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Transfer and Detection of Freshly Isolated or Cultured Chicken (*Gallus gallus*) and Exotic Species' Embryonic Gonadal Germ Stem Cells in Host Embryos

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Abstract

The management of captive avian breeding programs increasingly utilizes various artificial reproductive technologies, including in ova sexing of embryos to adjust population sex ratios. Currently, however, no attention has been given to the loss of genetic diversity following sex-selective incubation, even with respect to individuals from critically endangered species. This project evaluated the possibility of using xenotransfer of embryonic gonadal germline stem cells (GGCs) for future reintroduction of their germplasm into the gene pool. We examined and compared the host gonad colonization of freshly isolated and 3 day (3d) cultured donor GGCs from chicken and 13 species of exotic embryos. Following 3d-culture of GGCs, there was a significant increase in the percentage of stem cell marker (SSEA-1, -3, -4) positive cells. However, the percentage of positive host gonads with chicken donor-derived cells decreased from 68% (fresh) to 22% (3d), while the percentage of exotic species donor-cells positive host gonads decreased from 61% (fresh) to 49% (3d-cultured). Donor GGCs from both chicken and exotic species were localized within the caudal endoderm, including the region encompassing the gonadal ridge by 16 hours post-injection. Furthermore, donor-derived cells isolated from stage 36 host embryos were antigenic for anti SSEA-1, VASA/DDX4 and EMA-1 antibodies, presumably indicating maintenance of stem cell identity. This study demonstrates that GGCs from multiple species can migrate to the gonadal region and maintain presumed stemness following xenotransfer into a chicken host embryo, suggesting that germline stem cell migration is highly conserved in birds.

Keywords

xenotransfer; gonadal ridge; chimeric gonad; host embryo; GGC

INTRODUCTION

In recent years, new techniques for the management of captive bird breeding programs, such as in ova sexing, have been developed and are now at the verge of widespread use in avian conservation programs. Such tools will not only allow for more flexibility in the management of captive populations, but also potentially prevent the loss of genetic diversity without increasing the number of individuals. Management of captive avian breeding programs increasingly includes the in ova sexing of embryos to adjust population sex ratios. In fact, the Association of Zoos and Aquariums' Gruiformes taxonomic advisory group endorses using in ova sexing to correct skewed sex ratios of cranes (Gruidae; AZA Gruiformes Taxon Advisory Group, 2008). In addition, multiple zoo, conservation, and research groups have developed routine techniques for in ova blood collection (Langenberg et al., 1997; Nuechterlein and Buitron, 2000; Dutton and Tieber, 2001; Lecomte et al., 2006; Kaleta and Redmann, 2008; Jensen et al., 2012) and rapid DNA sexing (Walsh et al., 1991; Griffiths et al., 1998; Jensen et al., 2003). Due to resource and space constraints, most institutions will discard eggs if the sex ratio of a species is skewed. While the establishment of in ova sexing protocols prevents some loss of genetic diversity, no plan exists to preserve the genetic diversity from individuals removed from incubation. If the germplasm from removed embryos could be cryopreserved or cultured, and in the future reintroduced into the gene pool, the population would recover that genetic diversity. Previous groups have demonstrated that neither cryopreservation (Naito et al., 1994a; Takei et al., 1997; Tajima et al., 1998; Moore et al., 2006) nor in vitro culture (Chang et al., 1995; Chang et al., 1997; Park et al., 2003a; Park et al., 2003b; Ge et al., 2009; Shiue et al., 2009) impacts the ability of transferred chicken embryonic primordial germ stem cells (PGCs) or embryonic gonadal germline stem cells (GGCs) to produce donor-derived offspring. In addition, several studies have demonstrated the ability of one species' PGCs or GGCs to colonize the gonadal ridge of another species, including chicken (*Gallus gallus*) PGCs to helmeted guinea-fowl (*Numidea meleagris*) hosts (van de Lavoie et al., 2006), common pheasant (*Phasianus colchicus*) GGCs to chicken hosts (Kang et al., 2008), and chicken PGCs to domestic duck (*Anas domestica*) hosts (Liu et al., 2012).

