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Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos

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SUMMARY

Stage-X blastoderms, within intact eggs from White Leghorn hens, were exposed to 500-700 rads of γ radiation from a ^{60}Co source prior to injection, into the subgerminal cavity, of approximately 100 or 200-400 dispersed cells from stage-X blastoderms isolated from eggs laid by Barred Plymouth Rock hens. Embryos developing past day 14 of incubation and hatched chicks were assessed for donor and recipient cell contribution to the melanocyte population through examination of black and yellow down pigmentation, respectively (Barred Plymouth Rocks have a recessive allele at the *I* locus while the White Leghorns have a dominant allele at the *I* locus). Of the 809 embryos injected with approximately 100 cells, 192 developed past day 14 and black pigmentation, indicating somatic chimerism, was observed on 118 of the 192 (58%) embryos and chicks. Of the 296 embryos injected with 200-400 donor cells, 86 developed past day 14 of incubation. Somatic chimerism was observed on 55 of the 86 (64%) embryos and chicks. To test for germline chimerism, birds surviving to maturity were mated to Barred Plymouth Rocks. Five somatically chimeric females were produced when approximately 100 cells were injected, and one was a germline chimera. Six somatic female chimeras were produced following the injection of 200-400 cells, three of which proved to be germline chimeras by the presence of Barred Rock chicks among their offspring. Two of the nine males produced by injecting approximately 100 cells were germline chimeras. Five of 6 somatic male chimeras, produced by injection of 200-400 cells, have sired black chicks; furthermore, one of the roosters produced by the injection of 200-400 cells has sired only donor-derived chicks. These data indicate that irradiation of the recipient embryo, prior to injection of the donor cells, consistently yields somatic and germline chimeric chickens. The ability to insert donor cells into the germline provides a powerful new tool that will facilitate molecular and cellular manipulation of the developing chick embryo.

Key words: germline chimera, chicken, embryo

INTRODUCTION

Eggs and spermatozoa are derived from a specialized lineage of cells that is separated at an early stage of embryonic development from cells that form somatic tissues (Nieuwkoop and Satasurya, 1979). In the chicken, fertilization occurs within 1 hour after ovulation and embryonic development is initiated 3 hours later as the male and female pronuclei fuse (Perry, 1987). The first cleavage furrow develops within 2 hours as the ovum enters the shell gland. The shell is deposited during the next 18-20 hours and, when the egg is laid, the embryo is composed of 40 000-80 000 morphologically undifferentiated cells designated as stage-X by Eyal-Giladi and Kochav (1976). The precise timing of differentiation of the germline during embryonic development has yet to be established but it is thought to occur at approximately stage-X (Ginsburg and Eyal-Giladi, 1987). Access to the population of cells in the newly laid, but unincubated egg, that differentiates into the lineage giving rise to the gametes of the adult chicken, would provide a powerful tool to probe the complex interactions that regulate sexual differentiation. In addition, cells in the germline lineage are attractive candidates for genetic modification because manipulation of their DNA, which could include specific gene targeting, would ensure transmission of the genetic alteration to the next generation. Although we have previously demonstrated that germline chimeras can occasionally be produced by injecting dispersed blastodermal cells from stage-X embryos into recipients at the same stage of development (Petitte et al., 1990, 1993), manipulations of the cells in the germline have been thwarted because techniques to direct the developmental destiny of isolated chicken blastodermal cells into functional gametes in chimeric chickens have been unavailable. By combining isolated blastodermal cells that give rise to functional gametes in the adult chicken with recipient embryos whose development has been compromised by exposure to irradiation, we have now developed a proce-

dure that yields germline chimeras consistently and repeatably. The predictable incorporation of donor cells into the germline of chimera makes these birds ideal intermediates through which genetic alterations in dispersed blastodermal cells made in vitro might be transferred to the germline in vivo.

