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Characteristics of Long-Term Cultures of Avian Primordial Germ Cells and Gonocytes[1]

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ABSTRACT

Avian cell lines derived from germinal crescent primordial germ cells and gonadal gonocytes with long-term proliferative capacity in vitro and their subsequent rates of colonization and germline transmission are described. In general, male cultures proliferate more rapidly than female cultures although both can be developed into cell lines of $>2 \times 10^6$ cells, at which time, they can be grown indefinitely and a cell bank can be established. All the cell lines injected into embryos transmitted through the germline with the percentage of germline transmission of both male and female cell lines varying from single digits to the high 90s. The derivation of these primordial germ cell and gonadal cell lines and the subsequent robustness of germline transmission validates these cells as suitable for establishment of lines of chickens bearing novel genetic modifications.

avian, germline transmission, primordial germ cells, transgenic chickens

INTRODUCTION

Murine embryonic stem cells (ESCs) have been available for more than three decades but equivalent cell lines from other species have been difficult to find. To date, pluripotential cell lines from cattle, pigs, sheep, and other domestic animals remain elusive. Even in closely related species such as rats, pluripotent ESCs have only recently become available. The first indications that pluripotential cell lines could be derived from avian embryos came from the observation that transfer of blastodermal cells from one embryo to another yielded both somatic and germline chimeras [1, 2]. Subsequent studies showed that the ability to contribute to somatic tissues was maintained in cells with an ESC phenotype, but these cells were unable to contribute to the germline [3]. However, cell lines derived from primordial germ cells (PGCs) maintained their ability to contribute to the germline although they are unable to contribute to somatic tissues [4].

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The impetus to develop pluripotential cell lines from chickens has been the production of transgenic animals [5–10]. The first report on the establishment of chicken PGC lines that grew indefinitely, could be genetically modified, and would transmit through the germline was published in 2006 [4] and was later confirmed by Macdonald et al. [11] and Choi et al. [12].

The existence of two distinct stem cell lines, somatically committed ESCs and germline committed PGCs, has been shown only in chickens and therefore, these cells are a unique (and unexploited) resource for developmental biologists interested in the mechanisms separating the somatic and germline lineages. Both cell lines can be genetically modified. In the case of the ESCs, the genetic modifications are expressed in chimeric animals [13]. In the case of PGCs, the genetic modifications are expressed in the offspring of chimeras [4, 11, 14–16]. Here we show the relationships between parameters of the cells in culture, the ability of the cells to colonize the germline of chimeras during embryonic development, the likelihood that they will contribute to the germline, and surrogate measures of germline transmission derived from expression of green fluorescent protein (GFP) incorporated into transgenes.

MATERIALS AND METHODS

Birds

All the animal studies were approved by the Director-General of the Management of Nature Conservation, Department of the President's Affairs. All the experiments using birds were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Science Societies, 3rd edition.

Chickens Expressing GFP

The embryos used in these studies were from a line of chickens expressing GFP. This line was produced at Crystal Bioscience by deriving PGCs and transfecting them as described by van de Lavoir et al. [17]. This GFP line was designated as 165-2. Transfected cells were expanded and injected into recipient embryos to create G0 chimeras. Mating G0 chimeras to wild-type hens produced G1 founder animals from which the line of birds expressing GFP was established.

Deriving PGCs and Gonocyte Lines

Culture medium. The germ cell culture medium consisted of KnockOut DMEM (Invitrogen), 40% buffalo rat liver (BRL)-conditioned KnockOut DMEM [4], 7.5% fetal bovine serum, 2 mM GlutaMax, 1× nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 1% Pen-Strep (all from Invitrogen), 2.5% chicken serum (Sigma), and supplemented with 6 ng/ml recombinant murine SCF (rmSCF) and 4 ng/ml recombinant human FGF (rhFGF) basic (R&D Systems).

Retrieval of PGCs from the germinal crescent of GFP-positive embryos. Fertilized eggs were incubated for 30 h until stages 6–8 (H&H) before germinal

TABLE 1. Derivation of chicken PGC and gonocyte lines from germinal crescent and gonad, respectively.[a]

Source[b]	Sex of embryos	No. of embryos	No. of male lines (% efficiency)[c]	No. of female lines (% efficiency)[c]
Gonad	Male and female	83	4 (10)	2 (5)
Gonad	All female	21	–	1 (5)
GC	Male and female	51	8 (32)	2 (8)
GC	All female	16	–	4 (25)

[a] A culture is determined to be a cell line if it is made up of a minimum of 2×10^6 cells derived from a single embryo.
[b] GC, germinal crescent.
[c] To obtain the efficiency of cell line derivation when both male and female embryos were used, a 1:1 sex ratio was assumed.

crescents were collected. Briefly, after removing the egg white, the GFP-positive embryo was positioned on the top of the yolk. A drop of 0.2% trypan blue was injected under the embryo, to visualize the position of the embryo. A filter paper ring was placed on the embryo with the anterior part of the embryo inside the ring. The paper ring with part of the embryo attached to it was removed from the yolk and placed in PBS. The germinal crescent was excised from the embryo under a stereo microscope using a pair of 29-gauge syringe needles. The germinal crescent was put in 200 μl of germ cell culture medium and gently pipetted before being transferred to a 48 well containing 10^5 irradiated BRL feeder cells. Feeder cells were irradiated prior to use by exposure to 12 000 rads.

