Production of Transgenic Chimeric Chicken from Cryopreserved Primordial Germ Cells and its Validation by Developing shRNA Transgenic Chicken Chimera

D Divya
Directorate of Poultry Research
R Shukla
Directorate of Poultry Research
RN Chatterjee
Directorate of Poultry Research
G Sagar
Directorate of Poultry Research
A Rajendra Prasad
Directorate of Poultry Research
Tarun K Bhattacharya (bhattacharyatk@gmail.com)
Directorate of Poultry Research

Research Article

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Abstract

Primordial germ cells (PGCs) are precursors of gametes in birds. For ex-situ conservation and production of transgenic birds, there is a limitation for preservation of oocytes in birds as compared to other mammalian species. To overcome those limitations, PGCs have been used as candidate cells, which have been cryopreserved and manipulated and used as to produce transgenic birds. In this study, cryo-preserved PGCs were used to produce transgenic birds. The protocol for production of transgenic birds with cryo-preserved PGCs was developed and the success rate for production of transgenics 16.7% in the protocols established in the study. The same gene transfer protocol through PGCs was validated by transferring shRNA molecule of SREBP-1 gene to the host genome to produce transgenic chimeric birds and the success rate for production of transgenic chimeric chicken was 40%. Finally, it is concluded that a standard protocol for ex-situ conservation of birds through PGCs and production of transgenic birds from cryo-preserved PGCs and knock down birds from PGCs were developed. It may be suggested that these protocols for resurrecting live birds from cryo-preserved PGCs may be applied as model for protecting the endangered birds from their extinction.

Introduction

Conservation of any chicken population can be accomplished by either *ex situ* or *in situ* methods. The e*x situ* method has certain advantages over *in situ* method. The *ex situ* method can be employed to save the existing native, endangered, threatened and improved breeds of chicken, and use in production of genetically modified birds etc.[1,2]. On the other hand, *in situ* conservation needs huge monetary and infrastructure to maintain birds and protect from diseases and other calamities in the farm. The *ex-situ* conservation in birds may be carried out by cryo-preserving the cells which can be revived into live birds. In chicken, cryo-preservation of sperm has been successful, but cryo-preservation of oocytes has limited success as the collection of oocytes is tedious and requires sacrificing the hens. Further, *in-vitro* fertilization in birds is also not much successful due to lack of availability of good quality oocytes. Under such scenario, cryo-preservation of another type of cells, called as primordial germ cells (PGCs), which are diploid in nature and have multiplication ability have been successful to revive the live birds[1,2.]

Primordial germ cells are highly specialised cells that are precursors of gametes in birds and other animals[3,4]. The PGCs are divided through meiosis and developed to haploid sperm and oocytes. Ultimately, sperm and oocyte develop organism upon fertilisation. They transmit genetic and epigenetic information across the generations and ensure the survival of a species indicating its potentiality in maintaining generations across the species. PGCs are developed from pluripotent epiblast and differentiated into functional gametes in the adult animals. Chicken PGCs (cPGCs) are found in the blood vessels of the chicken embryos during Hamburger-Hamilton (H&H) stage 13-15[5,6]. The number of PGCs transported to the germinal crescent through blood vessels are decreased gradually from H&H stage 13-15 onwards[7,8]. The PGCs in the germinal crescent are ultimately differentiated into either oocytes or spermatozoa. The resultant oocytes/spermatozoa may be utilized upon fertilization to develop live birds. The PGCs and their resurrection method is to be optimized efficiently so that PGCs can be preserved and can be used further to develop live animals during exigencies. Hence, the objectives of this study were to (a) standardize protocol to preserve PGCs and to resurrect cryo-preserved PGCs to live birds and (b) to validate the protocol to produce chicken chimera.

Results

Characterization of PGCs. PGCs were collected from the 60h old embryos during the HH embryonic stage of 13 to 15. PGCs were cultured on the chicken fibroblast feeder cells with conditioned media at 37°C and 5% CO_2 (Fig. 1). The PGCs were characterized by cell surface markers such as CXCR, VASA, SOX, BLIMP, POUV, MYC, KLF4 and NANOG. These markers were expressed in the surface of PGCs. Expression of these markers were detected by real time PCR (Table 1 and Fig. 2). However, these markers were not expressed in the matured fibroblast cells, which were used as feeder cells. The PGCs were also analyzed with alkaline phosphatase staining where PGCs took red color stain and differentiated cells did not take any stain (Fig. 3). The PGCs were also differentiated with PAS staining where the cell nuclei took blue colour stain and other polysaccharide, glycogen, collagen, phospholipids and basal membrane stained purple (Fig. 3).

Table 1

Expression of surface markers of PGCs (40-dct) in chicken.