MATERIALS AND METHODS

Donor embryos were obtained from inter se matings of Barred Plymouth Rocks that are homozygous recessive at the *I* locus. Recipient embryos were obtained from inter se matings of White Leghorns that are homozygous dominant at the *I* locus. Donor cells were obtained by collecting stage-X blastoderms from freshly laid, unincubated eggs using filter paper rings (Petitte et al., 1990). The area pellucida from each embryo was dissected while submerged in Dulbecco's phosphate-buffered saline (PBS) containing 5.6 mM glucose (PBS-G; Gibco BRL, Burlington, Ontario). Isolated area pellucidae were transferred into fresh PBS-G, rinsed once in calcium- and magnesium-free PBS containing 2% (v/v) chick serum (2% CS-PBS-CMF) and dissociated with 0.25% trypsin (Sigma Chemical Company, St. Louis, Missouri) in 2% CS-PBS-CMF containing 0.04% (w/v) EDTA at 4°C. Dissociation of the cells was halted by suspending the cells in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) Fetal Bovine Serum (DMEM-FBS) prior to injection into recipients.

Recipients were prepared by exposing freshly laid, unincubated eggs to approximately 500-700 rads of irradiation from a ^{60}Co source. The embryo was accessed subsequently through a window cut into the long axis of the egg at its widest sector. The shell was removed to expose an 8 mm × 8 mm area of the shell membrane and then a 4 mm × 4 mm window was cut into the membrane to expose the embryo. Approximately 100 or 200-400 cells in 2-5 μl of DMEM-FBS were injected into the subgerminal cavity using a finely drawn micropipet. The window was closed by aligning a piece of freshly isolated shell membrane to overlap the exposed shell membrane of the recipient egg without contacting the shell. The membranes were allowed to adhere by exposure to air and a second piece of membrane was placed over the hole in the shell. When the outer covering of shell membrane was dry, it was glued to the shell with cement for plastic models and, after approximately 1 hour, was covered with Opsite™ surgical membrane (Smith & Nephew, Montreal). The eggs were placed into a conventional forced air incubator maintained at 37.5°C and 50% relative humidity and turned through 90°/hour for 19 days after which they were transferred to a conventional hatcher at 36.9°C and 85% relative humidity until hatch. Somatic chimerism was evaluated in embryos developing past 14 days of incubation or at hatch by the presence of black pigmented down; those that lacked black down were designated as putative chimeras. Somatic and putative chimeras surviving to sexual maturity were mated to Barred Plymouth Rocks and the distribution of black and yellow offspring was recorded to assess the contribution of the donor and recipient lineages to the germline, respectively (Fig. 1). The frequency of germline chimerism when approximately 100 or 200-400 donor cells were injected into irradiated recipients was compared to the frequency of germline chimerism when approximately 100 donor cells were injected into a non-irradiated recipient. Fisher's Exact Test was used to identify statistically significant differences between these frequencies.

The appropriate exposure of recipients to irradiation, prior to injection of donor cells, was determined by exposing freshly laid, unincubated, White Leghorn eggs to 0, 552, 691, or 898 rads of irradiation from a ^{60}Co source (Atomic Energy of Canada). Embryos were subsequently incubated at 37.5°C and 50% relative humidity for 48, 72 and 96 hours or allowed to complete development and hatch. Development of irradiated embryos was compared to development of embryos that were not irradiated but incubated for the same length of time. An estimate of embryonic development in hours of incubation under standard incubation conditions and according to the classification of Hamburger and Hamilton (1951) was assigned to irradiated and non-irradiated