Retrieval of gonocytes from gonads of GFP-positive embryos. Fertilized eggs were incubated for 6–7 days, and gonads were isolated from stages 28–30 (H&H) embryos. Pairs of gonads from GFP-positive embryos were individually transferred to 300 μl of PBS, and 100 μl of 0.25% trypsin/ethylenediaminetetraacetic acid was added and incubated at 37°C for 10 to 15 min. After adding 300 μl of media, individual gonads were triturated, centrifuged at $400 \times g$ for 5 min, resuspended in 400 μl of PGC culture medium and plated into one well of a 24-well plate. After 3–4 h, most of the somatic cells attached, and the floating cells containing gonocytes were transferred to 48 well plates containing a feeder layer of irradiated BRL cells.

Derivation of cell lines. Both PGC lines and gonocyte lines were derived and cultured by modifying the protocols described by van de Lavoir et al. [4] using a feeder of irradiated BRL. In germinal crescent cultures, PGCs became apparent after 3–4 days, and yolk materials disappeared after 7–10 days. In the gonadal cultures, gonocytes were apparent immediately after the start of culture although many somatic cells were also present. Cultures were passaged every 2–3 days, and at each passage, the number of somatic cells decreased and pure cultures of PGCs or gonocytes were obtained in 1–2 wk. When enough cells were present to obtain an accurate count, PGCs and gonocytes were passaged and seeded at 125 000 cells/ml. All the cultures were grown at 37°C and 5% CO_2.

PCR Sexing of Embryos and Cell Lines

Genomic DNA was prepared from embryonic tissue and blood, sperm, and cultured cells using the alkaline lysis method [18] or using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instruction. The PCR conditions for sex determination and eGFP (enhanced GFP) identification were: a total volume of 15.0 μl PCR mixture containing 50–100 ng of DNA template, 0.5 μM of each primer, and 7.5 μl 2× PCR Master Mix (Fermentas Life Sciences) containing 0.05 units/μl Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, and 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP). The mixture was amplified in a thermo cycler (Gene Amp PCR System 9700; Applied Bio Systems) using the following conditions: one cycle of 95°C for 4 min, 40 cycles of 95°C for 30 sec, 58°C for 35 sec, and 72°C for 40 sec, and one cycle of 72°C for 10 min. Fifteen microliters of PCR products were subjected to electrophoresis using 1.5% agarose gel containing ethidium bromide at 100 V for 1 h, and the resolution of the bands was observed under ultraviolet light.

The primers used for sex determination were USP1, 5′-CTA TGC CTA CCA C (A/C) T TCC TAT TTG C-3′, and USP3, 5′-AGCTGGA (T/C)TTCAG (A/T)(C/G)CATCTTCT-3′, to amplify a nonrepetitive region in W chromosome from females resulting in a 380-bp product. For eGFP, the primers were eGFP-F1, 5′-AAG GAC GAC GGC AAC TAC AAG ACC-3′, and eGFP-R1, 5′-CTT GAT GCC GTT CTT CTG CTT GTC-3′, which yielded a 180-bp product. The primers used were actin-F3, 5′-ATG CGC ATA AAA CAA GAC GAG ATT-3′, and actin-R3, 5′-GGG GAC TGT AAA GCC TTC ATT CAC-3′, which amplify a region in the β-actin housekeeping gene used as internal controls for both sex determination and eGFP. The product size was 446 bp [19].

Testing the Germline Transmission Potential of Cultured PGCs and Gonocytes

Production of chimeras. PGCs or gonocytes were collected and resuspended in manipulation medium (CO_2-independent medium [Invitrogen], 10% fetal bovine serum, and 1% Pen/Strep) at a concentration of 3000 cells/μl. One microliter was injected into the dorsal aorta of stages 14–15 (H&H) chicken embryos through a 1 cm diameter window in the sharp end of the egg. The window was sealed by egg albumin and cling film (Majid Plastics). The injected eggs were set sharp-end down and incubated at 37.5°C, 60% humidity until they hatched.

Evaluation of PGC colonization in gonads. Putative chimeric chickens were sexed by PCR after hatching, and all the mixed sex chimeras and some same sex chimeras were euthanized to evaluate colonization. Chicks were euthanized within 1 wk after hatching, and their gonads were collected and then microscopically evaluated for the presence of GFP-positive spermatogonia or oogonia. The contribution of GFP-expressing cells in the germline was assigned a score from 0 to 100.

Identifying germline chimeras. To evaluate the functionality of cultured PGCs and gonocytes, semen was collected from the roosters injected with male cells, and the presence of GFP DNA was evaluated by PCR. From the roosters that were positive for GFP in semen, a cohort was selected and used to artificially inseminate wild-type hens. For the female cell lines, all the hens injected with female cells were inseminated with wild-type semen. Eggs from the germline mating were incubated for 7 days, and the embryos were screened for GFP using a fluorescent excitation lamp (BLS Ltd.).