Parameter	Marker genes							
	CXCR	VASA	SOX	BLIMP1	POUV	MYC	KLF4	NANOG
Expression (40-dct)	47.52	46.80	46.36	37.15	43.70	43.31	41.04	45.19

The immuno-cytochemistry study with SSEA-1, SSEA-4, TRA1-60 and TRA1-81 markers revealed the presence of these cell surface markers in PGCs (Fig. 4) and not in differentiated cells. We also observed the normal karyotypes of PGC chromosomes revealing both macro and micro-chromosomes (Fig. 5).

Transfection.

The PGCs were transfected with recombinant GFP-pAcGFPC1 construct which was used as marker for donor PGCs. The GFP expression was checked in transfected and non-transfected PGCs.

Cryo-preservation.

The transfected and non-transfected PGCs were multiplied through subsequent sub-culture of cells. Before initiating cryo-preservation of PGCs, the cell concentration was 1.92 X 10^6 cells/ml. The cultured PGCs were collected with DMEM media containing 3% and 5% DMSO and followed cooling protocol to reach the ultra low temperature at liquid nitrogen. In case of 3% DMSO containing media, after one month of cryo-preservation, the live PGC concentration was 82.4% while in case of 5% DMSO, the live cell concentration was 68.3%. Upon six months of cryo-preservation, in case of 3% DMSO, the live cell concentration was 81.6% while in case of 5% DMSO, it was 49.7%. Thus, higher concentration of DMSO was found to be toxic for PGCs of native indigenous chicken breeds making the number of dead PGCs being increased.

GFP expression. The GFP expression was observed in PGCs before and after cryo-preservation. The transfected PGCs showed 3.2 folds lower expression after one month of cryo-preservation conserved in media containing 3% DMSO, whereas after six months of cryo-preservation, the GFP expression was 4.6 folds lower as compared to fresh un-cryopreserved PGCs (Table 2). In case of 5% DMSO containing media, the GFP expression in cryo-preserved PGCs after one month of cryo-preservation was reduced by 10.2 folds and after six months of cryo-preservation, it was reduced by 21.3 folds.

Table 2

Fold change of expression of PGC marker genes in fresh PGCs as compared to the cryo-preserved PGCs.

Compared to	CXCR	VASA	SOX	BLIMP1	POUV	MYC	KLF4	NANOG
Day 30	1.09	1.02	0.97	0.08	6.77	8.85	2.44	7.26
Day 180	0.38	0.46	0.46	0.35	4.13	3.16	1.70	3.01

Transplantation of PGCs.

The six months old cryo-preserved transfected PGCs as treatment, and non-transfected cryo-preserved along with un-cryopreserved PGCs as control were transferred to the recipient embryos of HH stage 13 to 15 so that the birds developed by utilizing transfected PGCs could be detected through GFP marker.

Fertility of eggs for X-ray exposure did not differ among the groups with one or three or five times X-ray exposure, while these treatment groups showed lower fertility than the control groups (Table 3). The hatchability on fertile egg set basis was lower in treatment groups than the control groups. Among the treatment groups, one exposure group showed better hatchability over three or five exposures. The three and five exposure groups had similar hatchability on fertile egg set basis. Accordingly, the hatchability on total egg set basis was lower in treatment groups than the control groups. Among the treatment groups, one exposure showed better hatchability over other two treatment groups. The percentage of good chicks was also higher in one exposure group than the three or five exposure groups. In case of five exposure group, there were no good chicks hatched whereas in control groups, all the chicks were of good quality. In treatment groups, dead chicks and chicks with anatomical deformities were observed indicating good chicks in one exposure of X-ray over three or five exposures. Overall, five exposure of X-ray was found to be the worst method to make the embryo infertile as this protocol affected the viability of embryos causing the mortality and deformities in chicks.

Table 3

Hatching performance of the recipient embryos for production of chimeric chicks.

Protocol	No. of eggs incubated	No. of fertile embryos	No. of chicks hatched	Fertility (%)	Hatchability on fertile egg set basis (%)	Hatchability of total egg set basis (%)	Good chicks (%)	Dead or chicks with deformities (%)	GFP Positive chicks (No.)	Positive birds (%)
One pulse 36KV	12	6	4	50	66.6	33.3	75	25	2	50
Three consecutive pulses with 10 sec interval	12	6	2	50	33.3	16.6	50	50	2	100
Five consecutive pulses	12	6	2	50	33.3	16.6	0	100	0	0
Transfected without X-ray irradiation	12	11	10	92	90.9	83.3	100	0	0	0
Un-transfected PGCs without X-ray irradiation	12	11	10	92	90.9	83.3	100	0	0	0

Chimeric chicks.