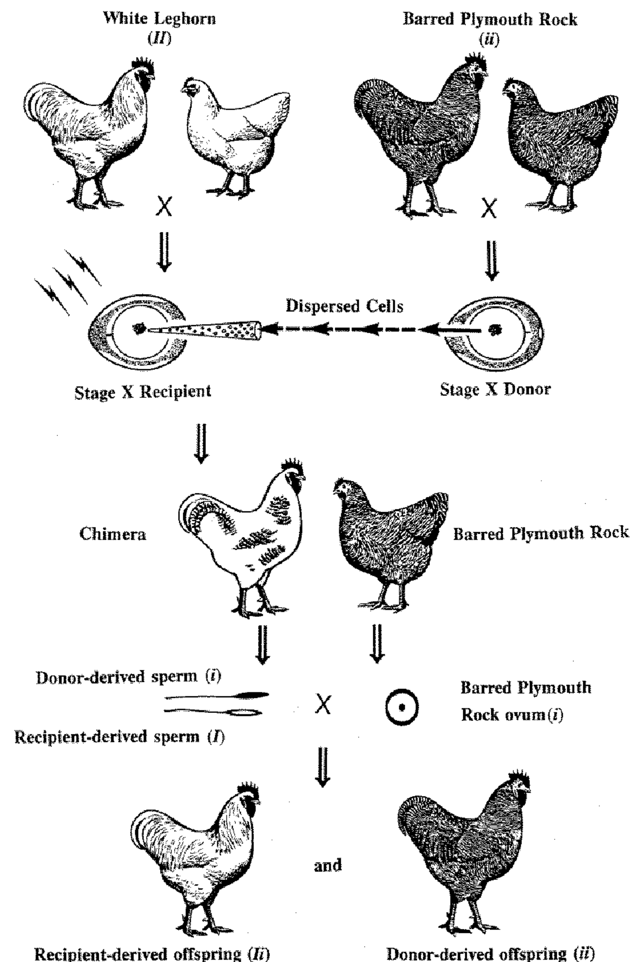

Fig. 1. The formation and analysis of chimeras. Donor embryos were obtained from inter se matings of Barred Plymouth Rocks that are homozygous recessive at the *I* locus. Recipient embryos were obtained from inter se matings of White Leghorns that are homozygous dominant at the *I* locus. Donor cells were obtained by isolating the area pellucida from stage-X blastoderms collected from freshly laid, unincubated eggs. These area pellucidae were dispersed into a single cell suspension prior to injection into recipients. Recipients were prepared by exposing freshly laid, unincubated eggs to 490-680 rads of irradiation from a ^{60}Co source. The embryo was accessed subsequently through a window cut into the long axis of the egg at its widest sector. Approximately 100 or 200-400 cells were injected into the subgerminal cavity of the recipient. The window in the egg was sealed and the eggs were incubated until hatch. Somatic chimeras were identified at hatch by the presence of black down. At sexual maturity, they were mated to Barred Plymouth Rocks and the distribution of black and yellow offspring was recorded to assess the contribution of the donor and recipient lineages to the germline, respectively.

embryos. Mortality and hatching rates were also compared between irradiated and non-irradiated embryos.

RESULTS

The development of embryos exposed to 552-898 rads was delayed relative to the development of embryos not exposed to irradiation and the extent of the delay was proportional to the exposure to irradiation (Fig. 2A-D). This delay in development was maintained throughout incubation and irradiated embryos emerged from the shell approximately one day later than non-irradiated embryos (data not shown). Of the embryos exposed to 552, 691 and 898 rads, 28.9%, 36.1% and 59.1%, respectively, did not hatch. By comparison 13% of the embryos that were not exposed to irradiation failed to hatch (Fig. 2E). These observations indicated that exposure of embryos to approximately 500-700 rads of irradiation prior to incubation would compromise development without reducing hatchability to impracticable levels. Chicks that developed from White Leghorn embryos exposed to 500 rads, but otherwise not manipulated, grew normally, possessed unimpaired fertility and produced only yellow chicks typical of the White Leghorn breed (data not shown).

Of 809 White Leghorn embryos exposed to 500-700 rads of irradiation and injected with approximately 100 donor cells, 192 developed past day 14 of incubation or hatched. Among the 192 embryos and chicks, somatic chimerism was evident on 118 (58%); on average 48% of the feather follicles of these somatic chimeras had been colonized by donor-derived melanocytes. In 2 hatched chicks, 75-90% of

