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High-grade transgenic somatic chimeras from chicken embryonic stem cells

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Abstract

Male and female embryonic stem (ES) cell lines were derived from the area pellucidae of Stage X (EG&K) chicken embryos. These ES cell lines were grown in culture for extended periods of time and the majority of the cells retained a diploid karyotype. When reintroduced into Stage VI–X (EG&K) recipient embryos, the cES cells were able to contribute to all somatic tissues. By combining irradiation of the recipient embryo with exposure of the cES cells to the embryonic environment in diapause, a high frequency and extent of chimerism was obtained. High-grade chimeras, indistinguishable from the donor phenotype by feather pigmentation, were produced. A transgene encoding GFP was incorporated into the genome of cES cells under control of the ubiquitous promoter CX and GFP was widely expressed in somatic tissues. Although cES cells made extensive contributions to the somatic tissues, contribution to the germline was not observed.

Keywords: Chicken; Avian; Transgenesis; Embryonic stem cells

1. Introduction

The ability to culture and genetically modify murine embryonic stem (ES) cells changed developmental biology from a largely descriptive science based on anatomy to an analytical science based on the tools of molecular biology. Among mammals ES cells have been available only in the mouse despite considerable efforts to obtain similar cell lines from cows, pigs, sheep, rats, rabbits and mink (Gardner and Brook, 1997; Prelle et al., 1999). With the exception of cultures of zebrafish embryo cells that possess ES cell like characteristics (Fan et al., 2004; Ma et al., 2001), embryonic stem cells have not been isolated from other vertebrates. The chicken is a particularly attractive animal from which pluripotential cells might be derived because it is a premier model in developmental biology (Stern, 2005) and because it has great potential for the production of pharmaceutical and industrial proteins in eggs (Ivarie, 2003; Lillico et al., 2005; Zhu et al., 2005).

Chicken blastodermal cells, retrieved from the area pellucida of Stage X (EG&K) embryos, contribute to all somatic tissues and the germline when transferred to the subgerminal cavity of a Stage X (EG&K) recipient embryo (Carsience et al., 1993; Kagami et al., 1995; Petitte et al., 1990) implying that at least some cells within this population have the ability to enter the germline. Area pellucidae, therefore, have been used as a starting cell population for the derivation of chicken embryonic stem (cES) cells. Although ES cell lines have been reported in the chicken, their use has not become widespread because their pluripotency after long-term culture has not been established (Etches et al., 1996; Pain et al., 1996; 1999; Petitte et al., 2004; Swanberg et al., 2004). Here, we report cell lines, derived from the area pellucida of Stage X (EG&K) embryos, that grow indefinitely, can be genetically modified, and express the genetic modification after contributing to the somatic tissues of chimeras.

2. Results

2.1. Morphology and growth of cES cells

Chicken ES cells became visible approximately 1 week after seeding the blastodermal cells. The cells were small with a large nucleus and a pronounced nucleolus (Fig. 1) and

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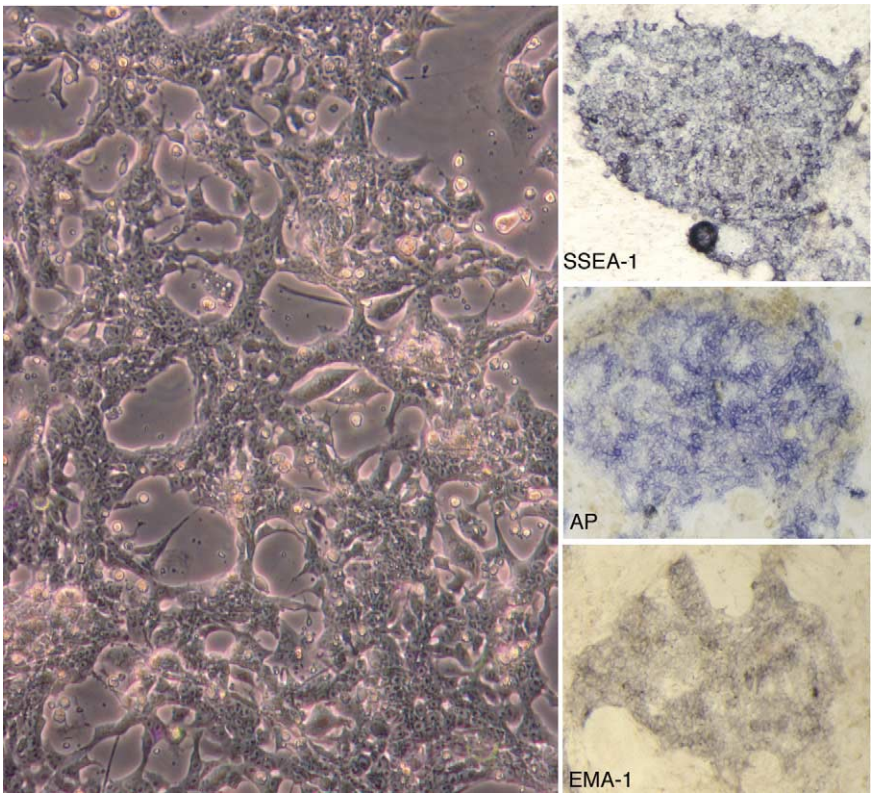

Fig. 1. Chicken embryonic stem cells in culture. Note the small size, large nucleus and prominent nucleolus. cES cells stain positive for the SSEA-1 and EMA-1 antigens, and for alkaline phosphatase.

grew in single layers with clearly visible individual cells. From 78 embryos that were seeded individually, 16 cell lines were grown to a sufficient cell number for cryopreservation and further expansion. Four cell lines were derived out of 30 cultures initiated on a 0.33 cm^2 insert; 3 out of 14 initiated on a 1.0 cm^2 insert; 3 out of 19 initiated on 0.33 cm^2 inactivated STO feeder cells and 6 out of 15 initiated on 1.0 cm^2 inactivated STO feeder cells. Of the 16 cell lines that were established, 10 were male and 6 were female. Chicken ES cells could not be maintained when seeded at lower densities ($<$40% confluency) and, therefore, the cells were grown to 80–100% confluency before they were passaged in a 1:2 ratio. Usually, cultures were passaged daily. To maintain optimum growth and to prevent differentiation, the cells were passaged by transferring 30–50% of the medium covering the cells into the new well. The cells were dispersed using either a short trypsin (0.25%) wash or a 1–2 min incubation in Ca/Mg free PBS and passaged in small clumps. As with mouse ES cells, cES cells were grown on a STO feeder layer but the concentration of the feeder layer needed to be lower (10^4 cells/cm^2 for cES cells vs 4×10^4 cells/cm^2 for mES cells). A dense feeder of STO cells negatively impacted cES cells' proliferation and no other feeder layer has been identified that supports growth of cES cells (data not shown). Although DMEM supported the growth of cES cells, the use of Knockout DMEM increased the growth rate, making it easier to obtain sufficient cells for transfection.

