LETTERS

Germline transmission of genetically modified primordial germ cells

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Primordial germ cells (PGCs) are the precursors of sperm and eggs[1]. In most animals, segregation of the germ line from the somatic lineages is one of the earliest events in development[2]; in avian embryos, PGCs are first identified in an extra-embryonic region, the germinal crescent, after approximately 18 h of incubation. After 50–55 h of development, PGCs migrate to the gonad and subsequently produce functional sperm and oocytes[3,4]. So far, cultures of PGCs that remain restricted to the germ line have not been reported in any species[5,6]. Here we show that chicken PGCs can be isolated, cultured and genetically modified while maintaining their commitment to the germ line. Furthermore, we show that chicken PGCs can be induced *in vitro* to differentiate into embryonic germ cells that contribute to somatic tissues. Retention of the commitment of PGCs to the germ line after extended periods in culture and after genetic modification combined with their capacity to acquire somatic competence *in vitro* provides a new model for developmental biology. The utility of the model is enhanced by the accessibility of the avian embryo, which facilitates access to the earliest stages of development and supplies a facile route for the reintroduction of PGCs into the embryonic vasculature. In addition, these attributes create new opportunities to manipulate the genome of chickens for agricultural and pharmaceutical applications.

Blood containing PGCs was collected from stage 14–17 (H&H; nomenclature used in ref. 7) Barred Plymouth Rock (BPR) chicken embryos and cultured in knockout (KO)-DMEM medium conditioned on buffalo rat liver (BRL) cells (which are known to produce leukaemia inhibitory factor), containing stem cell factor (SCF; 6 ng ml^{-1}) and human recombinant fibroblast growth factor (FGF; 4 ng ml^{-1}). PGCs were grown on a feeder of either Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) fibroblasts or BRL cells. Twelve cell lines determined to be male by the absence of the Xho repeat in the W chromosome[8] (data not shown) and two female cell lines were derived from 114 individual embryos. Cells in all of the lines display a round morphology, remain unattached (Fig. 1a) and can be successfully cryopreserved using conventional techniques. After 217 days in culture, karyotype analysis of one cell line (PGC13) revealed that it was diploid with a male ZZ sex chromosome constitution. To determine whether the cultured PGCs maintain their germ cell characteristics we analysed the expression of the germline-specific genes chicken vasa homologue (*CVH*)[9] and deleted in azoospermia-like (*DAZL*). Polymerase chain reaction with reverse transcription (RT–PCR) analysis of PGCs after 32, 143 and 197 days of culture showed expression of *DAZL* and *CVH* (Fig. 1b), whereas at 166 days of culture, western blot analysis showed production of the CVH protein in the cultured cells (data not shown). Expression of CVH and an ovomucin-like protein (OLP)