RESULTS

Derivation of Cell Lines

During the first week after isolation, the cultures typically grow well, irrespective of their origin. At 7–10 days, the rate of cell division in most cultures diminishes. Some cultures never recover while others adjust to the culture system and continue to proliferate. Generally, female PGCs divide at a slower rate than male PGCs and tend to form tight clumps, which seem to have a negative impact on their proliferative capacity. A culture of male cells was considered to be a good grower when it moved to a 24-well plate within 2 wk and then moved to a 12-well plate (around 100 000 cells) between 2 and 3 wk. Except for G23, which reached the 12-well stage at 2 wk, all of our female cultures took at least 3–6 wk before they were transferred to a 12-well plate. Although some cultures reached the 12-well stage within the ideal range of 2–3 wk, they did not continue to proliferate.

In our laboratory, a culture is granted cell line status when it is made up of at least 2×10^6 cells derived from a single embryo. While the precise minimum number of cells in a cell line is arbitrary, when a culture reaches 2×10^6 cells, it can be grown and expanded for an unlimited period of time, frozen for future use and thawed, or used for genetic manipulation. Table

TABLE 2. Efficiency of putative chimera production with male and female PGC and gonocyte lines.

Cell lines	No. of embryos Injected	Dead E7–E10 (%)	Dead E15 (%)	No. of embryos to hatcher	No. of chicks hatched	Hatch (%)	Hatch/injected embryos (%)
Male	366	35 (10)	27 (7)	304	226	74	62
Female	506	56 (11)	27 (5)	423	378	89	75

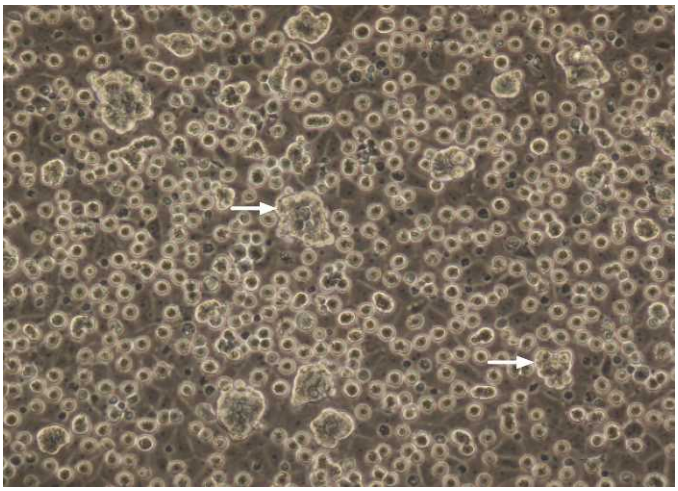

FIG. 1. Female PGC line G87 at 50 days in culture. Note the single cells and the clumps (arrows) that are very typical for a female culture. The cells are grown on a feeder of irradiated BRL cells. Original magnification ×100.

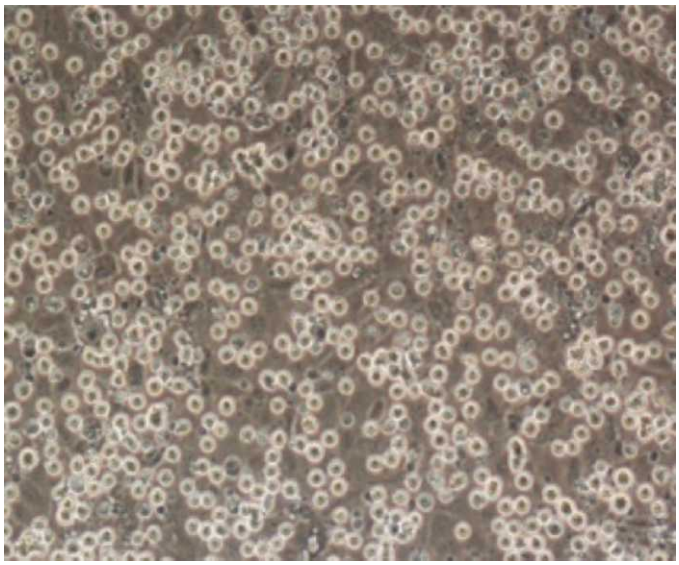

FIG. 2. Male PGC line G32 at 26 days in culture. Note the individual nature of the cells. The cells are grown on a feeder of irradiated BRL cells. Original magnification ×100.

1 shows the efficiency of cell line derivation from gonads and the germinal crescent. While the frequency of generating cell lines from germinal crescents appears to be higher than the frequency from gonads, the data is confounded because the gonocyte and PGC cultures were derived by different people.

Culture Morphology

The morphology of PGC lines and gonocyte cell lines is shown in Figures 1 and 2. In both cases, the cells are round and unattached. There is a difference between male and female cells irrespective of the source. In general, the male cultures are made up of single cells whereas the female cultures generally contain clumps of cells. These clumps can sometimes be dispersed by trituration, but at other times the clumps are too tight to be dispersed.

Efficiency of Chimera Production

The injected embryos were evaluated by candling between Embryonic Day 7 (E7) and E10 and at E15 before being transferred to the hatcher. The efficiency of hatching (Table 2) was 62% and 75% of all eggs injected for male and female cell lines, respectively.

