly, in some cases, the host accessibility can even be completely abolished or acquired by experimental modulation such as irradiation. First, both i^1 and i^3 albino strains can produce pigmented chimeras at a similar efficiency when non-cultivated blastula cells are used as donor. Only one of them, namely i^1 but not i^3, can generate pigmented chimeras from long-term cultivated ES cells, demonstrating that the pigment cell lineage exhibits donor source-dependent accessibility. Second, both albino strains have no detectable difference in the accessibility for ES cell contribution in many other cell lineages of the three germ layers, indicating that not all host cell lineages are equally accessible for ES cell

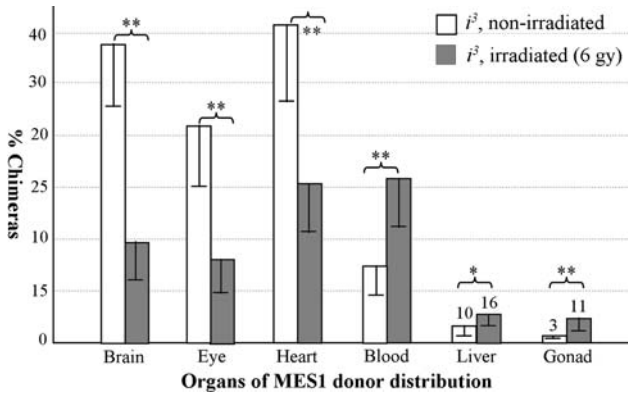

Fig. 9 Chimera formation in non-irradiated and irradiated i^3 host. Individual chimeras in 96-well plates were regularly monitored until hatching. In total, 361 and 237 MES1-derived chimeras produced in non-irradiated and irradiated hosts, respectively, were observed for data analysis. *Numbers* above *columns* are real numbers of rare chimeras. Shown here are the four organs with strong contribution of MES1 (brain to blood) and two organs with low contribution (liver and gonad). Values are means ± SD (*error bars*) of eight batches of independent experiments. $*P < 0.05$, $**P < 0.01$

contribution. Third, we show that the pigment cell as the best detectable of many host cell lineages can alter its accessibility/inaccessibility by host irradiation. Finally, we show that the use of compromised host blastulae significantly enhances the quantitative ES cell contribution in certain somatic cell lineages.

γ-irradiation has been used to compromise host embryos for improving chimera formation in chicken and medaka. In chicken, the use of irradiated recipients for injection of freshly isolated blastoderm cells resulted in a significantly increased degree of chimerism [23]. In medaka, host irradiation led to a 14-fold increase in the degree of chimerism from non-cultivated blastula cells [25]. We obtained a 25- to 78-fold increase in the degree of pigmented chimerism by using the long-term cultured MES1 cells. Our work corroborates and extends the previous reports by revealing a more profound boosting effect of host irradiation on ES cell cultures than on non-cultivated blastula cells.

The average degree of pigmented chimerism in i^1 is 11% for non-cultivated blastula cells (see Table 2 of [21]) and 0.08% for MES1 cells (this study; [10]). Therefore, the long-term cultured MES1 is 140 times less efficient than non-cultivated blastomeres, a difference similar to the smaller permissive input cell number in ES cell transplantation [10] compared to blastomere transplantation [21]. This would explain why no germline chimera has so far been obtained from long-term ES cell cultures in fish. Notably, even on a 25-fold increase by irradiation, the degree of chimerism for ES cells (2%) is merely 1/6 of the 11% for non-cultivated blastomeres in normal i^1 hosts. Future work is needed to test more efficient approaches towards production of ES cell-derived germline chimeras.

Sublethal irradiation compromises viability and proliferation. This general compromising effect has been made responsible for improved chimera formation in irradiated hosts of chicken [23] and medaka [25]. However, we have shown that host irradiation—besides changing the accessibility of the pigment cells lineage—also differentially affects the quantitative ES cell contribution to various organ systems. Specifically, irradiation reduces contribution to some organs (e.g., heart) but increases contribution to others (e.g., blood). It seems that ES cells that would otherwise be committed to a particular organ change their distribution to other organ types. It deserves to be noted that major organs of ES cell contribution, namely brain, eye, and heart, are the largest in cell number and are formed early during development. It is likely that irradiation produces an embryonic environment, which might delay lineage commitment of both host cells and donor ES cells to the major organs, and/or concurrently favors them to contribute to the more late-developing organs such as the liver that is formed at later stages. In support of this notion is the observation that irradiation indeed leads to early developmental delay in medaka host embryos (this study; [25]). Alternatively, irradiation might also comprise the accessibility of certain cell types in the major organs and prevent ES cell contribution, as is the case for pigment cells in the i^1 host. It is also possible that both mechanisms operate.

The developing gonad consists of somatic cells and PGCs. At present, it is not clear whether certain MES1 derivatives residing within the gonad are PGCs, because the low frequency of gonad colonization and high embryonic/post-hatching lethality prevented a functional test by germline transmission. Although the timely appearance, larger size, and location of these gonadal MES1 derivatives are not different from those of GFP-labeled PGCs in living embryos [32, 35, 37], the accessibility of the medaka germline to contribution by ES cell cultures remains an open question, because medaka PGCs might be formed by maternal factors [30]. Future work will determine whether MES1 expresses germ plasm components essential for PGC specification.

A striking finding is that the pigment cell lineage shows host-dependent accessibility to ES cell contribution, and that this accessibility is either abolished or acquired by host irradiation depending again on the host strain. Although it is unclear whether these phenomena are also present in other animals, this finding demonstrates necessary cautions for testing and interpreting pluripotency in vivo, and underscores the need to use several host strains for chimera production. More importantly, this finding provides important insights into the mechanisms underlying the accessibility of particular host cell lineages and into experimental approaches for manipulating host accessibility to ES cell contribution.

Acknowledgments We thank Dr. W. Thomas (Würzburg, Germany) for providing the irradiation facility, Dr. Y. Wakamatsu (Nagoya, Japan) for supplying ST2 medaka, and Q. Zeng for breeding fish. This work was supported by the Biomedical Research Council of Singapore (R-08-1-21-19-585, R-154-000-371-305 and SBIC-SSCC C-002-2007), the Ministry of Education of Singapore (R-154-000-285-112), the Lee Hiok Kwee donation fund (R-154-000-153-720), Deutsche Forschungsgemeinschaft, and the Commission of the European Union.

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