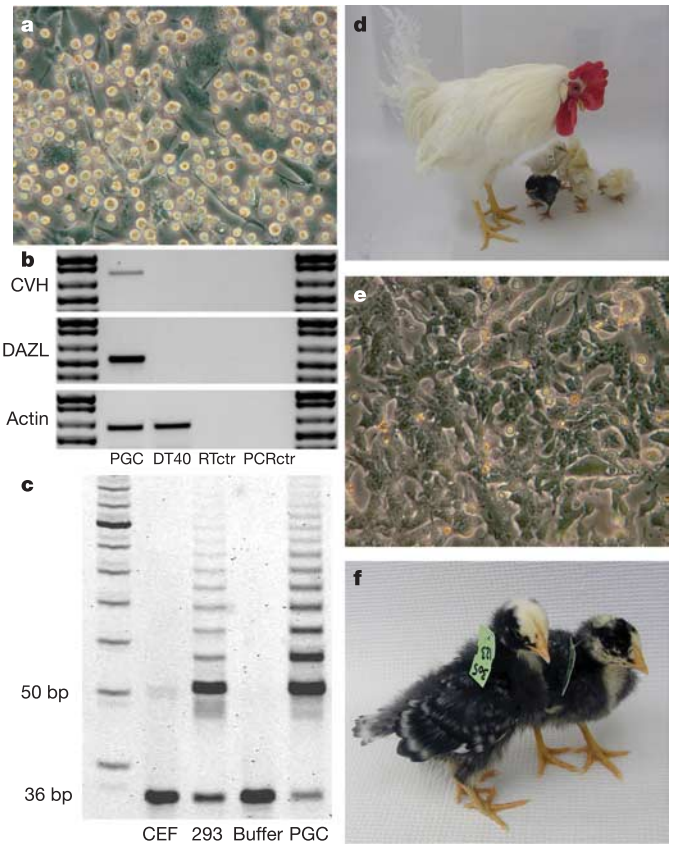

Figure 1 | Characteristics of PGC cell lines *in vitro*. **a**, PGCs are not attached, maintain a round morphology and are grown on a feeder layer of STO cells. **b**, *CVH* and *DAZL* expression in PGC13 and DT40 cells. Expression of both *CVH* and *DAZL* was observed in PGCs whereas DT40 cells did not express either *CVH* or *DAZL*. **c**, TRAP assay. Repeat sequences are visible in PGCs and the positive control cells (293), indicating the presence of telomerase. The 36-bp band in the negative control chicken embryonic fibroblast (CEF) lane is the internal PCR template. **d**, A WL is homozygous dominant at the dominant white locus (I/I). When bred to a BPR hen (i/i) all offspring from a WL rooster will be white (I/i). A black chick demonstrates that cultured PGCs derived from a BPR embryo (i/i) colonized the germ line. **e**, Chicken EG cells derived from PGCs in culture. EG cells are small, have large nuclei (light grey) and pronounced nucleoli. The EG cells were derived from PGCs isolated from black-feathered BPR embryos. **f**, Chimaeras obtained after EG cell injection into stage X (EG&K) white-feathered WL embryos. Somatic chimaerism is evident by the black feathers.

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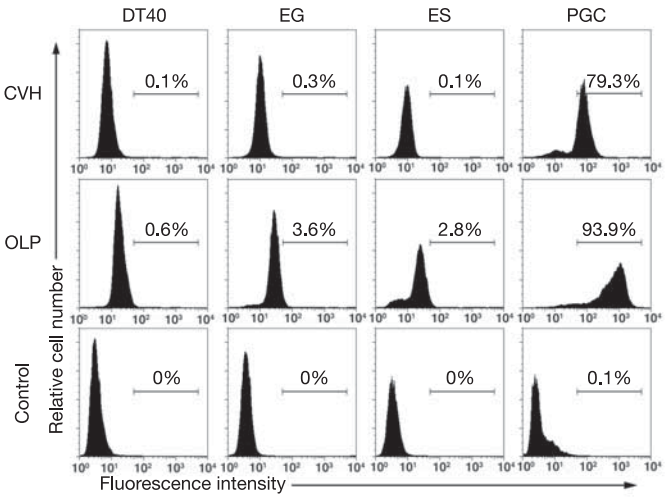

Figure 2 | FACS analysis of DT40 cells, ES cells, EG cells and PGCs, stained with antibodies against CVH and OLP. The DT40, ES and EG cells were negative for both markers, whereas a large majority of PGCs stained for both CVH and OLP.

that is expressed on the surface of PGCs during migration and colonization of the gonad[10] were examined by fluorescence-activated cell sorting (FACS) at 44 and 65 days for PGC102, and at 229 and 280 days for PGC13. The FACS profiles of PGCs, chicken embryonic stem (ES) cells[11] and chicken embryonic germ (EG) cells revealed that the 1B3 antibody recognizes the ovomucin-like protein only on PGCs and that the expression of CVH is likewise restricted to PGCs (Fig. 2). By comparison, cells expressing an ES cell phenotype or an EG cell phenotype, and DT40 cells that express a pre-B-cell phenotype[12], did not express these antigens. Between 7% and 20% of the cells in the PGC cultures did not stain for either protein, indicating that some cells in each culture do not express a PGC phenotype. It is possible that the starting population was heterogeneous; however, a small part of the PGC population regularly attaches to the feeder layer and differentiates. This differentiation is also seen in clonally derived cell lines, indicating that the non-stained cells are differentiated PGCs. A telomeric repeat amplification protocol (TRAP) assay performed on PGCs that were in culture for 196 days demonstrated the presence of telomerase, which is a key characteristic of immortal cell lines (Fig. 1c). The presence of telomerase is consistent with the long-term growth of the PGCs in culture.