2.2. Chicken ES cells express markers of pluripotency

The cES cells were positive for telomerase (Fig. 2A), which is characteristic of immortal cell lines. The cES cells stained for the pluripotency markers SSEA-1, known to be expressed on mouse EC and ES cells (Solter and Knowles, 1978), EMA-1, which recognizes an epitope on primordial germ cells in mice (Hahnel and Eddy, 1986; Pain et al., 1996), and alkaline phosphatase (AP), which is expressed by mouse, human, and chicken ES cells (Henderson et al., 2002; Pain et al., 1996) (Fig. 1). Both SSEA-1 and EMA-1 are expressed by chicken ES cells after short periods in culture (Pain et al., 1996). The cells expressed ERNI (Fig. 2B), a gene reported to be expressed in cES cells (Acloque et al., 2001).

2.3. Chicken ES cells are diploid

The age of the cells in culture, evaluated karyotypically, ranged from a minimum of 52 days for ES cell line 426 to a maximum of 236 days for ES cell line 50. The proportion of diploid cells in 11 of the lines ranged from 42 to 78% (Table 1). The higher end of the range is close to the 80–85% modal karyotype found in primary chicken embryo fibroblasts (Chang and Delany, 2004). In all cultures the most frequent karyotype was diploid (Fig. 2C) and the most frequent aneuploid condition was monosomy followed by nullosomy. Trisomy was observed in five lines while tetrasomy and pentasomy were

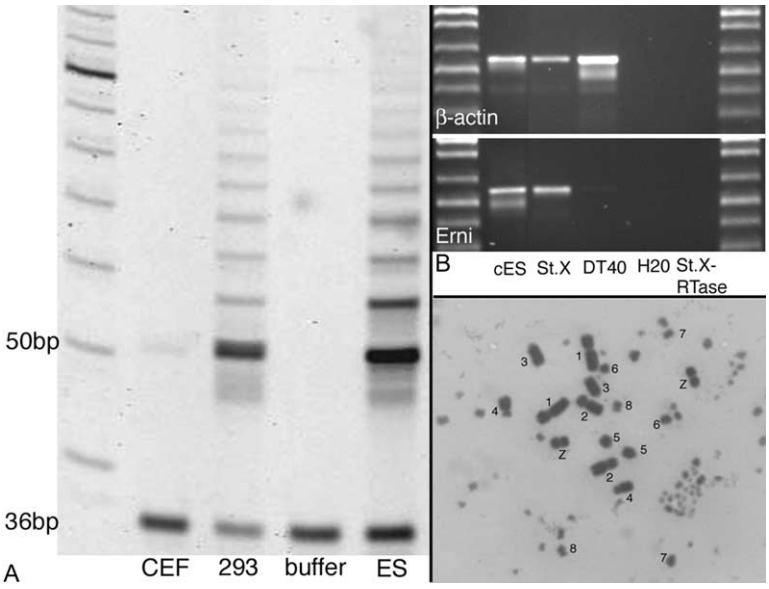

Fig. 2. (A) Telomeric repeat amplification protocol (TRAP) assay. In the lanes representing cES cells (ES) and the positive control (transformed human kidney cell line 293), repeat sequences are visible indicating the presence of telomerase in the cell extracts. In the chicken embryonic fibroblast (CEF) and buffer control lanes, only a 36 bp band representing the internal control PCR template is apparent. (B) ERNI expression in cES cells and DT40 cells as determined by RT-PCR. Expression of ERNI was observed in an aliquot of cES cells and the Stage X embryo while DT40 cells did not express ERNI. (C) Karyotype of a representative cES cell line (CX27J) in culture for 105 days. The macrochromosomes GGA 1-8 and the sex chromosomes (ZZ) are identified.

present in one line. The most frequent aneuploid conditions involved GGA 4 and Z.

2.4. Genetic modification of cES cells

Although cES cells resemble mouse ES (mES) cells at the single cell level, mES cells form compact colonies whereas cES cells spread out in a single, loosely connected layer and individual cells can dislodge during medium changes. To prevent colonies from mixing the cells are seeded into 1 cm^2 wells after transfection to obtain clonally derived colonies.

Table 1
Karyotypic analysis of cES cell lines grown in culture for various periods of time

Cell line[a]	Sex	Days grown in culture[b]	Days since transfection[c]	Diploid %[d]
439	Male	87		72 (36/50)
429	Male	120		73 (40/55)
426	Male	52		64 (34/53)
426	Male	118		60 (24/40)
323/CX27J	Male	105	33	74 (39/53)
323/CX27H	Male	115	45	57 (29/51)
328/UR4B	Male	235	82	78 (31/40)
440	Female	81		77 (39/51)
440/OV42	Female	125	44	56 (28/50)
440/OVH	Female	160	98	70 (37/53)
50/UR6N	Male	236	73	47 (20/43)
50/TB01D	Male	251	69	42 (23/55)

Cells were analyzed for GGA1-4 and the sex chromosomes.
[a] Parental cell line and transfected cell lines derived from the parental line.
[b] Total days cell line has been in culture.
[c] Days cell line has been in culture since the date of transfection.
[d] Percent diploid is calculated as total number of diploid cells per total number of cells examined.

Stable integration of the CX-GFP-Puro transgene into the genome of cES cells was demonstrated by Southern analysis in two cES cell lines (TB01 and TB09) (Fig. 3). In some of the cell lines used to evaluate the potential of cES cells to alter sexual differentiation of chimeras, the CX-GFP-Puro transgene was incorporated as part of a larger (approximately 45 or 150 kb) transgene (data not shown).