The current project investigated the ability of GGCs from a diverse set of species to successfully colonize chicken host gonads. The combination of in ova sexing, cryopreservation, and GGC xenotransfer could have a significant impact on captive avian population management by controlling sex ratios and reintroducing germplasm into the gene pool years or decades later.

MATERIALS AND METHODS

All experiments in this study were reviewed and approved by the San Diego Zoo Global IACUC (assurance# A3675-01) and in compliance with the "Guidelines for the use of animals in research." The taxonomic classification and naming of birds is based on Sibley et al. (1988) and Sibley and Monroe (1990) and Birdlife International (www.Birdlife.org). Embryos were staged following the Hamburger and Hamilton (1951) descriptions.

Donor cell isolation and vital-dye staining

Embryonic gonads from donors including chicken (*Gallus gallus*), and the following exotic species: mountain peacock-pheasant (*Polyplectron inopinatum*), Reeves's pheasant (*Syrmaticus reevesii*), Victoria crowned pigeon (*Goura Victoria*), pheasant pigeon (*Otidiphaps nobilis*), Mindanao bleeding-heart (*Gallicolumba crinigera*), coronated fruit-dove (*Ptilinopus coronulatus*), beautiful fruit-dove (*Ptilinopus pulchellus*), Mariana fruit-dove (*Ptilinopus roseicapilla*), great blue turaco (*Corythaeola cristata*), white-throated kingfisher (*Halcyon smyrnensis*), Papuan lorikeet (*Charmosuna papou*), black-necked swan (*Cygnus melancoryphus*), and white-rumped shama (*Copsychus malabaricus*), were visually sexed during dissection at stages 34–40. Donor embryo gonads were enzymatically dissociated by collagenase:DNAse (31 mg/mL:3.9 mg/mL) at 37°C for 30–60 minutes to obtain a single cell suspension. The GGCs were stained with the cellular membrane dye PKH67 for whole embryo photomicroscopy, or PKH26 for flow cytometry and immunocytochemistry. Dyes were used according to manufacturer's recommendations (Sigma-Aldrich).

Donor cell culture

Chicken and exotic species donor cells were injected into hosts fresh or after culture at 2×10^6 cells/mL for 3–18 days in modified DMEM/F12 medium (Germ cell Culture Medium: 15% FBS, 1% pen/strep, 2 mM L-glutamine, 40 ng hrbFGF, 1000U LIF, 0.1 mM 2-mercaptoethanol, 10 mM forskolin, 1 mM Na pyruvate, 1 mL non-essential amino acids per 500 mL medium, pH 7.2; (Freshney et al., 2007)) at 40°C in 6% CO₂. The 3-day culture is used to keep the cells alive and to maintain their stem cell identity while the host embryos develop to stage 14–17, when donor cells can be transferred. Less than 4% of cells exhibited active mitosis as detected using a proliferation assay (data not shown). Cultured cells were recovered by removal of suspended cells following gentle agitation of the medium to prevent collection of plated somatic cells.

Incubation

Unincubated fertilized chicken eggs used as hosts or donors were obtained from McIntyre Egg Ranch (Lakeside, CA). Fertilized exotic eggs that were scheduled for euthanasia were collected opportunistically from San Diego Zoo Global's Avian Propagation Center. Host chicken embryos were incubated at 37.5°C and 60–70% humidity in a forced air incubator (GQF manufacturing Co.). Exotic embryos were incubated at 37.8°C and 70% humidity in a forced air incubator (Grumbach, Lyon Technologies Inc.). Donor chicken and exotic embryonic gonads were collected between stages 34–40 (approximately 40–70% of the incubation period).

Donor cell injection

Fresh or cultured cells were injected as previously described (Naito et al., 1994b; Ono et al., 1996; Roe et al., 2013). A standard belt sander was used to make a 1.5 cm² opening in the host embryo shell, and 10,000 chicken or exotic donor gonadal cells suspended in 2–10 µL M199 medium (Mediatech, Inc.) were injected into the vitelline vein of unsexed chicken embryos at stages 14–17. Injections were performed with a SteReo discovery. V12

microscope (20– 25× magnification; Zeiss), and a 40–60 µm beveled glass needle mounted on a microinjection/micromanipulation setup (IM-9B Narishige, Kite-R WPI Inc.). Following injection, the egg was sealed using Parafilm (Pechiney Plastic Packaging, Co.) melted to the shell. The host embryos were incubated, as described above, opening down until reaching stages 34–36. Control embryos were not opened or injected.