The chimeric chicks (Fig. 6) were identified by PCR and sequencing of GFP gene fragment of pAcGFPC1 construct, which was transferred through PGCs into the recipient embryos (Fig. 7). In the chicks of treatment group, we observed presence of GFP expression in the gonadal cells, but in other tissues such as leg muscle, liver and gizzard, we did not find presence of GFP expression. The level of GFP expression varied among the chicks, but, in all the chicks of the

treatment groups, we found the GFP expression in gonadal cells (Table 4). In gonads of the chicks of the control groups, we did not find presence of GFP expression indicating the importance of protocol making the embryos infertile so that the PGCs of host embryo get distracted and inactivated.

Table 4

GFP marker gene expressed in the gonads of the chimeric chicks.

Protocol	GFP expression (40-dct) in gonads
One pulse (36KV)	47.3
Three consecutive pulses (36 KV) with 10 sec interval	41.5
Five consecutive pulses (36 KV)	0
Transfected without X-ray irradiation	0
Un-transfected PGCs without X-ray irradiation	0

Sequence of 256 bp GFP gene fragment amplified from genomic DNA isolated from gonads of chimeric chicks:
GCTGAATGGCGATGTGAATGGCCACAAGTTCAGCGTGAGCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCT(

FORWARD PRIMER

GCTGAATGGCGATGTGAATG…….

The GFP expression in gonads of chicks derived from three exposures of X-ray irradiation group did not differ significantly from one exposure group. Our results indicated better efficiency of transfer of donor PGCs from three exposure group than one exposure group. In case of one exposure group, there might be the chance of presence of both host and donor PGCs in which donor PGCs do have GFP construct integrated at their genome and host PGCs do not have other exogenous construct. In case of three exposure, there may be chance of greater population of donor PGCs showing higher GFP expression. In case of five exposure group, the health of the embryos was most adversely affected and may have induced mutation in viability genes causing hatching of dead chicks with fully developed organs. We suggest that the three X-ray exposures may be the best protocol for resurrection of cryo-preserved PGCs into live transgenic birds.

Validation of protocol by developing chimeric anti-SREBP-1 shRNA chicks.

Chicks were hatched both in the treatment (Fig. 8) and control groups (Table 5). In the treatment group, 2 chicks were hatched, which was confirmed by PCR and sequencing (Fig. 8) and both were found as normal without having any anatomical deformities. The hatching percentage at the treatment group was 40 %. In the control group, one chick was hatched and the hatching % was 20%. In the chicks (day old age) of treatment group, we observed presence of shRNA construct in the gonadal cells, but in other tissues such as leg muscle, liver and gizzard, we did not find the construct indicating that the transgene has been sheded in the gonadal cells only. Thus, through PGCs mediated transfer, we have developed chimeric chicks possessing the anti-SREBP-1 shRNA construct where efficiency of obtaining positive birds with respect to the total chicks hatched in the treatment group was 100%. However, with respect to the total eggs used in the study under treatment group, the percentage of obtaining shRNA possessing birds was 40%.

Sequence of 282 bp fragment of pENTRU6-shRNA construct amplified from the genomic DNA isolated from the gonads of chimeric chicks:
GCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGT

FORWARD PRIMER

GCTTCAGCAGAGCGCAGA…….

Table 5

Hatching performance of the SREBP-1 shRNA chimeric and control chicks.

Protocol	No. of eggs incubated	No. of fertile embryos	No. of chicks hatched	Fertility (%)	Hatchability on fertile egg set basis (%)	Hatchability of total egg set basis (%)	Good chicks (%)	Dead or chicks with deformities (%)	shRNA Positive chicks (No.)	Positive birds (%)
Donor PGCs transfected with shRNA and recipient embryo exposed with 3 pulses of X-ray exposure	5	3	2	60	67	40	100	0	2	100
Control-I (Donor PGCs non-transfected with shRNA and recipient embryos exposed with 3 pulses of X ray exposure)	5	2	1	40	50	20	100	0	0	0
Control-II (Donor PGCs non-transfected with shRNA and recipient embryos not exposed with 3 pulses of X ray exposure)	5	2	1	40	50	20	100	0	0	0

Discussion

We isolated the PGCs from the chicken embryos, which were characterized with several markers and cytochemistry, and the results are similar to the findings of earlier studies[9-17]. The chromosomes revealed both macro and micro-chromosomes in PGCs, which was in agreement with the earlier reports[18]. The PGCs were cryopreserved in liquid nitrogen for which the protocol with 3% DMSO was standardized to have better revival and viability, which was in agreement with the previous reports[19-21]. The six months old cryopreserved PGCs were used for resurrection so that cryo-preserved PGCs may be revived and the birds may be produced. The cryo-preservation of PGC may be further used as conservation method in chicken, which later be revived to produce live birds[22].