2.5. Contribution of cES cells to Stage X (EG&K) chimeras

Chicken ES cells injected into uncompromised Stage X (EG&K) recipient embryos contributed to feather chimerism but the frequency and extent was low. The contribution was increased after compromising the recipient embryos by either coring or irradiation but was maximized when both methods were combined (Table 2). Although coring produced high-grade chimeras it demanded more technical expertise and led to a higher mortality of embryos. The frequency and extent of feather chimerism became more predictable if cES cells were exposed to embryonic diapause following injection. Not only did chicken ES cells remain viable at the 15 °C temperature, they were widely incorporated into the recipient embryo and at hatch the frequency and extent of feather chimerism was consistently high (Table 2). In some cases, chimeric chicks were indistinguishable from pure Barred Plymouth Rock chicks (Fig. 4). By manipulating the method of compromising the recipient, a high frequency and extent of chimerism was maintained independent of the age of the cell lines.

2.6. FACS analysis of somatic tissues

Eighteen chimeras made with ES cells carrying a stably integrated copy of pCX-GFP-Puro were analyzed for the

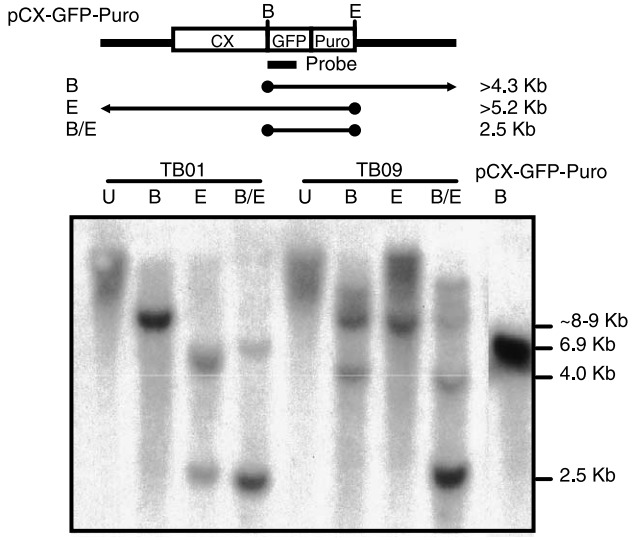

Fig. 3. Southern analysis of cES cell lines transfected with pCX-EGFP-Puro. Genomic DNA from either TB01 or TB09 was digested with BamH I (B) and EcoR I (E) enzymes as indicated. The presence of 8–9 kb DNA bands in B digestion of TB01 and in B digestion and E digestion of TB09 suggests stable integration of the transgene. The origins of the 2.5 kb band in E digestion of TB01 and the 4 kb band in B digestion of TB09 are unknown and possibly due to the presence of additional copies of the transgene. The pCX-EGFP-Puro digested with B was used as a positive control and a reference of the size of the transgene (6.9 kb). U, uncut; B, BamH I; E, EcoR I.

contribution of GFP-positive cells to selected tissues. The fraction of cells in each tissue sample that was fluorescing above a set level of autofluorescence is listed in Table 3. In general, chimeras with extensive, donor-derived feather pigmentation have higher proportions of GFP expressing cells in muscle, liver, and brain. In some cases, however, the correspondence between the estimates of cES cell contribution to somatic tissues by feather pigmentation and FACS analysis was low, indicating that recruitment of donor and recipient cells into lineages during differentiation was random. The observation that cES cell derived melanocytes can populate the majority (if not all) of the feather follicles was corroborated by FACS analysis of brain samples, which revealed that in some chimeras more than 90% of the cells were expressing GFP.

2.7. Contribution of cES cells to Stage IV–IX (EG&K) embryos

After injection of arginine vasotocin, 90% of the hens laid uterine eggs containing embryos from Stage IV to IX (EG&K). All of the hens returned to their normal laying pattern within 48 h. Irradiation and coring of Stage IV–VI (EG&K) embryos reduced viability to impractical levels ($<1\%$ hatchability), whereas Stages VII–IX (EG&K) embryos were less sensitive to compromising. Hatchability was reduced from 44% (11/25) in non-compromised recipients to 28% (31/109) after exposure to 660 rad of gamma irradiation and to 35% (6/17) after coring. For chimera production, irradiation was more effective than coring, approximately doubling the proportion of somatic chimeras to 86% (49/57) and increasing the average extent of feather chimerism from 32 to 43%. Low viability prevented evaluation of exposure to the embryonic diapause at 15 °C for Stage IV–VI (EG&K) recipients and needed to be reduced to 1–2 days for Stage VII–IX (EG&K) embryos. Although a high proportion of embryos was chimeric, the average extent of chimerism was lower than that of chimeras made with Stage X (EG&K) recipients (43 vs 67%).

2.8. Sex conversion

To determine if female (ZW) recipients could be converted into males by the presence of large populations of male (ZZ) cES cells, phenotypic males made from male ES cells were analyzed at 4–5 weeks of age for the presence of the W-chromosome Xho I repeat. Of the 29 phenotypic males, 22 exhibited more than 80% down pigmentation at hatch. The W-chromosome was not identified in any of the males indicating that sex conversion had not occurred. Of 55 female chimeras injected with male ES cells and with an average extent of $\geq 65\%$ black down feathers at hatch, 20 developed male secondary sex characteristics during sexual maturation. At the time of euthanasia these birds had well developed testes. During histological examination of the testes of 14 birds, all of the Sertoli cells appeared to be donor derived (i.e. were expressing GFP), whereas donor derived germ cells were not observed (Fig. 5). Although these birds had well developed testicular components in their gonads, their Wolffian ducts had atrophied during development and their oviducts were retained. Of the 35 birds that retained a female phenotype, 15

Table 2
Frequency of somatic chimerism after injection of cES cells into recipient embryos compromised by different methods

Treatment to compromise the recipient embryo	Time cells in culture	Embryos injected (*n*)	Embryos and chicks evaluated (*n*)	Chimeras (*n*)	Frequency of chimerism[a] (%)	Extent feather chimerism[b] (%)	Chicks > 65% black feathers (%)
None	4–106 days	764	347	83	24	26	17 (20)
Mechanical removal of cells	6 months	128	65	34	52	26	3 (9)
Irradiation	6–7 months	316	124	71	57	39	14 (20)
Irradiation and mechanical removal of cells	7–9 months	243	74	61	82	65	35 (57)
Irradiation and 4–7 days in embryonic diapause	40–214 day	2753	978	761	78	67	472 (62)

[a] Percentage of evaluated embryos that were chimeric.
[b] The average extent of feather chimerism of all chimeras evaluated.