To demonstrate that PGCs maintain their restriction to the germ line, cells from eight male cell lines that were in culture for a minimum of 35 days and a maximum of 110 days were injected into stage 13–15 (H&H) White Leghorn (WL) embryos, which are post-gastrulation embryos with 19–27 somites and a functional vasculature. Twenty-four male chimaeric chicks were reared to sexual maturity. Each of these chimaeras displayed the white-feathered phenotype of the WL breed (Fig. 1d). All of the male chimaeras transmitted the PGC phenotype (recognizable by black feathers) to the next generation, demonstrating that the cells retained their ability to colonize the germ line (Table 1 and Fig. 1d). The rate of germline transmission of these birds ranged from <1% to 86%, which is similar to the rates of germline transmission obtained in single-sex chimaeras after the transfer of freshly isolated PGCs[13]. Female PGCs in culture for 47 and 66 days were transmitted to the next generation by female chimaeras at frequencies up to 69% (Table 1), although these cell lines could not be maintained beyond 109 and 77 days, respectively. The variability of germline transmission among hens and roosters injected with aliquots of the same pool of cells may be due to variation in the number of injected PGCs that colonize the germ line and differential expansion of each of the cells within the gonad. Germline transmission of male PGCs has not been observed in 1,625 offspring of 14 female putative chimaeras, and female PGCs did not colonize the germ line of three male putative chimaeras that produced 2,739 offspring when mated to BPR hens. Similarly low rates of germline transmission were observed in mixed-sex chimaeras produced with freshly isolated PGCs[13], indicating that the development of germ cells is impaired in mixed-sex chimaeras. To evaluate the reproductive capacity of offspring derived from cultured PGCs, male and female progeny were mated together. Fertility ranged from 53% to 85% and the hatch rate of fertile eggs ranged from 79% to 96%, indicating that the reproductive capacity of offspring derived from PGCs in culture for 40 days is normal.

Conventional electroporation protocols that we use routinely to introduce genetic modifications into chicken ES cells failed to yield genetically modified PGCs. To obviate potential silencing of transgenes, two copies of the HS4 insulator sequence from the chicken β-globin locus[14] were inserted 5′ and 3′ of the transgenes and genetically modified clones were isolated. PGCs carrying an HS4-β-actin-EGFP-β-actin-puro-HS4 transgene (see Methods) were injected after 134 days in culture into stage 13–15 (H&H) embryos and eight roosters were bred to BPR hens to evaluate germline transmission. Seven of the eight roosters transmitted the PGC-derived genotype through the germ line at frequencies varying from 1% to 92%. Eighty of the 163 black-feathered offspring (49%) exhibited green fluorescence (Fig. 3b), indicating mendelian segregation of the transgene among PGC-derived offspring. Southern blot analysis confirmed that chicks expressing GFP were derived from the transgenic PGC line (Fig. 3a) and all of the chicks carrying a copy of the transgene expressed GFP (data not shown). We have also obtained transfected male cell lines carrying an HS4-ERNI-neo or an HS4-β-actin-puro transgene and have hatched live chicks carrying an HS4-β-actin-neo transgene from PGCs in culture for 267 days and an HS4-CAG-EGFP-CAG-puro transgene from PGCs in culture for 238 days.