Early donor cell migration localization

Stage 14–17 embryos were injected with fresh or 3d-cultured PKH67 stained chicken or exotic donor gonadal cells as described above. Following cell transfer, the embryos were incubated for 6–16 hours in ova (as described above) before donor cell localization at stage 17–19. Whole embryos were removed from the yolk, as previously described by Chapman et al. (2001), using filter paper rings, allowing the vitelline membrane to remain stretched. A SteReo discovery. V12 with a dual 470 LED and a filter set 38 intermediate tube, an AxioCam MRm camera (Zeiss) and the ZEN image software (Zeiss) were used to collect images of PKH67 stained cells.

Whole embryo immunohistochemistry—Stage 16–19 control embryos were removed from the yolk and associated membranes. Embryos were fixed in Dietrick's fixative (10% formalin, 30% ETOH, 2% glacial acetic acid) for 30 min, rinsed in blocking solution (PBS, 0.1% triton-x, 0.05% tween 20, 0.5% BSA), incubated in blocking solution for 30 min, followed by overnight incubation in blocking solution and FITC conjugated SSEA-1 (1:100, eBiosciences) at 4°C and rinsed in blocking solution prior to visualization. Antibody positive endogenous GGCs were visualized using a SteReo discovery. V12 microscope equipped, as described above.

Late donor cell migration localization

Whole gonads from host embryos injected with PKH26 stained donor cells were removed at stages 34–36, visually sexed using a SteReo discovery. V12, and prepared for flow cytometry and immunocytochemistry. Following removal, gonads (both testes or left ovary) were dispersed in collagenase:DNase (31 mg/mL:3.9 mg/mL) at 37°C for 30–60 min to obtain a single cell suspension.

Flow cytometry—Flow cytometry analysis of 50,000 cells from each control and injected embryo was used to determine the percent of PKH26 positive cells. The flow cytometer data were collected using CellQuest and a FACSCalibur (BD Biosciences) and analyzed using FlowJo (Tree Star Inc.). Front scatter (FSC) and side scatter (SSC) gating were used to eliminate endogenous red blood cells and cellular debris. FL-1 and FL-2 gating were used to gate positive cells, while excluding the high gonadal cell auto-fluorescence.

Immunocytochemistry—The expression of stem cell-specific antigens on cells from fresh or cultured chicken and exotic embryo gonads was determined by staining with the stem cell-specific antibodies SSEA-1, SSEA-3, and SSEA-4 (Jung et al., 2005; DHSB, University of Iowa, eBiosciences) diluted 1:400 and detected by direct FITC conjugation (). Flow cytometry was used to determine percent positive cells.

Co-localization

Following flow cytometry, cells from a subset of positive host and control embryos were incubated for 30 minutes in blocking solution, stained with FITC conjugated anti SSEA-1 (eBiosciences), VASA (Bioss Antibodies) or unconjugated EMA-1 (DHSB, University of Iowa) antibodies at room temperature for 30 minutes, followed by staining for 10 minutes in Hoechst 33342 nuclear stain. Co-localization of anti SSEA-1, VASA or EMA-1 antibodies with PKH26 and Hoechst 33342 positive staining was visualized on an Eclipse 80i (Nikon) with a Digital Sight and DS-Fi1 camera (Nikon). The anti SSEA-1, VASA and EMA-1 antibodies have previously been shown to detect avian germline stem cells (Jung et al., 2005; Nakamura et al., 2007; Tsunekawa et al., 2000, Bioss Antibodies website).

Statistical analysis

An embryonic gonad was considered positive when the percentage of PKH26 positive cells observed by flow cytometry exceeded two standard deviations above the average for the non-injected control embryos. The percent of positive donor-derived cells in host gonads was determined by subtracting the percent of cells in the positive flow cytometer gate of experimental gonads from the average of the non-injected controls. The two-tailed Fisher's exact test, paired or un-paired two-tailed t-test, Chi-square, and two-way ANOVA were used to determine statistical significance (Prism 6, GraphPad Software, Inc.); a p-value of 0.05 or less was considered significant.