The cryo-preserved PGCs were transplanted in the blood vessels of recipient embryos. The recipient embryos were of mainly two types, one was treatment groups and other one was control groups. In the treatment groups, we exposed embryos with (a) one, (b) three and (c) five exposures of X-ray. In case of control groups, (a) it was lack of exposure and use of transfected PGCs and (b) lack of exposure and use of un-transfected PGCs. In treatment groups, single exposure of X-ray on embryo showed better hatchability and lower anatomical deformities of chicks. As the number of X-ray exposure increased, the hatchability of eggs was decreased with higher incidence of death and deformities in chicks[23]. We suggest that three exposures of X-ray of 36KV may produce better quality of chicks with good health condition possessing donor type germ cells. Though single exposure showed better hatchability and quality of chicks, the transfer and deposition of germ cells in the recipient embryo was comparatively poor than the three exposure group being indicated in the GFP marker gene expression. It is stated that the recombinant GFP construct was transfected into the PGCs as positive markers for confirmation of presence and functionality of donor PGCs. As donor PGCs were the viable ones, they were prevailed in the recipient embryos being determined through the GFP expression in the gonads of chicks of the treatment groups. Thus, the chimeric chicks having properties of donor PGCs were hatched, which was in agreement with the earlier reports, where chimeric chicks were developed from fresh PGCs[8, 24-32]. However, in our study, we have standardized the protocol of resurrection of cryo-preserved PGCs into live birds which can further be used as efficient protocol for production of transgenic birds and for conservation of important chicken breeds/lines.

Further, using this technology, we have developed chimeric chicken possessing anti-SREBP-1 shRNA construct. This shRNA construct has potential to minimize the expression of SREBP-1 gene, which is involved in synthesis of cholesterol and tri-glycerides through *de-novo* lipid biosynthesis pathway. By such validation study, we have perfected the protocol to produce chimeric chicks. As our aim was to establish the protocol to produce chimeric chicks, we sacrificed the chicks to collect the gonads for screening of positive birds. We have not raised the positive birds to study gene knock-down by transferring shRNA constructs into the host genome. Earlier, transgenic knock-down birds were developed through other methods by transferring shRNA constructs for the specific genes [33-43]. Thus, the protocol to develop shRNA based transgenic birds have been established through PGC mediated method.

It is concluded that the protocols for development of chimeric chicks from cryo-preserved PGCs and development of gene knock down chicken through PGC based method were established in chicken. These protocols can be used further to resurrect the birds from donor PGCs and to develop chimeric birds for further development of transgenic chicken to produce high value proteins of therapeutic/neutraceutical importance and to improve the performance of birds.

Methods

Animals and fertilized eggs.

The PB-2, a pure line broiler chicken line was used in the present study. The PB-2 birds are used to produce Krishibro commercial colour broiler chicken for meat purpose[44]. A total of 60 fertile eggs of PB-2 were collected from the Institutional farm of ICAR-Directorate of poultry Research, Rajendranagar, Hyderabad, India. We grouped all the eggs into three treatment groups, one control group with transfected PGCs and one un-transfected un-irradiated control group. We kept 12 eggs each in each treatment group and 24 eggs were set in the two control groups. There were three treatment groups comprising of Group I (One time X-ray exposure of 36KV), Group II ((Three times X-ray exposures of 36KV each with 10 sec interval) and (Five times X-ray exposures of 36 KV each with 10 sec interval) while control groups were lack of any X-ray exposure. The whole experiment was approved by the Institute Animal Ethics Committee (IAEC) of ICAR-Directorate of poultry Research, Rajendranagar, Hyderabad, India. The work was approved by the Institute Biosafety Committee (IBSC) of ICAR-

Directorate of Poultry Research, Hyderabad, India and by the Review Committee of Genetic Manipulation (RCGM), Department of Biotechnology, Government of India. All methods in this study were performed following the guidelines and regulations of IAEC, IBSC and RCGM.

Collection of PGCs.

The fertile eggs were incubated in the Egg incubator at 37.5^{o}C and 85 to 90mm Hg of relative humidity for 18 days. The automatic turning was set in the machine for 6 times a day. The temperature and humidity were monitored closely in the incubator. On 3rd day of incubation i.e. 60 hours of incubation of eggs, a window was prepared by cutting egg shell in the broad end of the egg with a diamond pen. The piece of shell and shell membrane was removed from the egg and embryonic blood vessels were observed in the embryo (Fig. 1). All the blood vessels were collected from the embryo and kept in the eppendorf tube. The collected blood containing PGCs were counted in the Neubeur chamber after staining the cells with Trypan blue stain. The viability of the PGCs were also observed with this staining.