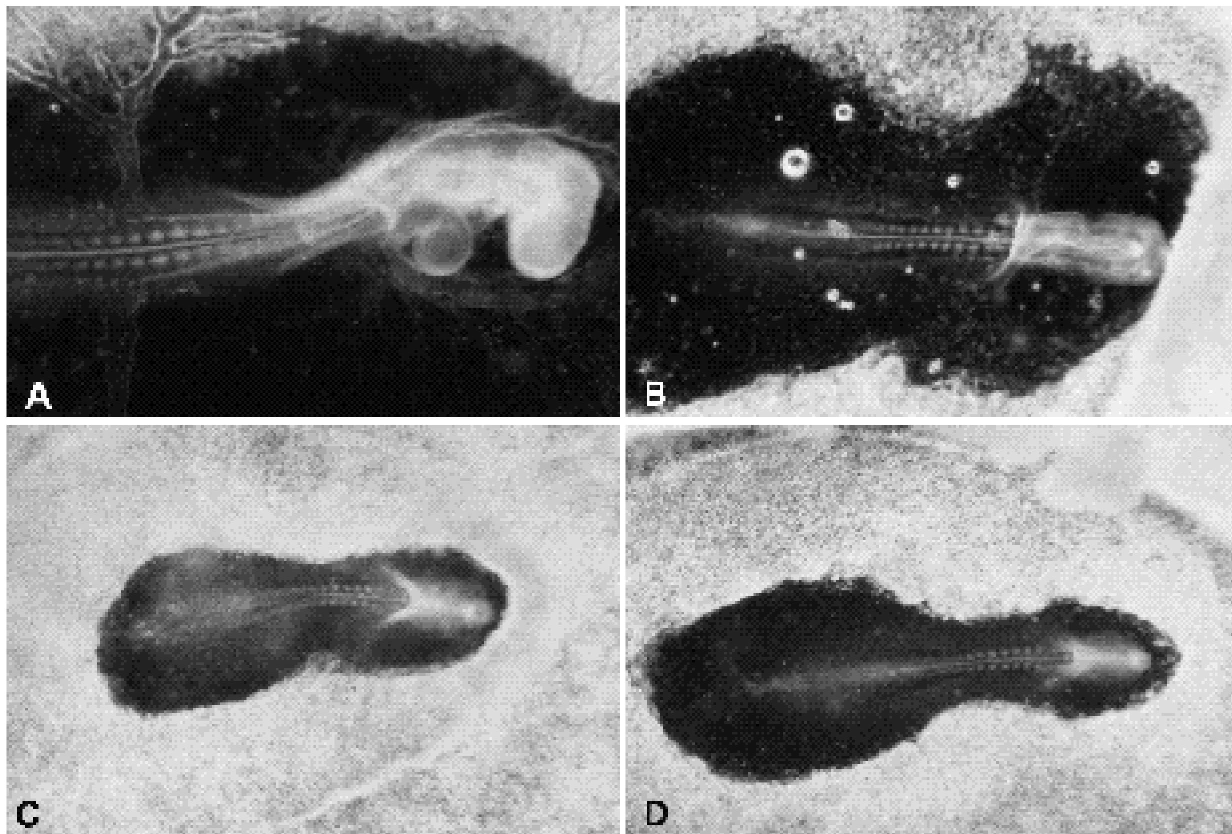

Fig. 2. Development of chick embryos, exposed to (A) 0, (B) 552, (C) 691 and (D) 898 rads from a ^{60}Co source, after 48 hours in an incubator at 37.5°C and 50% relative humidity. The embryo in A has a well developed vascular system, the head has undergone flexion and torsion and at least 12 pairs of somites are visible. In B, differentiation of the vascular system is delayed, the embryo has not undergone flexion and torsion and 11 pairs of somites can be distinguished. In C, the heart has not developed and only 7 pairs of somites are visible. In D, the anterior intestinal portal is less well developed than in C. Embryonic mortality was increased from 13% without exposure to irradiation to 59.1% when the embryos were exposed to 898 rads (E). The dose of irradiation that was chosen (500-700 rads) induced a moderate delay in development (approximately 24 hours) without increasing embryonic mortality to impracticable levels.