RESULTS

Stem cell marker analysis before and after culture

The percent of chicken embryonic testes cells that were positive for the SSEA-1, SSEA-3, and SSEA-4 markers increased significantly between fresh and 3d-culture from 2.33% to 14.44% ($t = 15.1$, $df = 2$, $P = 0.004$ t-test), 4.22% to 44.94% ($t = 201.2$, $df = 2$, $P < 0.0001$), and 1.73% to 96.01% ($t = 305.7$, $df = 2$, $P < 0.0001$), respectively (Fig. 1A). The percent of chicken embryonic ovarian cells positive for the SSEA-1, SSEA-3, and SSEA-4, markers increased between fresh and 3d-culture from 2.55% to 16.52% ($t = 3.6$, $df = 2$, $P = 0.071$) and significantly from 3.21% to 38.82% ($t = 6.2$, $df = 2$, $P = 0.025$), and 2.30% to 94.55% ($t = 133.0$, $df = 2$, $P < 0.0001$), respectively (Fig. 1B). A similar increase in stem cell marker expression was seen in black-necked swan and Reeve's pheasant embryonic gonads that were analyzed fresh and after 3d-culture (Fig. 1C, D). Following 15d culture of black-necked swan embryo gonads, stem cell marker positive cells decreased to or below those of fresh (data not shown). In addition, SSEA-1, SSEA-3 and SSEA-4 antigenicity were evaluated in freshly isolated embryonic gonads from Himalayan monal (0.87%, 2.86%, 2.41%), crested wood partridge (0.87%, 1.02%, 0.80%), and Papuan lorikeet (2.57%, 9.26%, 5.68%). However, the species-specific variability observed in the percent of embryonic gonadal cells that are SSEA-1, -3, and -4 positive appears to be variable across species. This variation may indicate species-specific differences in the number of germline stem cells, or possibly in the germline cell development, as previously proposed (D'Costa and Petite, 1999).

Immunohistochemical localization—Six hours post-injection, fluorescent donor-derived cells from chicken and Midanao bleeding heart were visible in the caudal region of stage 17 embryos (Fig. 2).

At 16 hours post-injection, donor-derived cells from Reeve's pheasant and chicken embryos were observed in most of the endoderm of the caudal area of the embryo, including the gonadal ridge area within the midgut fold (Fig. 3A–F). Donor-derived cells were not observed in the lateral body wall or around the somites or spinal cord (Fig. 3A, B). Immunohistochemical detection of SSEA-1 positive cells in non-injected control embryos was limited to the gonadal ridge in the midgut wall (Fig. 3G, H).

Chicken embryo GGC transfer—Of the 49 host embryos injected with fresh chicken donor cells, 31 (63%) survived to analysis, yielding 21 (67.7%) positive embryos, with 0.039 to 0.378% donor-derived cells per positive gonad. Of the 36 embryos injected with 3d cultured chicken donor cells, 32 (89%) survived to analysis yielding seven (22%) positive embryos with 0.041 to 0.148% donor-derived cells per positive gonad. Host embryos injected with fresh or 3d-cultured donor cells had a statistical difference in the number of positive cells ($P = 0.0004$, Fisher's exact test). No difference was observed in the number of donor-derived cells within positive host gonads when injected with fresh or 3d-cultured cells ($t = 0.5$, $df = 26$, $P = 0.62$ t-test; Table 1, Fig. 4A). The total number of cells in a pair of stage 36 embryo testes averaged $2.04 \times 10^6 \pm 2.2 \times 10^5$ and $2.00 \times 10^6 \pm 1.9 \times 10^5$ for the left ovary (Roe et al., 2013). This yielded a range of approximately 800 to 7600 and 800 to 3000 colonized donor-derived cells in hosts injected with fresh or 3d-cultured cells, respectively.