Preparation of feeder cells.

Primary chicken embryo fibroblast cell culture was prepared from 10 day old chicken embryos following the standard method[35]. The fibroblast cells were cultured in DMEM mixed with 10% FBS, 100U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2 in a CO_2 incubator. Cells were cultured for 36 h and reached about 90% confluency for further use as feeder cells.

Culture of PGCs.

The blood containing PGCs were spread over the feeder cells in the 25 cm^2 cell culture flask. The conditioned media was prepared in another flask containing adherent fibroblast cells with 70 to 90% confluency. The DMEM with 10% FBS and 100U/ml penicillin and 100 µg/ml streptomycin was used for culturing fibroblast cells. The fibroblast cells were cultured for 36 h and the media was collected as conditioned media expectedly having the growth factors, nutrients and other factors essential for growth of cells. After pouring blood cells on the feeder cells, the flask was incubated in the CO_2 incubator at 37^{o}C and 5% CO_2 for 5 to 6 days. However, on every 3 days, media was changed with fresh conditioned media. After 6 days of incubation, the PGCs were harvested very carefully with cell scrapper to collect PGCs.

Transfection of PGCs.

The PGCs were prepared for transfection by washing with only DMEM media which was devoid of FBS and antibiotic. The GFP-pAcGFP1-C1 GFP construct (Takara Bio-Sciences) was transfected into PGCs by electroporation with Gene pulsar (Biorad). Electroporation was performed at 160 mV and 1 pulse for 25 msec. Upon transfection, the PGCs were seeded on feeder cells with conditioned DMEM and incubated in CO_2 incubator at 37^{o}C and 5% CO_2 for 6 days. On every 3 days interval, media was changed with fresh conditioned media for better cellular growth. The transfection was assessed with real time PCR for analyzing GFP expression in the PGCs.

Cryo-preservation of cPGCs.

The PGCs both transfected and un-transfected were taken in cryo-vials containing DMEM with 10%FBS and DMSO. Two concentrations of DMSO namely, 3% and 5% were tested for optimization of cryo-preservation protocol for preserving PGCs. Before keeping PGCs in the media, the media was prepared by mixing with DMSO. After PGCs kept in the cryo-vial containing media mixing with DMSO, the tubes were placed at -20^{o}C for 6 h in the -20^{o}C refrigerator after which the vials were transferred to -40^{o}C deep freezer for 6 h. Then, the vials were kept at -80^{o}C deep freezer for 12 h. The vials were ready for transfer to LN_2 container. The vials were placed in the tube holder and the tube holders were dipped into the liquid Nitrogen in the LN_2 cryocan for cryo-preservation.

Alkaline phosphatase staining.

Cell lysate containing cultured primordial germ cells were placed on a glass slide and a smear was prepared, air dried and fixed on slide with fixative viz. 4% paraformaldehyde in PBS for 90s as per manufacturer's protocol (Millipore's ES Cell characterization kit, Cat. No. SCR001). The fixative was aspirated and rinsed with rinse buffer (TBST). Then, staining solution was added on the smear and incubated for 15 min in dark at room temperature and rinsed with rinsing buffer. Then, the colonies were counted with red PGCs against number of differentiated colourless colonies.

PAS staining.

Slides of PGCs were prepared and fixed PGCs rinsed once with distilled water and incubated with Reagent I (Periodic acid solution) for 5 min as per the protocol of PAS staining kit (Millipore, Cat. No. 1.01646.0001). Then, smear was treated with reagent II (Schiff's reagent) for 15 min and with Hematoxylin solution (Modified according to Gill III) for 2 min. Then the slide was placed to graded ethanol concentration from 70 % to 96 % followed by 100% ethanol. Finally, the slides were mounted with xylene and observed for the PGCs where the cell nuclei stained blue and other polysaccharide, glycogen, collagen, phospholipids and basal membrane stained purple.

Immuno-cytochemistry.

Cell lysate containing PGCs were smeared on a slide and air dried. After drying, they were fixed with a fixative (4% paraformaldehyde/PBS) for 20 min at room temperature and washed with rinse buffer (TBST) thrice each with 10 minutes incubation time. The cells were then, permeabilized with buffer containing 0.1% Triton X-100 for 10 min and blocked with 4% normal goat serum/PBS for 30 min at room temperature as per the manufacturer's protocol (Millipore's ES Cell characterization kit, Cat. No. SCR001). This was proceeded with incubation step with primary antibodies diluted each in 1:10 SSEA-1, SSEA-4, TRA1-60 and TRA1-81 for one hour at room temperature and washed thrice with TBST for 10 min incubation time in between. The cells were then incubated with secondary antibody for 1 hour at room temperature and washed thrice with rinse buffer. Finally, the slide was observed for the fluorescence under fluorescent microscope after mounting with mounting solution (xylene) under a cover slip.