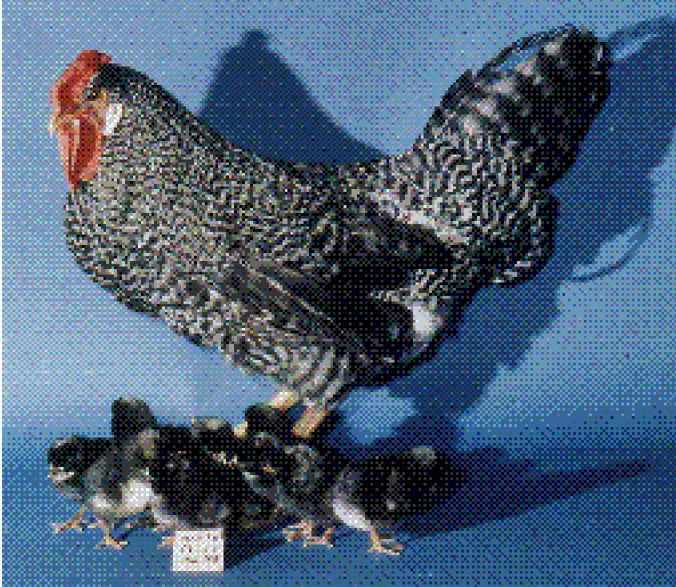

Fig. 3. Chimera number 042-195 and 11 of his offspring. This chimera was made by injecting approximately 400 dispersed blastodermal cells from stage-X Barred Plymouth Rock embryos into a White Leghorn recipient at the same stage of development. The recipient was exposed to 550 rads from a ^{60}Co source prior to the introduction of the Barred Plymouth Rock blastodermal cells. The body conformation and earlobes of this bird are typical of the White Leghorn breed but his plumage pigmentation, with the exception of a few white feathers, is indistinguishable from that of a Barred Plymouth Rock. The entire germline of this chimera is presumed to be donor-derived because he has produced only Barred Plymouth Rock offspring when mated to Barred Plymouth Rock hens (Table 1).

the melanocyte population was derived from the donor cell line.

Of 296 White Leghorn embryos exposed to 500-700 rads of irradiation and injected with 200-400 donor cells, 86 survived to a stage of development where somatic chimerism could be assessed. Donor-derived melanocytes were evident in 55 of the 86 (64%) embryos and hatched chicks and, on average, 66% of the feather follicles of somatic chimeras were colonized by donor-derived melanocytes. In a large proportion of hatched chicks (47%), more than 75% of the feather follicles were colonized by donor-derived melanocytes (Fig. 3).

Re-evaluation of chimeras that were produced previously in our laboratory using non-irradiated recipients, some of which have been described by Petitte et al. (1990, 1993), revealed that chimeras with donor-derived melanocytes were observed less frequently when the recipient was not exposed to irradiation. In a group of 102 embryos and chicks, produced by injecting approximately 100 donor cells from the central disk area of the stage-X embryo into non-irradiated eggs, only 33 (32%) exhibited black pigmentation in their down and, on average, only 29% of the feather follicles in these chimeras were colonized by the donor cell line. Chicks in which more than 75% of the melanocytes were donor-derived were not observed using non-compromised recipients.

The frequency of germline chimerism following injection of approximately 100 donor cells was significantly (P<0.001) increased from 2/106 to 3/24 by using an irradiated rather than a non-irradiated recipient. Whereas increasing the number of cells injected had no effect on the frequency of chimerism when non-irradiated recipients were used (Petitte et al., 1993), the frequency of germline chimerism was significantly (P<0.01) increased to 8/14 when 200-400 cells were injected into irradiated recipients. Using the ratio of germline to somatic chimeras as the basis for comparison, the frequency of germline chimerism increased significantly (P<0.01) from 2/21 when non-irradiated recipients were used, to 3/14 when approximately 100 cells were injected into recipients. Injecting 200-400 cells into irradiated recipients significantly (P<0.01) increased the ratio even further to 8/12.

When White Leghorn embryos were injected with approximately 100 cells from Barred Rock donors, 9 somatically chimeric males and 5 somatically chimeric females were produced and mated to Barred Plymouth Rocks. Two of the males have sired black chicks (Table 1) providing evidence that derivatives of the donor cells colonized the germline in these chimeras. Among the 5 somatically chimeric females one has produced a Barred Plymouth Rock chick (Table 1). Four putatively chimeric males and 6 putatively chimeric hens have produced only yellow chicks (Table 1) indicating that the donor-derived cells did not enter the germline in any of these birds.