The effects of donor/host sex on percent positive host embryo gonads ($\chi^2 = 0.056$, $df = 3$, $P = 0.997$ Chi-square) and percent donor-derived cells per positive host gonad (interaction: $F(1,17) = 0.03$, $P = 0.86$; host sex: $F(1,17) = 0.39$, $P = 0.54$; donor sex: $F(1,17) = 4.41$, $P = 0.051$, 2-way ANOVA) were not statistically significant, when injecting fresh donor cells (Fig. 5A). The effects of donor/host sex on percent positive host embryo gonads ($\chi^2 = 1.58$, $df = 3$, $P = 0.66$) and percent donor-derived cells per positive host gonad (interaction: $F(1,3) = 2.94$, $P = 0.19$; host sex: $F(1,3) = 2.38$, $P = 0.22$; donor sex: $F(1,3) = 1.76$, $P = 0.28$) were not statistically significant, when injecting 3d-cultured donor cells (Fig. 5B).

Exotic embryo species GGC transfer—A total of 148 host embryos were injected with exotic donor cells, of which 101 (68%) survived to analysis, yielding 50 (50%) positive embryos. Data were grouped for analysis as fresh, three to ten day culture, or greater than 10-day culture, according to donor cell treatment prior to injection. Forty-nine of the 74 (66%) fresh exotic donor cell embryo transfers survived to gonad collection. Of these, 30 (61%) were positive for donor-derived cells in the host gonad, ranging from 0.015 to 0.44% of total gonadal cells (Table 1). Thirty-nine of the 56 (70%) 3-10d exotic embryo transfers survived to gonad collection. Of these, 19 (49%) were positive for donor-derived cells in the host gonad, ranging from 0.037 to 0.27% of total gonadal cells. Thirteen of the 18 (72%) 10-18d-culture exotic donor cell injected embryos survived to gonad collection. Of these only 1 (8%) was positive for donor-derived cells in the host gonad, with 0.045% of total gonadal cells (Table 1). No significant difference was observed between the numbers of

positive host embryo gonads with fresh or 3-10d-cultured donor cells ($P = 0.28$ two-tailed Fisher's exact test). The number of positive host embryo gonads was significantly different when injected with fresh or 10-18d-cultured donor cells ($P = 0.001$), as well as with 3-10d and 10-18d-cultured donor cells ($P = 0.009$). No significant difference was observed in the number of positive cells within host gonads with fresh and 3-10d-cultured donor cells ($t = 0.39$, $df = 47$, $P = 0.70$, t -test; Fig. 4B). 10d-18d was not included in the statistical analysis of positive cells per positive gonad as only one embryo gonad was positive for donor-derived cells.

Co-localization—PKH26 stained donor-derived cells (fresh chicken Fig. 6A–D, fresh Reeve's pheasant Fig. 6E–H, 3d-cultured black-necked swan Fig. 6I–L, and 3d-cultured chicken Fig. 6M–T) from dissociated host gonads exhibited co-localization with the stem cell markers anti SSEA-1, VASA, EMA-1 antibodies and the nuclear stain Hoechst 33342. Dissociated control gonads were anti SSEA-1, VASA, EMA-1 antibody and Hoechst positive, but PKH26 negative (Fig. 6I–12).

DISCUSSION

Chicken embryos have historically been very important in the study of embryology, mainly due to their robustness and easy access. In addition, fertilized chicken eggs are easily obtainable in large numbers and can be maintained with minimal effort and cost, making them the ideal host species. It takes approximately 2.5 days for the host chicken embryo to reach stages 14–17, the point at which the endogenous primordial germ cells migrate through the circulatory system to colonize the gonadal ridge (Urven et al., 1989; Perez-Aparicio Francisco et al., 1998). Keeping the donor cells alive until transfer may be accomplished in several different ways, including: retaining the cells in the donor embryo, culture, or cryopreservation. The optimal method is dependent on the time between donor cell isolation and transfer.

The use of ART in the conservation of species whose populations are threatened or endangered is bound to increase in the future. In fact, the more endangered the species is, the greater the chance that ART will be utilized. One of the techniques that will become routine, especially for larger species of birds, is in ova sexing, which facilitates management of the sex ratio within a collection by selectively incubating embryos of the desired sex. While this is suitable for current flock management, the individuals not incubated still represent a loss of important genetic diversity. However, the use of GGC cryopreservation and xenotransfer could provide a highly effective long-term gene pool management tool that would make it possible to reintroduce an individual's genetic information back into the gene pool years or decades later.