Fig. 4. Two Barred Plymouth Rock chicks and two chimeric chicks produced by injecting Barred Plymouth Rock ES cells into irradiated White Leghorn recipients. The chimeric chicks are indistinguishable from the Barred Plymouth Rock chicks indicating extensive feather chimerism. The cells had been in culture for 250 days.

exhibited testicular components in the gonads. During histological examination of gonads from five high-grade male chimeras made with female (ZW) cells expressing GFP, no GFP-expressing Sertoli cells were observed. These data suggest that differentiation into Sertoli cells is restricted to cells with a ZZ genotype.

2.9. Contribution of cES cells to the germline

A total of 11 different cell lines, representing five parental cell lines, in culture from 36 days to 8 months, were analyzed for germline transmission. Chimeras were produced from Stage VI–X (EG&K) embryos using various methods of compromising the recipient embryo (see M&M) designed to maximize the extent of incorporation of cES cells. The proportion of diploid cells in the cell lines that were used to create chimeras for germline testing ranged from 42 to 78% (Table 1). Chimerism levels varied but the majority of roosters that were mated to determine germline transmission were high-grade chimeras as judged by feather pigmentation (Tables 4 and 5). After evaluating more than 54,000 embryos and chicks, cES cell derived offspring, as determined by black down, were not obtained from any of the roosters produced from ninety Stage X (EG&K) embryos (Table 4). Twenty-nine chimeric hens, all from parental line 50, were also evaluated and ES cell derived offspring were not observed among 1940 offspring (data not shown). Approximately 5000 offspring were screened from eleven male chimeras produced from pre-oviposition embryos (Table 5) and a further 364 offspring were obtained from 9 female chimeras (data not shown), but germline transmission was not observed.

3. Discussion

This is the first report of a pluripotent chicken cell line that can be routinely genetically modified, grows indefinitely in culture and contributes to somatic tissues after more than 9 months in culture. Here, we show stable incorporation of CX-GFP-Puro, a relatively small transgene that is expressed ubiquitously. We have also produced chicken ES cell lines that carry large (e.g. 45–150 kb) transgenes that direct tissue specific and developmentally regulated gene expression (Zhu et al., 2005). These technologies represent a significant advancement over transfecting ES cell lines with promoter trap vectors (Acloque et al., 2001) or viral constructs that are limited to transgenes comprising 8 kb or less (Chapman et al., 2005; McGrew et al., 2004). In many applications, it is desirable to create lines of birds that express a specific phenotype through germline transmission of a transgene. However, at least 6 months are required to produce G0 birds, rear them to sexual maturity and obtain the G1 offspring. In contrast, when transgenic cES cells are incorporated into high-grade somatic chimeras expression of the transgene can be evaluated within 8 weeks after the start of transfection. Four weeks are required to obtain a transfected cell line and another 4 weeks are required to produce chimeras for evaluation of expression levels and tissue specificity. At Origen we have used this system to evaluate the expression of constructs in the oviduct and the Bursa of Fabricius of perinatal chicks. In addition adult chimeras can be used to quickly evaluate production of novel proteins in eggs within 5 months. Substantial amounts of protein can be purified from the egg white of chimeric hens allowing characterization of the product of the transgene in less than half the time required using a germline transgenic technology (Zhu et al., 2005).

The lack of contribution to the germline in chimeras made with cES cells contrasts with the ability of chicken blastodermal cells to contribute extensively to the germline, even in low grade chimeras (Carsience et al., 1993; Kagami et al., 1995; Kino et al., 1997) but is consistent with the

Table 3
Analysis of chimeras made with GFP-transfected cES cells

Feather chimerism	GFP-positive liver cells (%)	GFP-positive brain cells (%)	GFP-positive leg muscle cells (%)	GFP-positive breast muscle cells (%)	Number of birds in group (*n*)
Control (0)	1.12	0.38	7.73	12.72	8
<15%	1.3	10.5	10.3	9.42	12
15–40%	7.74	53.07	58.95	21.02	4
100%	40.31	93.66	93.56	89.86	2

Chimeras were grouped according to the range of feather chimerism. The percentage denotes the fraction of cells in each tissue sample that were fluorescing above a set level.