Six somatically chimeric males, 6 somatically chimeric females and 2 putatively chimeric females produced by injecting 200-400 cells into irradiated embryos have been mated to Barred Plymouth Rocks (Table 1). Donor-derived cells were incorporated into the testes of 5 of the 6 male somatic chimeras (Table 1) and, in one case (042-195 in Fig. 3 and Table 1), all of the spermatozoa that sired chicks were descendants of donor cells. Three of the 6 somatically chimeric females contained donor-derived oocytes in their ovary (Table 1) whereas neither of the putatively chimeric females produced black offspring to date.

DISCUSSION

We have previously demonstrated that it is possible to make somatic and germline chimeras by injecting dispersed cells from a stage-X chick embryo into non-irradiated recipient embryos at the same stage of development (Petitte et al., 1990) and interspecific chimeras have been reported by Naito et al. (1991) and Watanabe et al. (1992). Subsequent experience with non-compromised recipients, however, has revealed that somatic chimeras are produced infrequently, donor-derived cells have colonized the gonads in only 2 of 106 chimeras and the transmission rate of donor-derived gametes from germline chimeras was less than 0.4% (Petitte et al., 1993).

By contrast, extensive colonization of the recipient by donor cells was promoted by exposing the recipient White Leghorn eggs to 500-700 rads from a ^{60}Co source within one hour after oviposition. Whereas only 32% of the embryos and chicks produced by the injection of approximately 100 cells into a non-irradiated recipient exhibited

Table 1. The number of donor- and recipient-derived offspring from matings to Barred Plymouth Rocks of somatic and putative chimeras made using compromised White Leghorn recipients

Description of the chimera	Approximate number of donor cells injected	Percentage of black pigmentation	Number of donor-derived offspring	Number of recipient-derived offspring	Percentage of donor-derived offspring
Somatic♂					
1585-1586	100	50	0	984	0
1583-1584	100	45	0	946	0
119-1592	100	30	0	830	0
101-102	100	30	0	833	0
103-104	100	85	0	616	0
114-116	100	20	0	212	0
142-143	100	35	0	752	0
1587-120	100	5	41	2147	1.9
105-106	100	45	104	1929	5.1
Somatic♀					
1579-1580	100	45	0	199	0
110-111	100	25	0	75	0
125-126	100	5	0	68	0
134-139	100	85	1	73	1.3
163-165	100	20	0	90	0
Putative♂					
1595-1596	100	0	0	809	0
1597-1598	100	0	0	688	0
121-122	100	0	0	607	0
167-168	100	0	0	612	0
Putative♀					
1589-1590	100	0	0	207	0
107-108	100	0	0	171	0
076-077	100	0	0	172	0
112-173	100	0	0	90	0
203-204	100	0	0	90	0
201-174	100	0	0	131	0
Somatic♂					
065-066	275	20	398	539	42.5
075-078	400	30	377	708	34.7
048-049	275	99	9	271	3.2
042-195	300	99	869	0	100.0
068-198	275	45	62	1001	5.8
082-083	250	20	0	449	0
Somatic♀					
050-055	275	99	1	43	2.3
021-022	350	85	3	54	5.3
028-029	300	3	15	45	25.0
046-047	300	60	0	96	0
032-033	300	95	0	113	0
034-035	300	35	0	103	0
Putative♀					
063-064	275	0	0	111	0
175-176	400	0	0	77	0

black pigmentation in their down, 58% and 64% of the chimeras injected with approximately 100 or 200-400 cells, respectively, into irradiated recipients exhibited donor-derived down pigmentation. The proportion of feather follicles colonized by donor-derived melanocytes was increased from 29% when about 100 cells were injected into non-irradiated recipients to 48% and 66% when approximately 100 cells and 200-400 cells, respectively, were injected into irradiated recipients.

The frequency of somatic and germline chimeras was unaffected by the number of cells that were injected into non-irradiated recipients (Petitte et al., 1992). By contrast, both the frequency and extent of incorporation of donor cells into somatic and germline lineages was increased when the number of cells injected into irradiated recipients was increased (Table 1). From these data we have concluded that irradiation compromises the ability of cells within the recipient embryo to divide and that the ratio of donor/recipient cells is increased as embryonic development proceeds during the first few hours or days of development. We assume that the relative rate of replication of cells in the chimera can favour colonization of any tissue

by the more rapidly dividing donor cells although we have direct evidence to support this assumption only for melanocytes and germ cells.