In this study, we demonstrated that the percent SSEA-1, -3, and -4 positive cells increased dramatically following 3-day culture of chicken, black-necked swan and Reeve's pheasant embryonic gonadal cells when compared to freshly isolated cells (Fig. 1). However, fresh chicken donor-cell transfers yielded a significantly higher percentage of positive host gonads when compared to 3d-cultured donor-cell transfers (Fig. 4, Table 1). Although the trend was similar for the exotic species transfers, the difference was not significant (Fig. 4, Table 1)

indicating that culture conditions may differ between species. Furthermore, a decrease in the percent positive hosts could be explained by greater proliferation of the chicken donor-derived cells within the chicken host environment, as intensity of the membrane-bound PKH dye decreases following each mitotic division.

Therefore, even though the observed decrease in percent positive host embryos suggest that GGCs should not be cultured following isolation, this hypothesis needs to be tested with a mitosis-resistant dye, such as green fluorescent protein. In addition, it is likely that some cellular reprogramming of donor cells occurs in the host embryo, as seen following transfer of adult testicular cells to embryonic host gonads (Minematsu et al., 2004; Jung et al., 2010; Roe et al., 2013). However, if reprogramming does not occur within the time the gonadal ridge migration signal is available, donor-derived cells may be unable to migrate and colonize, explaining the lack of larger numbers of donor-derived cells. Nevertheless, this study strongly indicates that at least a small percentage of donor-derived cells are indeed germline stem cells (SSEA-1, VASA, EMA-1 antigenic; Fig. 6), and that their migration to and colonization of the gonadal ridge is highly conserved across taxonomically distant species.

Transferred cells were observed throughout the embryo in the vascular system immediately following transfer (data not shown). By six hours post-transfer, both chicken and exotic species donor-derived cells were visible within the vasculature and the caudal region (Fig. 2). By 16 hours post-transfer, the fluorescent cells were detectable within the endoderm, including the gonadal ridge area within the midgut wall, but not in the lateral body wall or around the spinal cord or somites (Fig. 3). These data show that some donor-derived cells are capable of active migration out of the vasculature and into the midgut wall area and the gonadal ridge, although the transferred GGCs do not exhibit as tight a distribution as the endogenous GGCs (Fig. 3). The percent of endogenous anti SSEA-1, -3, and -4 antibody positive cells (2.3%, 4.2% and 1.7% in males and 2.6%, 3.2% and 2.3% in females) detected in chicken gonads at stage 36 were similar to the previously reported roughly 2% of stage 27 embryo gonad cells sorted by FACS using the SSEA-1 antibody (Mozdziak et al., 2005). The percent of donor-derived cells within stage 34–36 host gonads ranged from 0.015 to 0.44% and 0.039 to 0.38% for the exotic and chicken donors, respectively (Table 1). This low colonization rate of donor-derived GGCs could be explained by a low number of transferred cells maintaining or gaining the ability to accurately migrate to the host gonadal ridge, as exemplified by the diffuse distribution of donor-derived cells within the host midgut wall when compared to the anti SSEA-1 antibody positive cells (Fig. 3). Our study shows that only a fraction of the donor cells are capable of active and accurate migration. In the exotic donor species, the percent of anti SSEA-1 antibody positive cells ranged from 0.25 to 2.57%, or 25 to 260 GGCs per 10,000-cell transfer. In chicken donors, the percent anti SSEA-1 antibody positive cells ranged from 2.33 to 2.55%, or 233 to 255 GGCs per 10,000-cell transfer. The number of endogenous anti SSEA-1 antibody positive cells within a stage 36 embryo gonad is approximately 50,000 of the roughly 2×10^6 cells per gonad (both testes/left ovary; Roe et al., 2013) compared to the 300–8,800 (0.015 to 0.44%) exotic donor-derived cells and 800–7600 (0.040 to 0.38%) chicken donor-derived cells (Table 1). It is worth noting that after five mitotic divisions, 250 donor cells would number approximately 8000 cells. The amount of PKH26 dye available following four to five

mitotic divisions would be at the limit of detection in this model. Therefore, we may not be able to detect very successful transfers using membrane-intercalating dyes.