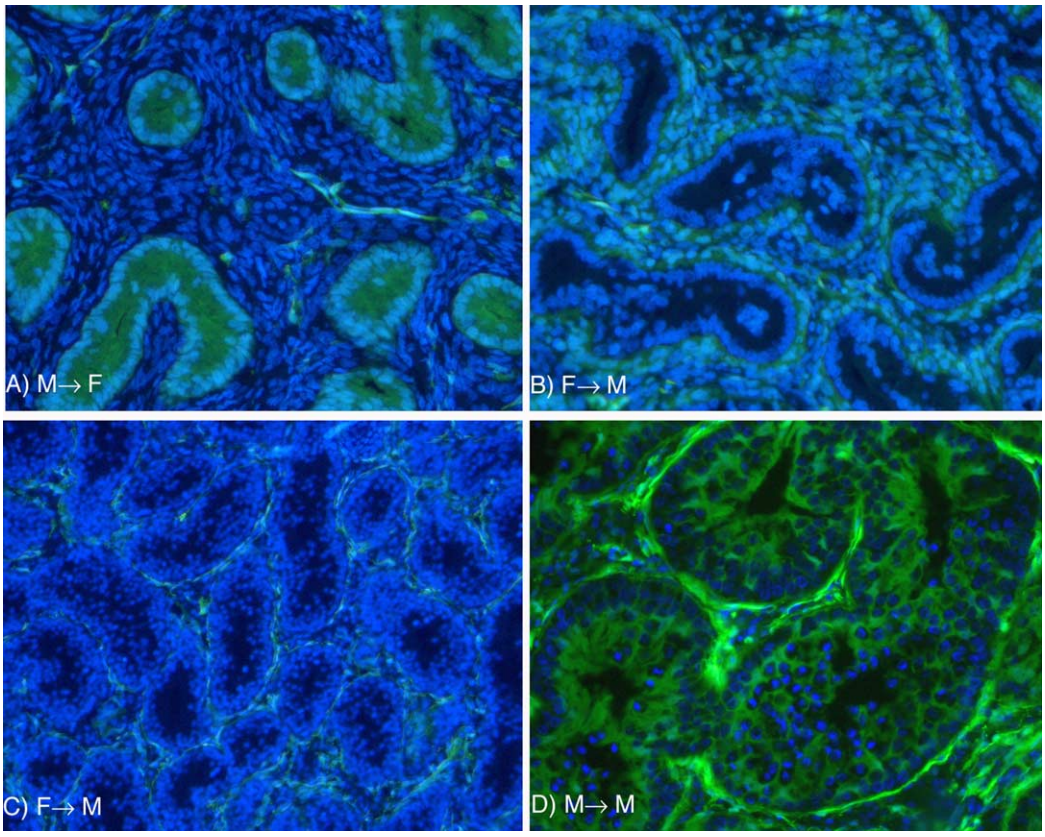

Fig. 5. Testicular sections of chimeras made with male and female GFP-positive cES cells stained with DAPI. (A) Testis from a female chimera showing extensive contribution of the male GFP-positive cES cells to the Sertoli cell population. The non-green recipient female cells contributed to the interstitial cell population but non-green Sertoli cells were not observed. (B,C) Sections through testes of male chimeras made with female cES cells. The female GFP-positive cES cells contribute to the interstitial tissue of the testis but no contribution was observed within the seminiferous tubules of an immature testis (B) or a mature testis (C). (D) Section through a testis from a male chimera made with male cES cells. The GFP-positive male cES cells contribute to both the Sertoli cells within the seminiferous tubules and the interstitial cells.

inability to retain germline transmission of cES cells cultured for more than 7 days (Pain et al., 1996; Petitte et al., 2004). In the mouse, high levels of somatic chimerism and a euploid karyotype are known to maximize the likelihood of obtaining germline transmission (Bradley, 1987). We therefore maximized the contributions of cES cells to somatic tissues using coring and irradiation of recipient embryos. Coring of the area pellucida has been used to produce sterile embryos (Kagami et al., 1997) and, therefore, should reduce the competition between recipient primordial germ cells (PGCs) and PGCs from donor cES cells for entrance into the germline. However, we did not observe germline transmission of the donor cES cells after coring the recipient embryo nor did we observe any sterile animals among the 16 chimeras that were reared to

Table 4
Male chimeras produced from Stage X (EG&K) embryos and tested for germline transmission through breeding

Parental cell line	Transfected line	Sex of cell line	Age cells (days)[a]	# Chimeras tested[b]	Feather chimerism[c] (%)	# Offspring evaluated[d]
50	No	M	180–193	6	51	1147
	Yes	M	201–260	25	81	13,058
323	Yes	M	111–220	21	93	6208
426	No	M	36–50	13	90	13,339
429	No	M	77–111	12	96	13,010
440	Yes	F	151–153	13	92	7211

The number of offspring produced from five different parental cell lines at various times following establishment of the cES cell culture. Germline transmission of the cES cell genotype was not observed.

[a] Age of cES cells at the time of injection into recipient embryos.

[b] Number of chimeras that were selected to be tested for germline chimerism.

[c] The average extent of feather chimerism of all the chimeras that were tested for germline chimerism.

[d] Total number of offspring evaluated to determine germline transmission of the tested chimeras. None of these chicks were derived from ES cell contributions to the germline.

Table 5
Male chimeras produced from Stage VI–IX (EG&K) embryos and tested for germline transmission through breeding

Bird ID	EG&K Stage	Parental cells	Feather chimerism (%)	#Offspring evaluated
HG15-14	VIII	50	87	498
HG17-13	VIII	50	40	419
HG17-15	VIII	50	33	670
245-07	VIII	323	93	349
245-09	VII	323	93	354
245-40	VIII	323	80	350
248-40	VII	323	93	331
271-06	IX	323	80	581
271-15	VII	323	67	242
HG56-04	IX	440	47	414
HG56-15	VIII	440	67	733

sexual maturity and tested for germline transmission following this procedure. The rapid pace of chicken embryonic development (from fertilization to 40,000–60,000 cells within 24 h) provides cES cells injected at Stage X (EG&K) an interval of only a few hours to differentiate from their ES cell state and assume the phenotypic state of a primordial germ cell. It is possible that this transition cannot occur within the short time frame available and, therefore, we increased the exposure of the cES cells to the embryonic environment by keeping the embryo in diapause after injection of the cES cells. By storing the Stage X (EG&K) embryos at 15 °C, embryonic diapause is maintained for several days until incubation. The cES cells remained viable during exposure to embryonic diapause and were able to extensively contribute to the recipient embryo, making it our method of choice for the production of chimeras. Although we were able to produce chimeras that, in some cases, were indistinguishable from the donor Barred Plymouth Rock breed of chicken, germline transmission was not observed. To maximize both the cES cells' incorporation into the recipient embryo and their exposure to the inductive environment in the early embryo, cES cells were injected into Stage IV–IX (EG&K) embryos. However, storage at 15 °C for one or two days was detrimental to embryos before Stage X (EG&K), fewer high-grade chimeras were produced and none of them transmitted the cES genotype through the germline.