Developmental Potential of Male Cell Lines

The developmental potential of four male cell lines was evaluated within 1 wk of hatching and again at sexual maturity. The early evaluation was conducted by assigning a subjective score following microscopic evaluation of GFP-positive cells in the male and female gonads of chimeras. Two cell lines were derived from the germinal crescent (G32 and G39) and two were derived from the gonads (S48 and S54). Figure 3 shows the colonization for the four male cell lines. The distribution of colonization frequencies was broad for both cell types (Fig. 3), ranging from very low (0%–9%) to very high (>75%). In the figures, we have pooled the embryos that had no colonization with the embryos that had very poor colonization (up to 9%). Often only a few GFP-positive cells were seen, and although cells had colonized the germline, for practical purposes, the colonization is zero. Table 3 shows the number of gonads that had no colonization and the ones that had very poor (i.e., <10%) colonization.

Table 3 lists the number of embryos evaluated for colonization and the number of roosters tested for the presence of GFP in their semen. GFP could be detected by PCR in semen from most chimeras although GFP-negative birds were detected in three of the cell lines. These data are consistent with the observation that some chimeras have very low rates of incorporation of the donor cell line (Fig. 3).

Table 4 lists the rate of germline transmission for males from which GFP was identified in semen by PCR following in ovo injection with the male cell lines. Every bird identified as a carrier of the donor cell line by PCR transmitted through the germline although the rate varied from 3% to 85%. There were no obvious differences between the rates of germline transmission for PGC and gonocyte cell lines. Because the rate of germline transmission in at least one member of the cohort of G0 chimeras from each cell line is greater than 10%, founders can be derived from each source with equal ease.

TABLE 3. Evaluation of chimerism in gonads and sperm after injection of male cells into male recipients.

			Colonization				
Cell line	Source[a]	Total gonads evaluated	0	<10%	No. of roosters evaluated	Semen negative by PCR (%)	Semen positive by PCR (%)
G32	GC	40	3 (7.5)	12 (30)	17	1 (6)	16 (94)
G39	GC	41	6 (15)	11 (27)	26	8 (30)	18 (70)
S48	Gonad	52	3 (6)	6 (12)	25	4 (16)	21 (84)
S54	Gonad	15	0 (0)	2 (13)	8	0 (0)	8 (100)

[a] GC, germinal crescent.