Previous work has demonstrated that the genetic sex of the host or donor PGCs does not influence the donor PGCs' migration or colonization during the early development (Ono et al., 1996). Likewise, this study did not detect a statistically significant influence of host and donor GGC sex on the colonization of the host gonad (Fig. 5). However, these studies only include the initial migration and colonization of the host gonad, prior to the pre-hatch gonadal organization. Several studies propose that transgender transfers most likely encounter developmental restrictions during the latter embryological stages of gonadal development (Tagami et al., 1997; Naito et al., 1998; Furuta et al., 1999; Naito et al., 1999; Yamaguchi et al., 2000), resulting in abnormal gonadal development including production of ovotestes or complete gonadal sex reversal with low donor-derived gamete production.

This study found no relation between the success of donor-derived cell migration and the host/donor taxonomic distance (Table 1) for the species used in this study, including donors from closely related families within the order Galliformes (*Polyplectron*, *Syrnaticus*) and from distant orders (Columbiformes, Musophagiformes, Coraciiformes, Psittaciformes, Anseriformes, and Passeriformes). This study was not designed to demonstrate if functional sperm could be matured from the donor-derived cells, but it did show that xenotransferred GGCs from multiple species could be localized to the gonadal region of the mid-incubation chicken host embryo. In addition, by stage 36 (mid-incubation) the donor-derived cells co-localized with the gonadal stem cell markers anti SSEA-1, EMA-1 and VASA antibodies suggesting that these cells maintained stem cell-ness.

This study demonstrated that xenotransfer of exotic species' GGC to chicken hosts is feasible and results in appropriate migration and colonization, and therefore could potentially be a part of future conservation programs for a wide variety of species.

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Fig. 1.

The change in percent of cells positive for expression of the stem cell markers SSEA-1, SSEA-3, and SSEA-4 in freshly isolated cells and following 3d-culture. Different letters denote statistical significant difference between fresh (grey bars) and 3d-culture (black bars). Chicken samples are triplicate cultures each containing three sets of testes (**A**) or three left ovaries (**B**); black-necked swan (**C**) is a single culture containing gonads from two males and two females; Reeve's pheasant (**D**) is a single culture containing gonads from one male and four females. Results are expressed as mean $\pm$ sem.

Fig. 2.

Stage 17 host embryos 6 hours post-injection of freshly isolated PKH67 stained embryonic gonadal germline stem cells from chicken (**A**) and Mindanao bleeding-heart (**C**). (**B**) and (**D**) show close-ups of (**A**) and (**C**), respectively. The PKH-stained cells can be seen throughout the caudal area. Control embryo stage 16 (**E**), nt: neural tube, s: somites, arrow: PKH positive cells, scale bar represents 200 micron.

Fig. 3.

Stage 19 host embryos 16 hours post-injection of freshly isolated PKH67 stained embryonic gonadal germline stem cells from Reeve's pheasant (**A–D**) and chicken (**E, F**). Control embryo (**I**) and control embryo following SSEA-1 antibody staining for endogenous germline stem cell localization (**G, H**). (**B, D, F, H**) show close-ups of (**A, C, E, G**), respectively. The PKH stained cells can be seen throughout the caudal endoderm, including the gonadal ridge. lb: limb bud; bracket: gonadal ridge and positive cells, scale bar represents 200 micron.

Fig. 4.

The percent of host gonads with detectable chicken embryo donor-derived cells and percent of donor-derived cells following injection with fresh or 3d-cultured cells (**A**). The percent of host gonads with detectable exotic embryo donor-derived cells and percent of donor-derived cells following injection with fresh and 3d–10d-cultured cells (**B**). Grey bars represent percent positive gonads. Black bars represent percent positive cells per positive gonad. Percent positive cells per positive gonad results are expressed as the mean $\pm$ sem. Different letters denote statistical significance.

Fig. 5.