The failure to colonize the germline may be an inherent attribute of the cells or it may be a consequence of the very rapid pace at which the germline segregates from the somatic tissues. It is generally believed that the germline is induced between Stage IX (EG&K) and Stage XIV (EG&K) (Karagenc and Petitte, 2000). For cES cells the window to enter the germline is likely closed within 8–10 h following injection, when the hypoblast is fully formed at Stage 2–3 (H&H). By Stage 4–6 (H&H) (18 h after the start of incubation), when the PGCs congregate in the germinal crescent, the germline is clearly an anatomically distinct lineage. This timeline contrasts sharply with that of mouse ES cells, which are exposed to the embryonic environment for 3 days before germline segregation occurs. Since gastrulation is initiated after germline segregation, cES cells have a longer period of time in the embryo before incorporation into the somatic lineages. Slowing the development of the recipient embryo by irradiation and exposing the cES cells to the diapause environment has enabled us to increase the exposure of cES cells to the embryonic environment and maximize the contribution of cES cells to somatic tissues. However, the rapid pace of embryonic development may be an insurmountable barrier preventing colonization of the germline by non-committed cells such as ES cells. The mitotic rate of blastodermal cells is approximately 1 h whereas cES cells divide at 24 h intervals. If several cell divisions are needed for a cell to undergo epigenetic changes that allow it to enter a germline committed fate, it would not be possible for cES cells to enter the germline.

The failure of cES cells to enter the germline may also be due to predetermination of the germline. In Drosophila the germline is already specified in the early cleavage stage embryo and Vasa is one of the genes essential for germ cell formation. The chicken Vasa homologue (CVH) has been reported to be present from conception and subsequently in the germline and it has been suggested that the chicken germline is determined by maternally inherited factors (Tsunekawa et al., 2000). However, it is unknown whether the presence of Vasa in the early chicken embryo is functionally important. Our observation that sterile embryos were not produced following coring, however, is inconsistent with the hypothesis that the germline is predetermined in birds.

A high proportion of cES cells in culture have a normal diploid karyotype and, in combination with our ability to return several thousand cells to recipient embryos, high-grade chimeras were produced from all of the cell lines. The ability of cES cells to contribute to somatic tissues after lengthy periods in culture contrasts with that of mES cells, which yield a lower level of both somatic and germline chimerism as the duration of culture increases (Nagy et al., 1993). However, we observed no change in the ability of the cells to contribute to somatic tissues after more than 200 days in culture (Table 4) but contributions to the germline were not evident, even after short periods (36–50 days) in culture. For example, the 426 and 429 cell lines were chromosomally stable, less than 4 months old and produced high-grade chimeras but they did not contribute to the germline. While the karyotype analysis did not include the entire genome, the proportion of normal cells should be compared to the same analysis of primary embryonic fibroblasts, which shows that 80–85% of the cells are normal (Chang and Delany, 2004). Even if only 10% of the cells were normal, between 500 and 1000 cells with a normal karyotype would be introduced. The same number of freshly collected blastodermal cells yields germline transmission (Carsience et al., 1993). Although it seems unlikely that the inability of cES cells to contribute to the germline is due to the accumulation of genetic defects, it is theoretically possible that there are genetic rearrangements that prevent the cells from entering meiosis since the small size of most chicken chromosomes restricts evaluation of the karyotype to chromosomes 1–4 and the sex chromosomes. We, therefore, evaluated sections of gonads of 48 cES cell chimeras at various stages of

sexual development for the presence of GFP positive spermatogonia. Although GFP expressing cells were seen that morphologically looked like germ cells, we were unable to unequivocally demonstrate the presence of the CVH protein in these cells. We therefore concluded that cES cells had not developed into functional germ cells.

Although cES cells contributed extensively to somatic tissues, sex conversion was never observed. The reproductive organs, (i.e. ovary, testes and efferent ducts) and phenotypic sex developed according to the genetic sex of the recipient embryo. However, after sexual maturation, secondary sex characteristics, (e.g. comb, wattle and feather structure) sometimes developed according to the sex of the cES cells, especially in female chimeras made with male cES cells. A substantial number of female birds changed their sexual phenotype, became masculinized and did not lay eggs despite the presence of an oviduct. In contrast, when male chimeras were made with female cES cells, feminization was less pronounced although some male chimeras produced sperm for only a few weeks following sexual maturation. The absence of sex conversion following introduction of cES cells contrasts with the frequent occurrence of sex conversion when chimeras were made with blastodermal cells (Kagami et al., 1995). Blastodermal cells contribute to both the germline and somatic tissue suggesting that interactions between germ cells and somatic cells of the same sex might be required for sex determination in birds.

We have applied the term 'chicken ES cell' to these cells recognizing that they do not have all of the attributes of murine ES cells. However, their ability to contribute to somatic tissues and the germline in vivo has been more extensively characterized than similar attributes of human ES cells. The application of ES cell terminology to the human cell lines is so firmly established in both the scientific and popular literature that we believe any attempt to introduce a more scientifically accurate terminology will obfuscate rather than clarify the situation. We suggest therefore, that the term ES cell be retained because the cells are of embryonic origin, they are self renewing in culture and they give rise to ectodermal, mesodermal and endodermal derivatives in vivo

Chicken ES cells are a unique avian cell type that can be genetically modified, kept in culture indefinitely and retain their ability to contribute to somatic tissues. We have not been able to determine if the inability to enter the germline is due to inherent characteristics of these cells, to the rapid pace of embryonic development or to predetermination of the germline. However, even with the absence of germline transmission, these cells are important tools that complement the availability of the sequence of the chicken genome (Wallis et al., 2004) and the emerging field of comparative genomics. These tools can be applied to molecular analyses of the earliest stages of development, to the elucidation of highly conserved sequences that control fundamentally important processes in vertebrate development and to the anticipated production of novel proteins in the eggs of chickens (Alper, 2003; Ivarie, 2003). In combination with the extensive anatomical database that accumulated during the 19th and 20th centuries (Stern, 2005) and the remarkable accessibility of the avian embryo, the existence of cES cells provides new opportunities to study the earliest aspects of vertebrate development.