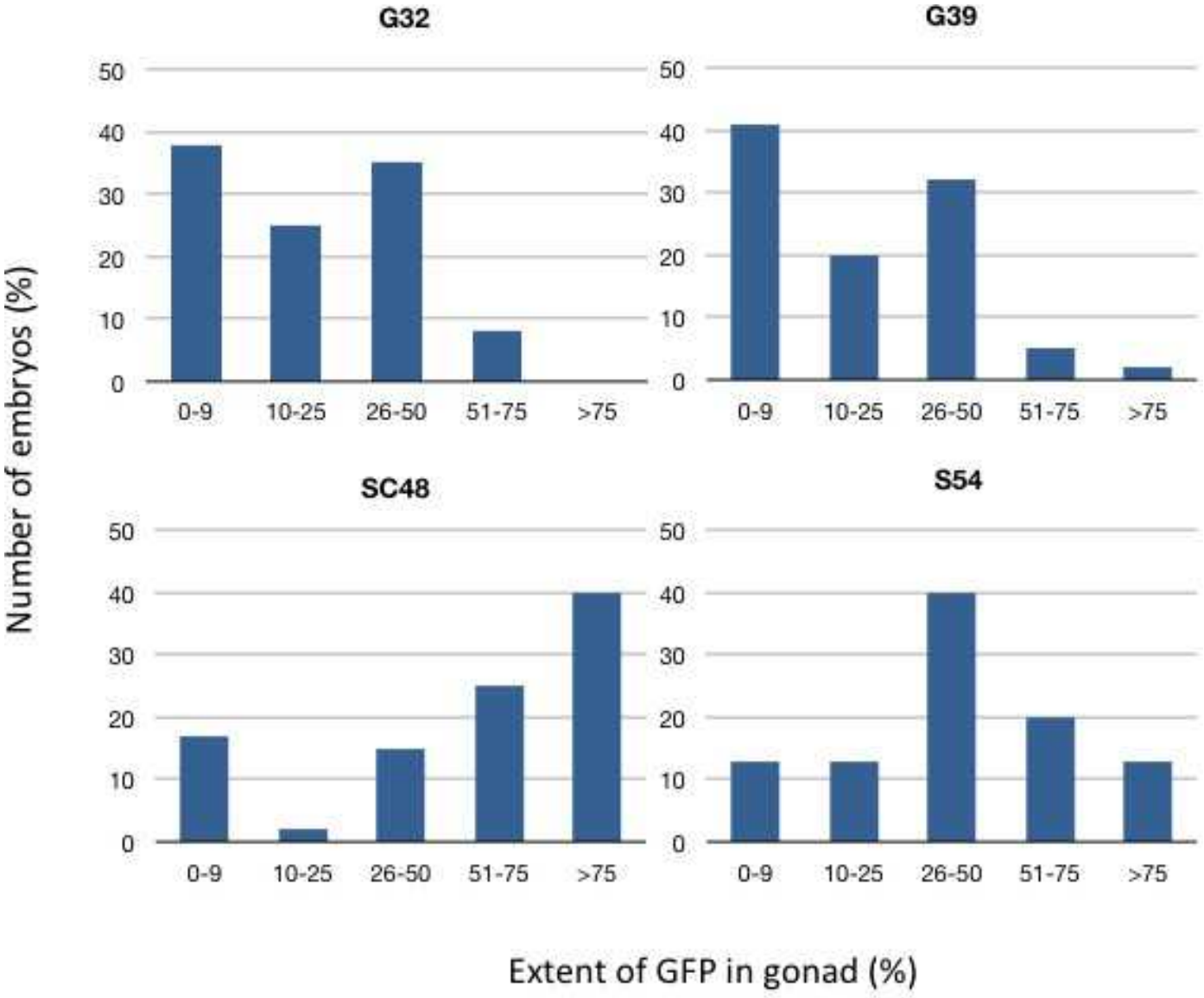

FIG. 3. Colonization of male and female gonads by GFP-positive male germ cell lines. The evaluations were made within 1 wk after hatching.

Developmental Potential of Female Cell Lines

The contribution of female cell lines to the germline was evaluated within 1 wk of hatching by assigning a subjective score following microscopic evaluation of the male and female gonads of chimeras. Three cell lines were derived from the germinal crescent (G23, G57, and G87) and three were derived from the gonads (S3, S122, and K23). Each cell line contributed to the germline, although the rate of incorporation ranged from low to high for both PGCs and gonocytes (Figs. 4 and 5).

Rates of germline transmission were established for the six female cell lines by mating to wild-type males and observing the phenotype of the offspring. In contrast to male cell lines, which were screened for the presence of GFP in semen, all the hens were selected to establish rates of germline transmission for female cell lines. Hence, it is not possible to compare the rates of germline transmission of male and female cell lines. Although some hens injected in ovo with cells from the six female cell lines transmitted through the germline, there were many hens that never transmitted the donor cell line (Table 5). The rates of germline transmission of the transmitting hens varied from 2% to 100% (Table 6).

DISCUSSION

Growth Characteristics of a Cell Line and Colonization of the Germline

Because not every embryo produces a cell line and not every chimera transmits through the germline (see Tables 1, 4, and 6), it is necessary to start with several embryos, triage cell lines with inappropriate growth characteristics, screen putative males using indirect measures of germline transmission, and test the germline competence of several lines to obtain donor-derived offspring. In general, female PGCs and gonocytes do not grow as well as their male counterparts, irrespective of the origin. Although this paper only shows growth characteristics of germinal crescent PGCs and gonadal germ cells, the same growth characteristics hold true for PGCs derived from the area pellucida (Stage X, EG, and K) and the vasculature (stages 14–16 H&H, data not shown). While the reasons for the sex difference in growth characteristics is unknown, it appears to

TABLE 4. Rates of germline transmission of male cell lines in chimeric roosters selected by PCR for the presence of GFP in semen.

Rooster ID	Injected cell line	Age cell line (days)	No. of eggs set	No. of fertile eggs	Fertility (%)	No. of GFP-positive offspring	GFP-positive embryos (%)	Germline transmission (%)[a]
504	G32	42	140	131	94	3	2	5
513	G32	42	103	99	96	3	3	6
541	G32	49	85	58	68	6	10	21
544	G32	49	148	128	86	29	23	45
545	G32	49	186	169	91	14	8	17
304	S48	70	144	124	86	47	38	76
327	S48	70	144	129	90	12	9	19
369	S48	76	176	157	89	27	17	34
573	S48	63	139	128	92	25	20	39
578	S48	63	121	102	84	7	7	14
344	S54	68	156	135	87	5	4	7
352	S54	68	114	107	94	5	5	9
353	S54	68	93	66	71	1	2	3
363	S54	68	124	114	92	2	2	4
375	S54	68	150	135	90	8	6	12
370	S54	68	138	103	75	3	3	6
629	G39	37	140	128	91	29	23	45
639	G39	37	126	108	86	37	34	69
644	G39	37	191	175	92	74	42	85
690	G39	48	204	183	90	45	25	49
694	G39	48	117	109	93	26	24	48
696	G39	48	145	99	68	37	37	75
710	G39	55	129	126	98	42	33	67
723	G39	55	188	167	89	13	8	16

[a] The donor embryos were heterozygous for GFP, and therefore the rate of germline transmission is calculated by doubling the frequency of embryos expressing GFP.