A small proportion of PGCs attaches spontaneously to the feeder layer and assumes the morphology of ES cells, which is shown in

Table 1 | Germline transmission of primordial germ cells injected into the vasculature of stage 14–15 (H&H) embryos

Cell line	Sex	Time in culture (days)	No. of cells injected	No. of putative chimaeras tested	Germline transmission* (%)
PGC13	Male	40	1,200	3	0.1,1.5,17
PGC13	Male	110	2,500–3,000	5	1,1,1.5,3,84
PGC21	Male	44	1,500	3	10,16,21
PGC34	Male	47	3,000	3	42,74,80
PGC35	Male	35	3,000	7	15,23,47,61,80,85,86
PGC51	Male	47	3,000	1	11
PGC54	Male	47	3,000	4	0.5,2,20,24
PGC80	Male	29	3,000	1	55
PGC84	Male	50	3,000	1	70
PGC56	Female	66	3,000	5	1,3,6,52,69
PGC85	Female	47	3,000	10	0,0,1,1,1,4,5,10,11,12

*Each value represents the rate of germline transmission of one chimaera.

ref. 11. By removing FGF, SCF and chicken serum these cells can be expanded and are called EG cells (Fig. 1e). Without these components and with an increase in the percentage of conditioned medium, the culture conditions are the same as those used for long-term culture of ES cells derived from blastodermal cells[11]. Chicken EG cells are observed in both newly derived and clonally derived transgenic PGC lines. Southern blot analysis of EG cells derived from a clonal GFP-positive PGC line (Fig. 3a) indicates that EG cells originate from the PGCs and are not derived from a contaminating population of cells in blood samples from stage 14–17 (H&H) embryos from which the cultures originated. When EG cells were injected into stage X (EG&K; nomenclature used in ref. 15) embryos, which are the functional equivalents of mammalian blastocysts, they contributed extensively to various somatic tissues (Fig. 1f and Supplementary Fig. 1). Of the 140 WL embryos injected with EG cells, 30 survived until at least embryonic day (E)14, and 20 embryos (67%) exhibited the black feathering of the BPR breed that was used to derive the EG cells. Four of these chimaeras with extensive feather pigmentation have been bred to evaluate contributions to the germ line. One black chick was observed among 4,490 offspring, indicating that some capability to colonize the germ line is retained within the culture. In general, however, the ability of EG cells to colonize the somatic tissues and their inability to colonize the germ line is similar to that of chicken ES cells[11].

To determine whether cultured PGCs contribute to somatic tissues, tissues from chicks injected with GFP-positive PGCs were analysed by histology. With the exception of four green cells in a brain sample, cells expressing GFP were not present in other tissues, demonstrating that cells in a PGC culture remain restricted to the germ line. When PGC13 cells, after 209 days of culture, were injected into the subgerminal cavity of 111 stage X (EG&K) embryos, 20 embryos survived to E14 but black feathers were not observed, indicating that PGCs do not contribute to somatic tissues, even when injected at very early stages of development. However, three out of four roosters injected with PGCs at stage X (EG&K) transmitted them through the germ line at frequencies of 0.15%, 0.2% and 0.45%, indicating that they can colonize the germ line of early stage embryos. In contrast, chicken ES cells contribute substantially to somatic tissues but not to the germ line[11].

We describe a novel system for the production of transgenic chickens using PGCs. To our knowledge this is the first description in any species of PGCs that can be grown indefinitely *in vitro* and be genetically manipulated while retaining their commitment to the germ line. Combined with the extensive anatomical database describing development in the chick embryo and the ease of access to the earliest stages of development[16], the ability to make genetic changes to chickens will provide a unique resource to address important issues in developmental biology. In addition, the ability to use cultured PGCs to make changes to the germ line of chickens has enormous implications for industrial[17] and agricultural applications of avian transgenic technology.