PGC marker genes expression by real-time PCR.

The transfected and un-transfected PGCs before initiating cryo-preservation, and one and six months after cryo-preservation were taken in separate eppendorf tubes. The cells were washed with 1% DEPC treated PBS. Total RNA was isolated from the PGCs of transfected and un-transfected groups using Trizol following standard protocol[45]. The cDNA was synthesized with random hexamer primers (Verso cDNA synthesis kit, Cat. No. AB1453A). The real-time PCR was performed in Stratagene Mx3000P thermal cycler using platinum Maxima SYBR green mix (Applied Biosystems). The *GAPDH* gene was used as internal control for which one pairs of primers, namely, QGAPDHF: 5'-CTGCCGTCCTCTCTGGC-3' and QGAPDHR: 5'-GACAGTGCCCTTGAAGTGT-3' for GAPDH gene was designed from the chicken cDNA sequences of the *GAPDH* gene (Accession No. AF047874) with DNASTAR software to co-amplify a 119 bp *GAPDH* fragment[34]. The surface markers of PGCs such as Nanog, SOX2, cPouV, Sox2, cMyc, Klf4 and cvh, BLIMP1, Myc and Klf4 were analysed for characterization of PGCs[46-48] (Table 6). Reactions were prepared in triplicate with a final volume of 25 µl containing 12.5 µL Maxima syber Green, 0.5 µL ROX reference dye, 0.2 µM of each primer, and 2 µl cDNA. The qPCR conditions included initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 1 minute, and extension at 72°C for 30 seconds. Following amplification, a dissociation melting curve analysis was conducted with programming the PCR machine from 55°C to 95°C to detect possible nonspecific products. Fluorescence threshold was determined by default method at 32.5% with Stratagene software (Stratagene, La Jolla, CA, USA). The threshold cycle (Ct) value of each sample was noted, and average Ct values of each sample in duplicate qPCR reactions were estimated[49].

ΔCt =Average Ct of target gene - Average Ct of reference genes (GAPDH)

Fold change of expression was calculated by the formula, $2^{-\Delta\Delta Ct}$.

Table 6

Primers of the PGC marker genes used in the experiment.

Name of gene	Primer Name	Primer sequence (5'-3')	Annealing temperature (Tm) (°C)	Fragment size (bp)
CXCR4	CXCR4F	GCCATTCTGGTCTGTGGATG	56	151
	CXCR4R	GGCATGGACTATTGCCAGGT		
VASA	VASAF	TGACTTATGTCCCCCCTCCT	56	103
	VASAR	GTAATGCTGCTGGAGGGTCA		
SOX2	SOX2F	ATAAATACCGACCCCGGAGG	56	102
	SOX2R	CGGTCGTCATGGTATTGGTG		
BLIMP1	BLIMP1F	AGCAACTGGATGCGCTATGT	57	108
	BLIMP1R	AGGGATGGGCTTAATGGTGT		
cPouV	cPouV_F	TCAATGAGGCAGAGAACACG	55	267
	cPouV_R	TCACACATTTGCGGAAGAAG		
cMyc	cMyc_F	GCACAGAGTCCAGCACAGAA	57	234
	cMyc_R	GTTCGCCTCTTGTCGTTCTC		
cKlf4	cKlf4_F	AGCTCTCATCTCAAGGCACA	56	233
	cKlf4_R	GGAAAGATCCACTGCTTCCA		
Nanog	cNanog_F	TTGGAAAAGGTGGAACAAGC	54	140
	cNanog_R	GGTGCTCTGGAAGCTGTAGG		

Karyotyping.

The PGCs were seeded on fibroblast feeder cells in the 6 well culture plate and the cells were allowed to grow for 72h. Then, the media was removed and cell pellets were taken for cytogenetic studies. The cell pellets were mixed with hypotonic solution (0.075 M KCl) and incubated for 7 minutes. Then, 1 ml freshly prepared fixative (Methanol:Glacial Acetic acid- 3:1) was added in the cell mix and centrifuged at 1000 rpm for 10 minutes. Most part of the supernatant was decanted and about 1 ml was left in the tube. Then, slides were prepared by dropping cells from 1.5 ft height and stained with 2% Giemsa stain to visualize the chromosome under microscope at 40X magnification.

Viability of cryo-preserved PGCs.