4. Materials and methods

4.1. Isolation and culture of embryonic cells

The area pellucida was isolated from Stage X (EG&K) Barred Plymouth Rock embryos according to Petitte (2004), washed twice with culture medium and dispersed using a 200 μl pipette into a single cell suspension. Single embryos were seeded on either polyester coated inserts (Transwell®, Costar) or mitotically inactivated STO cells seeded on gelatinized tissue culture plates at a concentration of 10^4 cells/cm^2. To obtain mitotically inactivated STO cells, the cells were grown to confluency, irradiated and cryopreserved at 10^7 cells/vial until further use. The cES cell culture medium consisted of conditioned DMEM or Knockout DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS), 1% pen/strep; 2 mM glutamine, 1 mM pyruvate, 1X nucleosides, 1X non-essential amino acids and 0.1 mM β-mercaptoethanol. Before use, the DMEM or Knockout DMEM medium was conditioned on buffalo rat liver (BRL) cells as described (Petitte, 2004). Briefly, after BRL cells were grown to confluency, medium containing 5% serum was added and conditioned for 3 days. The medium was removed and a new batch of medium was conditioned for 3 days. This was repeated once more and the three batches were combined, frozen at −20 °C, thawed and filtered to make the cES cell culture medium. Chicken ES cells were cryopreserved using standard procedures in 10% DMSO in medium containing DMEM + 25 mM HEPES or CO_2 independent medium (Invitrogen) supplemented with 10% serum.

4.2. Assignment of sex to cell lines and chimeras

The sex of newly derived cell lines was determined by PCR for the female specific W-chromosome Xho I repeat in pUGD0600 (Kodama et al., 1987) using primers W-Chr1 (5′-CGTGAGAAAAGTGGTAGTTC-3′) and W-Chr2 (5′-CCAAAAATACCACCTGTCTC-3′) to amplify a 276 bp fragment. As controls, primers Act-1 (5′-AACAAGACGAGATTGGCATG-3′) and Act-2 (5′-CTATCACTGGGGAACACAGC-3′) were used to amplify a 410 bp fragment in the endogenous chicken β-actin DNA. PCR reactions were performed with AmpliTaq Gold (Applied Biosystems) following the manufacturer's instruction. The presence of the W-chromosome repeat was also examined in phenotypically male chimeras to determine if male ES cells (ZZ) had induced male sexual differentiation in female recipient embryos.

4.3. Evaluation of pluripotency characteristics in vitro

4.3.1. Detection of cell surface epitopes and enzymes associated with pluripotency

To stain for cell surface epitopes, the cES cells were fixed in 4% paraformaldehyde. Anti-SSEA-1 (MC-480) and EMA-1 antibodies were obtained from the Developmental Studies Hybridoma Bank, Iowa University, USA. The staining procedure was carried out using an anti-mouse IgM avidin/biotin-conjugated alkaline phosphatase system (Vectastain ABC-AP kit, Vector Laboratories) according to the manufacturer's instruction. As controls, two wells were used in which either the primary or secondary antibody was eliminated. To detect the presence of alkaline phosphatase (AP), a kit (Sigma) based on the method of Ackerman (1962) was used.

4.3.2. Reverse transcriptase-PCR for Erni expression

mRNA was isolated from cES cells, a Stage X (EG&K) embryo, and DT40 cells (a chicken B cell line (Baba et al., 1985)) with the Oligotex Direct mRNA kit (Qiagen). cDNA was synthesized using the SuperScript RT-PCR System for First-Strand cDNA synthesis (Invitrogen). Primers Act-RT-1 (AACACCC-CAGCCATGTATGTA) and Act-RT-2 (TTTCATTGTGCTAGGTGCCA)

were used to amplify a 597 bp fragment from the chicken β-actin transcript (NM_205518). Primers Erni-1 GATCTAGATCCTCAAATGAAT and Erni-2 TCTTGGGCAACCTCTCCCC amplified a 594 bp fragment from the Erni transcript (AF218814). mRNA from a Stage X (EG&K) embryo without RTase was used as control for the RT-PCR reaction and water was used for the PCR control.

4.3.3. Telomerase detection

Chicken ES cells in culture for 266 days were grown without STO feeder cells for 2–3 passages over a period of 8 days to eliminate contamination with STO cells. The cES cells were trypsinized, pelleted and washed with PBS before being frozen at −80 °C until analysis. Cell extracts were prepared and analyzed for telomerase activity according to the manufacturer's directions using the TRAPeze Telomerase Detection Kit (Serologicals Corporation) which is based upon the Telomeric Repeat Amplification Protocol (TRAP) (Kim et al., 1994). The positive control consisted of the transformed human kidney cell line 293. The two negative controls were primary chicken embryonic fibroblasts (CEF), which are known to be telomerase negative (Swanberg and Delany, 2003) and lysis buffer. An internal 36 bp PCR control was present in all experimental and control reactions.

4.3.4. Karyotype analysis of cES cells

When cES cells were 50–80% confluent they were incubated in 0.1 μg/ml Colcemid (Karyomax, Gibco) for 1–1.5 h. The cells were trypsinized, pelleted and resuspended in 0.56% KCl solution. After 25 min, the cells were centrifuged for 6 min at 200 g and fixed in 3:1 methanol:glacial acetic acid. For metaphase analysis the cells were dropped on slides, stained with 6% Gurr's Giemsa and analyzed for the four largest pairs of macro-chromosomes (GGA1-4) and the sex chromosomes (ZZ or ZW).

4.4. Genetic modification of cES cells

4.4.1. Transfection

Chicken ES cells were transfected as adherent cells using a Petri-Pulser or in suspension using the BTX Electro Square Porator ECM 830 (Genetronics, Inc.). When the Petri-Pulser was used, adherent cells in a 9.5 cm^2 well were washed and 1 ml of electroporation buffer (Specialty Media) containing 2–5 μg of linearized DNA was added. The Petri-Pulser was set into the well and a 15 ms square wave pulse varying from 675 to 750 V/cm was given. After 10 min, 2 ml of medium were added and the culture was returned to the incubator. When cells were transfected in suspension, 10^7 cells in electroporation buffer were exposed to 8 square wave pulses of 2.8 kV/cm and 100 μs duration in an electroporation cuvette. After electroporation cells were seeded into 48 wells to limit the number of transfected clones to no more than one per well. Antibiotic selection was initiated by the addition of 1 μg/ml of puromycin or 100 μg/ml neomycin approximately 2 days after transfection. Resistant clones became visible 8–14 days after transfection and were transferred to new wells for expansion.