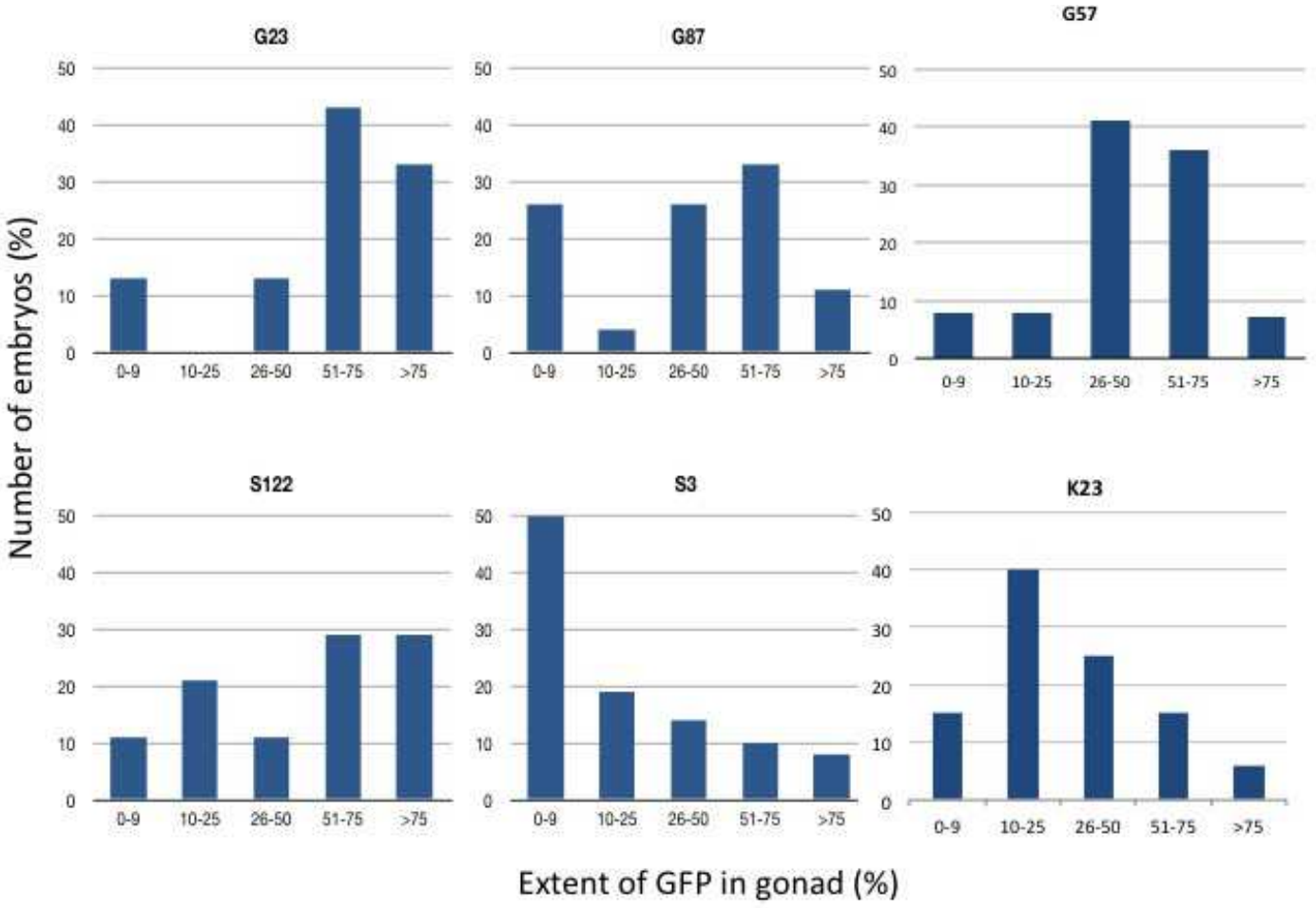

FIG. 4. Colonization of male and female gonads by GFP-positive female germ cell lines. The evaluations were made within 1 wk after hatching.

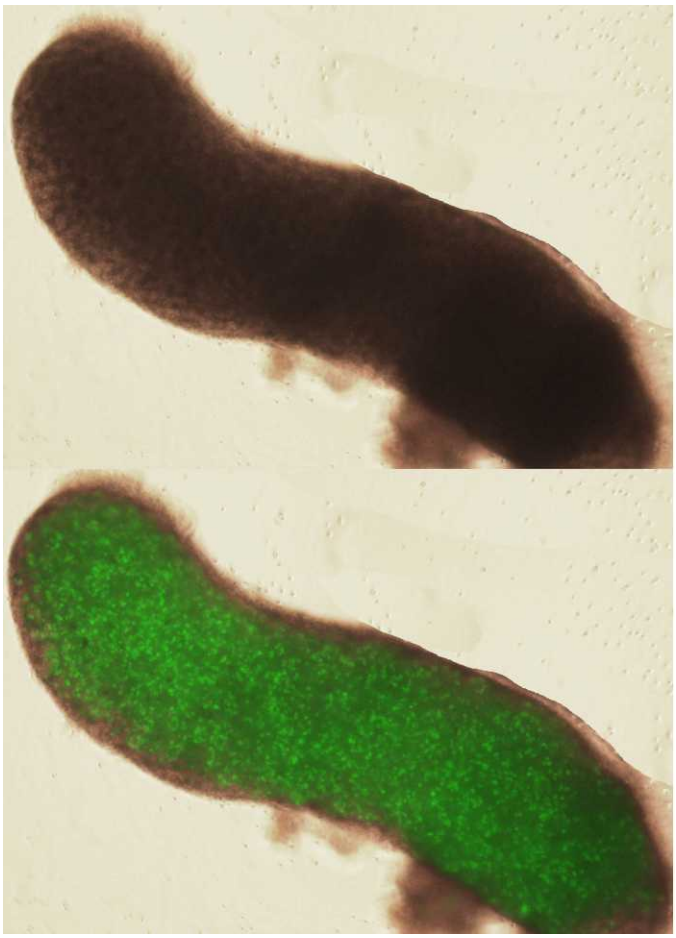

FIG. 5. Extensive colonization of a Day 2 testis by female G23 GFP-positive cells. Top: brightfield image of gonad. Bottom: brightfield image merged with the fluorescent image. Original magnification ×40.