Effect of donor and host sex on percent host gonads positive for donor-derived cells and percent donor-derived cells in positive host gonads following injections with fresh (**A**) and 3d-cultured (**B**) cells. Grey bars represent percent positive gonads. Black bars represent percent positive cells per positive gonad. Percent positive cells per positive gonad results are expressed as the mean $\pm$ sem.

Fig. 6. Co-localization of donor-derived cells (PKH26 positive) (**A, E, I, M, Q**) with the anti SSEA-1 (**B, F, J**), VASA (**N**), and EMA-1 (**R**) antibody stem cell markers. Hoechst staining (**C, G, K, O, R**) and bright field (**D, H, L, P, T**) show nuclear and cell morphology. Control embryo gonads were anti SSEA- 1 (**2**), VASA (**6**), and EMA-1 (**10**) antibody positive, but no PKH26 (**1, 5, 9**) positive cells were detected. Scale bar represents 20 micron.

Table 1

Donor cell origin and host colonization success
Effect of transfer using fresh and varied lengths of cultured donor cells from 13 exotic species, compared to fresh and 3d-cultured chicken donor cells.
Low numbers of injected host embryos are due to limited numbers of cells available from donor embryos or culture.

Order	Species	Donor cells	# Embryos injected	# Embryos surviving	# Positive embryo gonads	Range of positive cells
Galliformes	Mountain peacock-pheasant <i>Polyplectron inopinatum</i>	Fresh	10	5 (50%)	5 (100%)	0.054%–0.261%
	Reeves's pheasant <i>Symaticus reevesii</i>	Fresh	36	24 (67%)	14 (58%)	0.015%–0.208%
		3 day culture	19	16 (84%)	5 (31%)	0.042%–0.154%
		18 day culture	5	5 (100%)	0	–
Columbiformes	Victoria crowned pigeon <i>Goura victoria</i>	9 day culture	6	2 (33%)	2 (100%)	0.138%–0.225%
	Pheasant pigeon <i>Otidiphaps nobilis</i>	Fresh	4	3 (75%)	3 (100%)	0.088%–0.204%
	Mindanao bleeding-heart <i>Gallucolumba crinigera</i>	Fresh	8	6 (75%)	2 (33%)	0.035%–0.072%
	Coroneted fruit-dove <i>Ptilinopus coronulatus</i>	9 day culture	6	4 (67%)	3 (75%)	0.037%–0.070%
	Beautiful fruit-dove <i>Ptilinopus pulchellus</i>	Fresh	4	3 (75%)	1 (33%)	0.04%
	Mariana fruit-dove <i>Ptilinopus roseicapilla</i>	14 day culture	8	5 (63%)	1 (20%)	0.05%
	Great blue turaco <i>Corythaeola cristata</i>	Fresh	4	3 (75%)	2 (67%)	0.030%–0.041%
		6 day culture	7	5 (71%)	3 (60%)	0.040%–0.182%
		18 day culture	5	3 (60%)	0	–
	Coraciiformes	White-throated kingfisher <i>Halcyon smyrnensis</i>	Fresh	5	3 (60%)	2 (66.7%)
Psittaciformes	Papuan lorikeet <i>Charmosyna papou</i>	Fresh	3	2 (67%)	1 (50%)	0.10%
Anseriformes	Black-necked swan <i>Cygnus melancoryphus</i>	3 day culture	13	8 (62%)	4 (50%)	0.054%–0.274%
Passeriformes	White-rumped shama <i>Copsychus malabaricus</i>	7 day culture	5	4 (80%)	2 (50%)	0.052%–0.055%
Totals (not including <i>G. gallus</i>)		Fresh	74	49 (66%)	30 (61%)	0.015%–0.441%
		3–10 day culture	56	39 (70%)	19 (49%)	0.037%–0.274%

Order	Species	Donor cells	# Embryos injected	# Embryos surviving	# Positive embryo gonads	Range of positive cells
Galliformes	Domestic chicken Gallus gallus	10+ days in culture	18	13 (72%)	1 (8%)	0.05%
		Fresh	49	31 (63%)	21 (68%)	0.39%–0.378%
		3 day culture	36	32 (89%)	7 (22%)	0.041%–0.148%