4.4.2. Plasmid construction

The transgene designated as pCX-GFP-Puro was prepared in several steps. The PGK driven puromycin resistance gene cassette was released from pKO SelectPuro (Stratagene) by Asc I digestion. The fragment was blunted and Kpn I linkers were added before insertion into the Kpn I site of pMIEM (kindly provided by Dr J.M. Petitte), a GFP expression vector derived from the lacZ expression vector pMIWZ (Suemori et al., 1990) to produce pGFP-Puro. The GFP-Puro cassette was released from pGFP-Puro by BamH I and EcoR I digestion and inserted into the corresponding sites of pUC18 (Invitrogen) to yield pUC18-GFP-Puro. In a final step, the CX promoter which includes 384 bp of the CMV-IE enhancer, 1.3 kb of the chicken β-actin promoter, and a portion of the first chicken β-actin intron, was released from pCX-EGFP (Ikawa et al., 1995) by Sal I and EcoR I digestion and inserted into the Sal I and BamH I sites of pUC18-GFP-Puro in the presence of an EcoR I (null)-Xmn I-BamH I linker to produce the plasmid pCX-GFP-Puro. Prior to transfection into cES cells, the plasmid was linearized by Sca I digestion.

4.4.3. Southern blot analysis

Ten micrograms of genomic DNA were used in each digestion. The digested DNA was run on a 0.8% agarose gel (Invitrogen). The probe DNA was a 740 bp BamH I-Not I EGFP fragment from pCX-GFP-Puro labeled with [^{32}P] dCTP by the Megaprime Labeling System (Amersham). Hybridization was conducted with Rapid-hyb Buffer (Amersham).

4.5. Production of chimeras using Stage X (EG&K) recipients

Barred Plymouth Rock cES cells were injected into White Leghorn embryos, which are homozygous at the dominant white locus. The resulting chimeric chickens have black feathers that are derived from the cES cells and white feathers that are derived from the recipient embryo. Between 1 and 5 μl of cell suspension containing between 2000 and 20,000 cells were injected into the subgerminal cavity of the recipient embryos, although empirical evidence suggested that the greatest number of high-grade chimeras was obtained when 5000 cells were injected (data not shown). To allow easy access to the embryo during injection of the cES cells, Stage X (EG&K) recipient embryos were transferred to a surrogate shell, which was filled with albumin and covered with Saran Premium® wrap. The culture procedure was modified from systems II and III of Rowlett and Simkiss (1987); Perry (1988), and Borwornpinyo et al. (2005). The injected embryos were incubated for 3 days at 37.5–38 °C and 60% relative humidity. During this time they were rocked every 10 min through 90°, with their long axis perpendicular to the angle of rotation. After 3 days the embryos were gently poured into a second surrogate shell (30–35 g heavier) and the window was sealed with Saran Cling Plus®. Embryos were incubated until hatch at 37.5–38 °C and 60% humidity, with hourly rocking through 60° until day 10 of development. To increase the contribution of cES cells the recipient embryos were compromised by irradiating the eggs with approximately 660 rad from a cesium source (Carsience et al., 1993) within 6 h before injection of the donor cells. In some cases the center of the area pellucida of the recipient embryo was removed (coring) (Kagami et al., 1997) before injection of cES cells and some recipient embryos were compromised by combining irradiation and coring. Following injection of cES cells, the embryos were either placed in incubators within a few hours or were held in a temperature controlled room at 15 °C for 3–7 days to maintain embryonic diapause before development was initiated by incubating at 37.5 °C. While the embryos were maintained in diapause they were rocked through 90° at hourly intervals.

4.6. Production of chimeras using Stage IV–IX (EG&K) recipients

To acquire pre-oviposition embryos, 0.1–0.2 μg/kg of arginine vasotocin was injected into the wing vein 3–14 h before the predicted oviposition time. All pre-oviposition embryos were cultured using surrogate shells, as explained for Stage X (EG&K) embryos. Chicken ES cells were injected into either the subembryonic cavity or between the epiblast and the perivitelline layer. Embryos were compromised before injection as described for Stage X (EG&K) embryo recipients.

4.7. Evaluation of chimerism in somatic tissues by FACS analysis

Chimeras were produced using cES cells carrying the pCX-GFP-Puro construct. The age of the cells varied from 222 to 251 days (27–68 days after the start of transfection). As controls, chimeras produced with the same parental, non-transfected, cell line were evaluated. Chimeras were euthanized and samples of tissue were removed from liver, breast muscle, thigh muscle, and brain and placed in PBS. The tissue samples were then minced into fine pieces with scalpel blades and placed in a solution of collagenase, incubated at 37 °C for 30–60 min and then dispersed into a single cell suspension by trituration and filtration through a nylon mesh and/or centrifugation with or without a density gradient. For most samples 10,000 cells were analyzed, although, in a small number of samples, fewer cells were counted due to the low concentration of cells in the sample. For each set of samples a negative control sample was included. Cells fluorescing above a set level of fluorescence were expressed as a percentage of the total cells.

4.8. Evaluation of chimerism in somatic tissues by feather pigmentation

Feather chimerism was evaluated subjectively after hatch by one person. The extent of chimerism was recorded as the percentage of black feathering on the chick at hatch.

4.9. Evaluation of germline chimerism

Chimeric roosters and hens were grown to sexual maturity. Semen was collected from the roosters, diluted 1:1 with Beltsville poultry semen extender (Continental Plastics) and used to inseminate Barred Plymouth Rock hens. The chimeric hens were inseminated with semen from Barred Plymouth Rock roosters. Eggs were collected and stored for up to 7 days then incubated for 14 days, at which time the embryos were euthanized and the number of white and black chicks was recorded. One hundred and one chimeric roosters and 38 chimeric hens were tested for germline transmission of the cES cells. Eleven cell lines, varying in age from 36 days to 8 months and representing 5 parental cell lines, were tested.

4.10. Histological examination of gonads

To evaluate the contribution of GFP-expressing cES cells to the gonads of chimeras, gonads were retrieved immediately after euthanasia, washed in PBS and fixed in 4% paraformaldehyde overnight at 4 °C. After several washes in PBS, the gonads were incubated overnight in 30% sucrose at 4 °C. Blocks were prepared and frozen sections were cut at 5 μm.

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