The cryo-preserved PGCs were washed with PBS three times to make the cells free from DMSO. The cells were incubated with Trypan blue stain for 5 minutes. The stained cell mix was dropped on the Neubeur chamber and counted under sterio-zoom microscope. The stained cells were dead PGCs while unstained cells were live PGCs. The live and dead cells were counted and concentration of live cells enumerated.

Resurrection of chicken breed.

The cryo-preserved PGCs were taken out from the LN_2 container. The cells were washed first with PBS three times and then, washed with DMEM for three times. The cleaned PGCs were mixed with DMEM with 10% FBS, 100U/ml penicillin and 100 µg/ml streptomycin, which were incubated in the CO_2 incubator at 37^oC and 5% CO_2 for 6 h. Then, PGCs were washed with PBS two times and was made it ready for transfer.

The fertile eggs of three treatment groups having X-ray exposure on day1 and eggs of control group were already kept in the egg incubator for 60h to make it ready for accepting the PGCs. Three types of X-ray exposures namely, 36 KVA X-ray for 10s for 1 exposure, 36 KVA X-ray for 10s for 3 exposures and 36 KVA X-ray for 10s for 5 exposures were given to the day1 embryos following the standard protocol[50,51]. All the safety measures were taken during X-ray exposure. The window was made at the broad end of the egg with diamond pen and blood vessels were viewed. The prepared PGCs were taken in the disposable 1 ml syringe with 26 Gauze needle. The PGCs were injected in the blood vessels of the recipient embryo. The window was sealed with another egg shell and closed with wax. The eggs were kept in the Egg incubator at 37^oC and 85-90 mm of Hg relative humidity for 18 days. On 19th day, eggs were candled to determine the fertility of eggs and then, kept in the incubator again at 37^oC with 90mm of Hg relative humidity for 3 to 5 days. The chicks were hatched on 24th, 25th and even 26th day of incubation in the treatment groups while in the control group (without transfection), the chicks were hatched on 21st day.

Confirmation of positive knock-down birds by PCR and sequencing.

The chicks on day 1 were sacrificed following approved protocol of Institute Animal Ethics Committee (IAEC) and an incision was made on the mid-ventral side of the abdomen of the chicks. Digestive organs were taken out and liver and gizzard were removed. On both side of vertebral column above the kidneys, the gonads were located and gonads were collected in the DEPC treated eppendorf tube. All the chicks of both treatment groups and control group were sacrificed for collection of gonads and other tissues such as liver, gizzard, small intestine and thigh muscle. Genomic DNA was isolated from gonads of the chicks following standard protocol. Through PCR, the positive birds were screened and confirmed by sequencing (ABI prism 377). For PCR, a pair of primers (QGFPF: 5'-GCTGAATGGCGATGTGAATG-3' and QGFPR: 5'-CCTCGAAGAAGATGGTGCG-3') were designed from the GFP construct of pACFGPC1 vector for amplification of 256 bp fragment of the construct.

In addition, the gender of all the positive chicks were determined with gender specific primers (Patent No. 309612).

GFP expression study.

Total RNA was isolated from all the tissues including gonads, liver, gizzard, small intestine and thigh muscle using Trizol. The cDNA was synthesized with random hexamer primers (Verso cDNA synthesis kit, Cat. No. AB1453A). The quantification of mRNA expression of target gene (*GFP*) and internal control *(GAPDH)* were performed by real-time PCR with SYBR green dye. The *GFP* gene of fragment size 256 bp was amplified using QGFPF: 5'-GCTGAATGGCGATGTGAATG-3' and QGFPR: 5'-CCTCGAAGAAGATGGTGCG-3' and that of GAPDH (Accession No. AF047874) of size 119 bp by QGAPDHF: 5'-CTGCCGTCCTCTCTGGC-3' and QGAPDHR: 5'-GACAGTGCCCTTGAAGTGT-3'. The threshold cycle (Ct) value of each sample was noted, and average Ct values of each sample in duplicate qPCR reactions were estimated[37].

ΔCt =Average Ct of target gene - Average Ct of reference genes (GAPDH)

Fold change of expression was calculated by the formula, $2^{-\Delta\Delta Ct}$.

Development of SREBP-1 shRNA knock-down chicken

Preparation of construct.

One shRNA construct for sterol repeat element binding protein-1 (SREBP-1) gene was taken from an study conducted at our Lab under an *in vitro* hepatic primary cell culture study[52]. The shRNA molecule was cloned in pENTR/U6 vector (BLOCK-iT^{TM} U6 RNAi Entry Vector Kit, Invitrogen, Cat. No. K4944-00) and

the recombinant shRNA plasmid was multiplied manifold by culturing transformed DH5α *E. coli* cells to obtain a large volume of recombinant plasmid. The recombinant shRNA plasmid was linearized by digesting with *Pvu*I restriction enzyme following standard protocol (……).