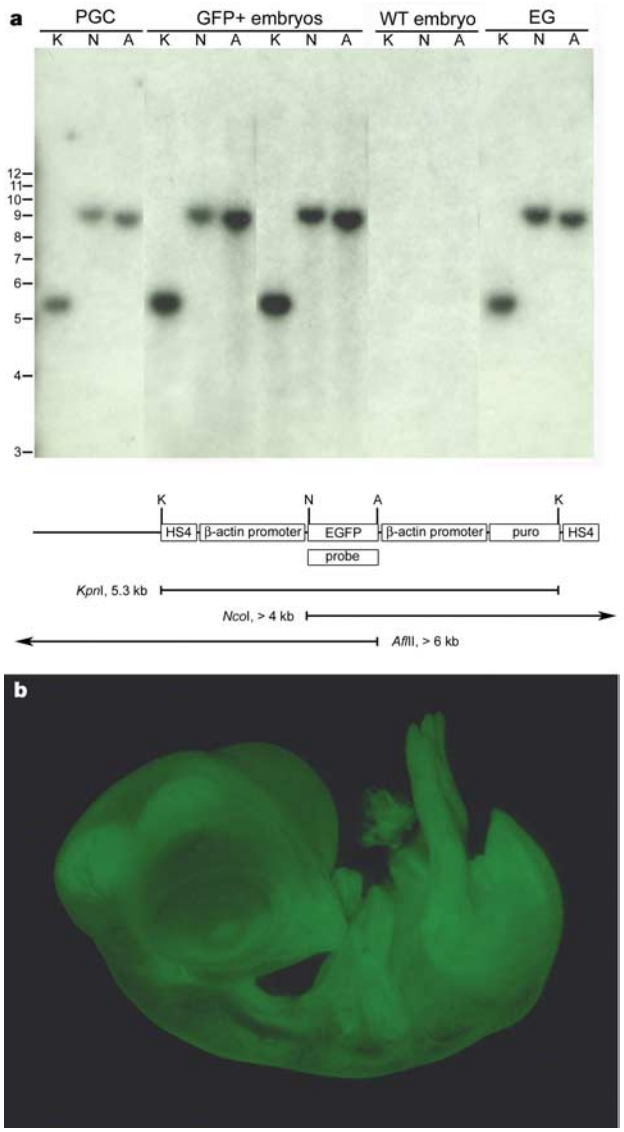

Figure 3 | β-Actin-EGFP is incorporated into the PGC genome and expressed in offspring. a, Southern blot analysis showing that a clonally derived, transfected PGC line can contribute to the germ line in chimaeric chickens and differentiate into EG cells. Top panel: samples of genomic DNA from PGCs transfected with the HS4-β-actin-EGFP-β-actin-puro construct, three embryos derived from a chimaeric rooster made with the transfected PGCs, and EG cells derived from the transfected PGCs were digested with restriction enzymes for detecting internal (*Kpn*I) and junction fragments (*Nco*I, *Afl*II) of the transgene insertion. The digested DNA was separated on a 0.7% agarose gel, blotted to nylon membrane, and probed with radiolabelled EGFP sequences. The sizes of the hybridizing fragments in kilobases (left axis) were identical in the PGCs, EG cells and two embryos that showed green fluorescence (GFP^+ embryos). A third, non-fluorescing embryo (WT embryo) showed no hybridization. Bottom panel: a schematic of the construct is shown, with the locations of the restriction sites indicated, and the expected restriction fragment sizes shown below. **b**, The offspring of a chimaeric rooster transgenic for the HS4-β-actin-EGFP-β-actin-puro transgene at embryonic stage 34 (H&H). GFP is expressed ubiquitously throughout the embryo. The image was captured on a Leica MZ16FA microscope fitted with a Leica DFC300FX camera and a PlanAPO 0.6× lens that were provided by Leica.

METHODS

Derivation, culture and cryopreservation of PGCs. One to five microlitres of blood was taken from the vasculature of stage 14–17 (H&H) embryos and deposited in single wells of a 96- or 48-well plate seeded with either mitotically inactivated STO cells (3×10^4 cells cm^{-2}) or BRL cells (10^5 cells cm^{-2}). After 1–2 weeks, red blood cells had died and PGCs became visible. Throughout derivation and culture, the cells were grown in KO-DMEM (Invitrogen) that was conditioned with BRL cells[18]. The medium was supplemented with 7.5% fetal calf serum (FCS), 2.5% chicken serum, 2 mM glutamine, 1 mM pyruvate, 1× nucleosides, 1× non-essential amino acids and 0.1 mM β-mercaptoethanol, 6 ng ml^{-1} SCF and 4 ng ml^{-1} human recombinant FGF. For passage, the cells and medium were removed into a centrifuge tube, pelleted by centrifugation at 300 *g* for 5 min, resuspended and seeded at a concentration of 25,000 cells cm^{-2}. For cryopreservation, the cells were resuspended in CO_2-independent medium containing 10% FCS, 1.0% penicillin/streptomycin and 10% DMSO. The vials were frozen at −80 °C and transferred to LN_2 after 24 h.