From the data presented in Table 1, it is evident that germline chimeric chickens can be made frequently and reliably using a recipient that has been compromised by exposure to irradiation. The production of chimeras from compromised recipients significantly increased the number of germline chimeras when an irradiated rather than a non-irradiated recipient was used, and increasing the number of donor cells from approximately 100 to 200-400 significantly improved the frequency of germline chimeras to more than 50%. In addition to improving the number of germline chimeras, the use of irradiated recipients also increased the frequency of transmission of gametes of donor-cell origin from the gonads of the chimeras. The 2 germline chimeras made using non-irradiated recipients yielded donor-derived offspring at a rate of less than 0.4%, whereas the minimum rate of transmission using compromised recipients was 1.3%, the median rate was 5.8% and the maximum rate was 100%.

Our results to date indicate that male chimeras are more likely to accommodate donor cells in the germline than female chimeras but this sex difference is not yet understood. In a previous study, male chimeras were also observed more frequently than females and female cells were usually excluded from adult male chimeras (Shaw et al., 1992). Further analysis of male germline chimeras made with female donor cells and compromised recipients are required to resolve this enigma.

All of the germline chimeras listed in Table 1 are also somatic chimeras but there is little correlation between the extent of feather pigmentation in somatic chimeras and the likelihood that donor cells will have colonized the germline. For example, hen 028-029 possessed only a small patch of black feathers on her neck, but approximately 1 in 5 oocytes was donor-derived (Table 1). The melanocyte population of males 048-049 and 042-195 was almost exclusively donor-derived (Table 1 and Fig. 3) but the proportion of functional, donor-derived spermatozoa in their semen was approximately 3% and 100%, respectively (Table 1). These data support the interpretation that entry of cells into the germline and into the ectoderm, from which the melanocytes are derived, occurs separately and independently in chimeras made with compromised recipients. In addition, the rates of proliferation of donor and recipient cells in the ectoderm and the germline may not be equal.

By our definition, putative chimeras show no evidence of donor-derived feather pigmentation and to date, no putative chimera produced in the currently reported experiments has proved to be germline. The lack of success in producing extensive somatic or germline chimerism in every embryo may be due to donor cells being injected into an inappropriate location in the irradiated egg and we are addressing this issue. Nevertheless, the ease with which large numbers of fertile eggs can be irradiated and injected with donor cells, and with which somatic chimeras can be identified, make the production of germline chimeric chickens a routine and practicable procedure.

The simplicity and practicality of obtaining germline chimeras using compromised recipients creates new opportunities for manipulation of the avian genome. Blastodermal cells can be frozen and retain their ability to form chimeras after they are thawed (Petitte et al., 1993; Naito et al., 1992); therefore, there is a unique opportunity to cryopreserve the genome of chickens using these readily available cells. Blastodermal cells can be transfected and have been shown previously to express their modified genotype in the non-compromised recipient (Brazolot et al., 1991). More recently, we have shown extensive incorporation of transgenic cells in compromised recipients (Fraser, Carsience, Clark, Etches and Verrinder Gibbins, unpublished results) indicating the possibility of using the compromised recipient as a vehicle to move genetically modified blastodermal cells from in vitro systems into the germline. We have also shown that blastodermal cells retain their ability to form germline chimeras when introduced into compromised recipients after 48 hours in culture (Etches, Toner, Clark, Vielkind and Verrinder Gibbins, unpublished results) allowing time for selection of some types of genetically modified donor cells. Genetically modified stocks can be dispersed quickly and inexpensively since founder chimeras can produce more than 1500 offspring in six months (e.g. 1587-120 in Table 1). Taken together, these data indicate that the compromised recipient has the potential to provide experimental biologists with a powerful new tool that will facilitate molecular and cellular manipulations of the developing chick embryo. To date, such manipulations have only been possible using the mouse as the vertebrate model. The application of these techniques to the chick embryo via transgenic germline chimeras will allow the amalgamation of classical information regarding development, gleaned from anatomical studies of the chick embryo, with the nascent understanding of the molecular control of cellular function during both embryonic and adult life.

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