be associated with the presence of clumps of cells in the female cultures that are difficult to separate into individual cells. Because in chickens, male and female germ cells only transmit through the germline of the same sex [4, 20] female cells are needed for certain applications [17]. For most applications, however, male germ cells are preferred because they are easier to grow, males mature more quickly than females, and many more offspring can be obtained by mating one male to several females. Although the range in the rate of germline transmission is similar for males and females, when producing transgenic birds, it is preferable to derive G1 founders from G0 males because the best candidates can be selected by PCR and semen production begins several weeks prior to the onset of egg production.

A cell culture was determined to be a cell line when 2×10^6 cells were obtained. In our experience, when this milestone is met, the cell line can be indefinitely expanded, the cells can be banked, and hence the cell line can be used repeatedly. Up until the milestone of 2×10^6 cells, a cell culture can still fail; several cultures in this study that reached close to 2×10^6 cells could not be maintained. This does not mean that these cell cultures lack the potential to colonize and transmit through the germline. In other studies, we have observed that these cells can transmit through the germline (data not shown), but the lack of robust growth means that these cultures cannot be used for transfections that rely on site-specific integrations or homologous recombinations, which require well over 2×10^6 cells. These cell line qualifications differ from those presented in several publications [12, 21, 22] and contrast with the characteristics of induced pluripotential cells [10], which are predicted but never shown to contribute to the germline. For practical reasons, more rapidly growing cells are preferred although there is no obvious relationship between growth rate of the cell line and the ability of the cells to contribute to the germline. The growth characteristics of the male cell lines that were tested were all very similar. In contrast, the female cell lines showed more variation in proliferative capacity and germline transmission. For example, line G23 showed rapid growth and good germline transmission, line G57 showed slow growth and good germline transmission, and lines K23 and S122 showed rapid growth and poor germline transmission.

Colonization of a Cell Line

Typically, we incorporate GFP into genetic constructs to facilitate estimation of the contribution of the cell line to the germline. In these experiments, we derived cell lines from GFP-expressing embryos. In late stage embryos and newly hatched chicks, GFP is a useful histological marker in the gonads of G0 animals at the time of hatch. Colonization of the gonad provides proof that the cells are PGCs and or gonocytes because germline competent cells are only found in the seminiferous tubules of males and the ovarian cortex of females (Fig. 3). However, the contribution of the GFP-positive PGCs and gonocytes to the gonad of recipients varies widely and does not always correlate to the subsequent rate of germline transmission. For example, the male PGC lines G32 and G39 both grew well, and 10^6 cells were present in the culture within 4 wk. However, approximately 40% of the gonads in embryos injected with these lines showed <10% incorporation, and very few gonads showed incorporation of more than 50%. In the case of G32, the germline transmission rate matched the colonization rate, but for G39, the germline transmission rate was higher than anticipated from the colonization rate. Evaluation of colonization by estimating incorporation of GFP-expressing cells in the gonad is subjective, and in our experience is generally an overestimate

TABLE 5. Evaluation of chimerism in gonads and by breeding after injection of female cells into female recipients.

			Colonization				
Cell line	Source[a]	No. of gonads evaluated	0%	<10%	No. of hens bred	Negative by breeding (%)	Positive by breeding (%)
G23	GC	40	4 (10)	9 (23)	23	9 (39)	14 (61)
G87	GC	27	4 (15)	3 (11)	22	10 (45)	12 (55)
G57	GC	59	5 (8)	0 (0)	17	1 (6)	14 (94)
S122	Gonad	28	3 (11)	0 (0)	17	13 (76)	4 (24)
S3	Gonad	102	18 (18)	32 (31)	21	12 (57)	9 (43)
K23	Gonad	48	5 (10)	2 (4)	19	18 (95)	1 (5)

[a] GC, germinal crescent.

TABLE 6. Rates of germline transmission of female cell lines in chimeric hens.[a]