RNA isolation and RT–PCR. A 750-base-pair (bp) fragment from the *CVH* transcript, a 536-bp fragment from the *DAZL* transcript and a 597-bp fragment from the chicken β-actin transcript were amplified by RT–PCR (Supplementary Table 1).

FACS analysis of PGCs, EG cells and ES cells. Cells were washed in PBS/2% FBS

and fixed in 4% paraformaldehyde for 5 min. Cell aliquots to be stained for CVH were permeabilized with 0.1% Triton X-100 for 1–2 min. Primary antibody was added for 20 min, cells were washed twice and incubated with secondary antibody (Alexa 488 anti-rabbit IgG for CVH and control and Alexa 488 anti-rabbit IgM for 1B3) for 15 min. Control cells were stained only with secondary antibody.

Telomerase detection. Primordial germ cells that were in culture for 196 days were pelleted and washed with PBS before being frozen at −80 °C until analysis. Cell extracts were prepared according to the manufacturer's directions using the TRAPeze telomerase detection kit (Serologicals Corporation)[19]. The positive control was the transformed human kidney cell line 293 and the negative control contained lysis buffer and an internal PCR control template.

Karyotype analysis. PGCs were incubated in 0.1 $\mu g\,ml^{-1}$ colcemid (Karyomax, Invitrogen) for 2 h. The cells were pelleted and resuspended in 0.56% KCl solution. After 25 min, the cells were centrifuged for 6 min at 200 *g* and the pellet was fixed in 3:1 methanol:glacial acetic acid. For metaphase analysis the cells were dropped on slides, stained with Giemsa and analysed for the four pairs of macro-chromosomes (GGA1– GGA4) and the sex chromosomes.

Transfection of PGCs. A total of 5×10^6 PGCs were resuspended in 400 μl electroporation buffer (Speciality Media) to which 20 μg of linearized DNA was added. One exponential decay pulse (200 V, with 900–1,100 μF) or eight square wave pulses (250–350 V, 100 μs) were given. After transfection the cells were grown for several days before neomycin (300 μg ml) or puromycin (0.5 μg ml) was added. In most cases, single colonies were derived by plating the cells at low concentration in 48-well plates after transfection.

Transgene construction. Transgenes were flanked by the insulator element from the chicken β-globin locus. The 250-bp core sequence of hypersensitive site 4 (HS4) was amplified from chicken genomic DNA by PCR (Supplementary Table 1) and a tandem duplication of the HS4 site was made by joining two copies of the PCR product, in the same orientation, resulting in 2× HS4. The 2× HS4 insulator was then inserted at both the 5′ and 3′ ends of the following constructs: β-actin-puro, β-actin-neo (gifts from J.-M. Buerstedde), β-actin-EGFP-β-actin-puro, ERNI-neo[20], CAG-EGFP-CAG-puro[21].

Production of chimaeras using PGCs. PGCs were injected using a 37-μm diameter needle into the anterior portion of the sinus terminales of a stage 13–15 (H&H) embryo. The injected embryos were transferred to a second surrogate shell for incubation until hatching[11,22].

Derivation of EG cells and production of chimaeras. When an EG cell phenotype became visible, the growth factors and chicken serum were removed from the medium. Chicken EG cells were grown in 80% BRL conditioned medium on irradiated STO feeders plated at 10^4 cells cm^{-2} (ref. 11). At confluency the cells were incubated in Ca/Mg free PBS to obtain small clumps and passaged 1:2 or 1:3. For the production of chimaeras the cells were trypsinized into a single cell suspension and resuspended at 5,000 cells μl^{-1}. One microlitre was injected into the subgerminal cavity of an irradiated stage X (EG&K) embryo. After injection the embryo was incubated for 3 days before being transferred to a second surrogate shell and incubated until hatching[22].

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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