Hen ID	Injected cell line	Age cell line (days)	No. of eggs set	No. of fertile eggs	Fertility (%)	No. of GFP-positive embryos	GFP-positive embryos (%)	Germline transmission (%)[b]
587	G23	69	28	21	75	2	10	19
584	G23	69	40	34	85	8	24	47
585	G23	69	37	36	97	15	42	83
590	G23	72	85	73	86	5	7	14
595	G23	72	54	54	100	7	13	26
602	G23	74	69	66	96	1	2	3
605	G23	74	66	55	83	1	2	4
619	G23	74	46	43	93	1	2	5
624	G23	74	55	51	93	4	8	16
627	G23	74	72	66	92	6	9	18
609	G23	74	34	34	100	4	12	24
621	G23	74	40	40	100	18	45	90
651	G23	79	46	46	100	4	9	17
664	G23	81	42	27	64	5	19	37
738	S3	111	62	58	94	1	2	3
737	S3	111	55	50	91	1	2	4
751	S3	111	50	47	94	1	2	4
741	S3	111	57	45	79	1	2	4
739	S3	111	75	71	95	2	3	6
731	S3	111	50	48	96	3	6	13
759	S3	113	45	39	87	3	8	15
793	S3	124	34	29	85	1	3	7
819	S3	130	67	54	81	1	2	4
930	G87	37	68	61	90	1	2	3
963	G87	39	63	61	97	1	2	3
943	G87	37	68	43	63	1	2	5
940	G87	37	55	47	85	2	4	9
964	G87	39	73	63	86	3	5	10
936	G87	37	82	75	91	4	5	11
938	G87	37	76	74	97	4	5	11
962	G87	39	51	45	88	3	7	13
922	G87	37	63	46	73	4	9	17
957	G87	39	76	59	78	6	10	20
952	G87	39	21	16	76	2	13	25
958	G87	39	58	47	81	11	23	47
Y867	S122	61	98	97	99	1	1	2
Y885	S122	69	102	71	70	1	1	3
Y860	S122	56	66	62	94	2	3	6
Y859	S122	56	75	60	80	3	5	10
3611	G57	37	47	37	79	6	16	32
3626	G57	37	41	35	85	5	14	28
3609	G57	37	33	29	88	4	14	28
3670	G57	37	59	49	83	15	31	62
3664	G57	37	58	36	62	13	36	72
3612	G57	37	42	25	60	3	12	24
3578	G57	37	65	48	74	13	27	54
3666	G57	37	17	15	88	1	7	14
3607	G57	37	32	27	84	14	52	100
3627	G57	37	58	48	83	6	13	26
3568	G57	39	26	24	92	8	33	66
3669	G57	39	50	48	96	13	27	54
3655	G57	39	46	33	72	6	18	36
3657	G57	39	52	23	44	6	26	52
3662	G57	39	47	44	94	12	27	54
3623	G57	39	57	51	89	2	4	8
Y931	K23	49	95	95	100	2	2	4

[a] Females that did not produce donor-derived offspring are not included in this table.
[b] The donor embryos were heterozygous for GFP, and therefore the rate of germline transmission is calculated by doubling the frequency of embryos expressing GFP.

in male gonads and an underestimate in female gonads because the germ cells are located in the cortex, which can be hard to visualize. To evaluate the colonization of G39 and other male cell lines, the female chicks were euthanized at hatch and their ovary was evaluated. Hence, the rate of germline transmission was probably underestimated in the male cell line G39 and was probably overestimated in the female cell lines G87 and G23. However, it is unlikely that overestimation of colonization in the gonadal female cell lines is the only reason that the frequency and rate of germline transmission was low. Experience has shown that cell lines with contributions of less than 10% in all peri-natal animals have low to undetectable levels of germline transmission in sexually mature birds, and therefore, we typically discard cell lines whose colonization does not surpass this level. Within a cohort of G0 chimeras made with germline competent cells, it is not uncommon to observe some animals without any contribution from the donor cells. In the absence of better information, these cases are

typically attributed to a faulty injection. However, in this study, the percent of chimeras that did not transmit through the germline was much higher in female chimeras compared to male chimeras. It is possible that the tendency for female cells to aggregate impairs the migration of individual cells to the gonad. However, considering that female cells behave differently in cell culture, there might be other biological reasons for the reduced developmental potential of female germ cells.

Germline Transmission

As shown in Table 4, the rates of germline transmission of male chimeras selected on the basis of GFP in semen samples ranges from 3% to 85%. In females, which cannot be preselected, about one half of the birds did not transmit through the germline (Table 5) and the rate of germline transmission varied from 2% to 90% (Table 6). Therefore, the final selection of males based on the presence of GFP in semen appears to be an effective screening tool. Although the range in the rate of germline transmission is similar for males and females, it is preferable to derive G1 founders from G0 males because the best G0 candidates can be selected by PCR and semen production begins several weeks prior to the onset of egg production.

Gonadal cell lines S48 and S54, colonized better than the germinal crescent lines but their germline transmission rate was lower than that of the germinal crescent line G39. S54 is noteworthy because all the gonads and all the sperm samples analyzed were positive for GFP. However, the germline transmission rates were only 4% to 12%. Possible explanations of this observation are either genetic drift and/or epigenetic changes within the culture that impair the ability of the cells to differentiate into functional sperm.

The success rate of establishing cell lines was higher when the germinal crescent was the starting tissue. Also, the germline transmission frequency and rates were better in the lines derived from the germinal crescent than in the gonadal lines. The difference was especially pronounced in the female lines. However, the cell lines from the germinal crescent and from the gonads were derived by different people and therefore, we cannot conclude that the tissue of origin influences the efficiency of derivation and developmental potential of a germ cell line.

Germ cells freshly isolated from the gonad have been shown previously to contribute to the germline after injection into recipient embryos [23, 24]. This study shows that these gonocytes can retain their potential in culture and contribute to the germline.

Unlike ESCs, which make extensive contributions to somatic tissues [3], and induced pluripotential cells, which make meager contributions to somatic tissues [10], PGCs do not contribute to somatic tissues. Their developmental fate can be altered by changing the culture conditions and creating embryonic germ cells, which contribute exclusively to somatic tissues [4]. The complete switch from commitment to the germline to commitment to somatic tissues is a unique developmental model in the search for a molecular understanding of the earliest events in vertebrate development.

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