Transfection of shRNA recombinant DNA into PGCs and its transfer.

The recombinant linear shRNA vector was transfected into PGCs by electroporation with Gene pulsar (Biorad). Electroporation was performed at 160 mV and 1 pulse for 25 msec. Upon transfection, the PGCs were seeded on feeder cells with conditioned DMEM and incubated in CO_2 incubator at 37^oC and 5% CO_2 for 6 days.

The fertile eggs of three treatment groups having X-ray exposure on day1 and eggs of control group were already kept in the egg incubator for 60h to make it ready for accepting the donor PGCs (shRNA vector transfected PGCs as treatment group and non-transfected PGCs as control group). The transfer was done through windowing technique already detailed above. The eggs were kept in the Egg incubator at 37^oC and 85-90 mm of Hg relative humidity for 18 days. On 19th day, eggs were candled to determine the fertility of eggs and then, kept in the incubator again at 37^oC with 90 mm of Hg relative humidity for 3 to 5 days. The chicks were hatched during 21st to 25th day of incubation.

Confirmation of positive knock-down birds by PCR and sequencing.

Gonads were collected from all the chicks hatched under the present study. Genomic DNA was isolated from gonads of the chicks following standard protocol. Through PCR, the positive birds were screened and confirmed by sequencing (ABI prism 377). For PCR, a pair of primers (pENTR/U6 FP: 5'-GCTTCAGCAGAGCGCAGA-3' and pENTR/U6 RP: 5'-CTCAATGCT CACGCTGTAG-3') were designed from the pENTR/U6 vector for amplification of 282 bp fragment of the construct.

Declarations

Data availability

The data has been provided in the article.

Acknowledgements

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Author contributions

DD & RS carried out the wet lab experiment. RNC prepared the tables. VG carried out the X-ray irradiation of fertile eggs. ARP conducted karyotyping of PGCs. TKB designed the study, analyzed all data and prepared the manuscript and the whole research work was conducted in the DBT funded research project of TKB.

Competing interests

The authors declare no competing interests.

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Figures

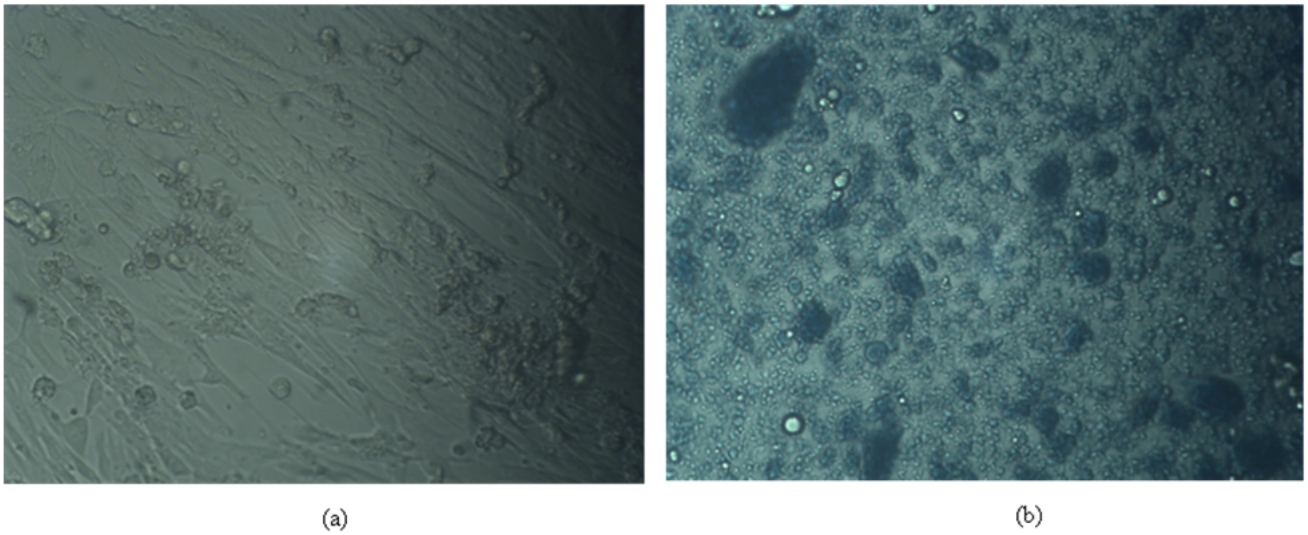

Fig. 1 Cultured PGCs. (a) Normal PGCs on feeder cells. (b) PGCs stained with trypan blue for counting live and dead PGCs. Dead PGCs took blue stain while un-stained PGCs were live cells.

Figure 1

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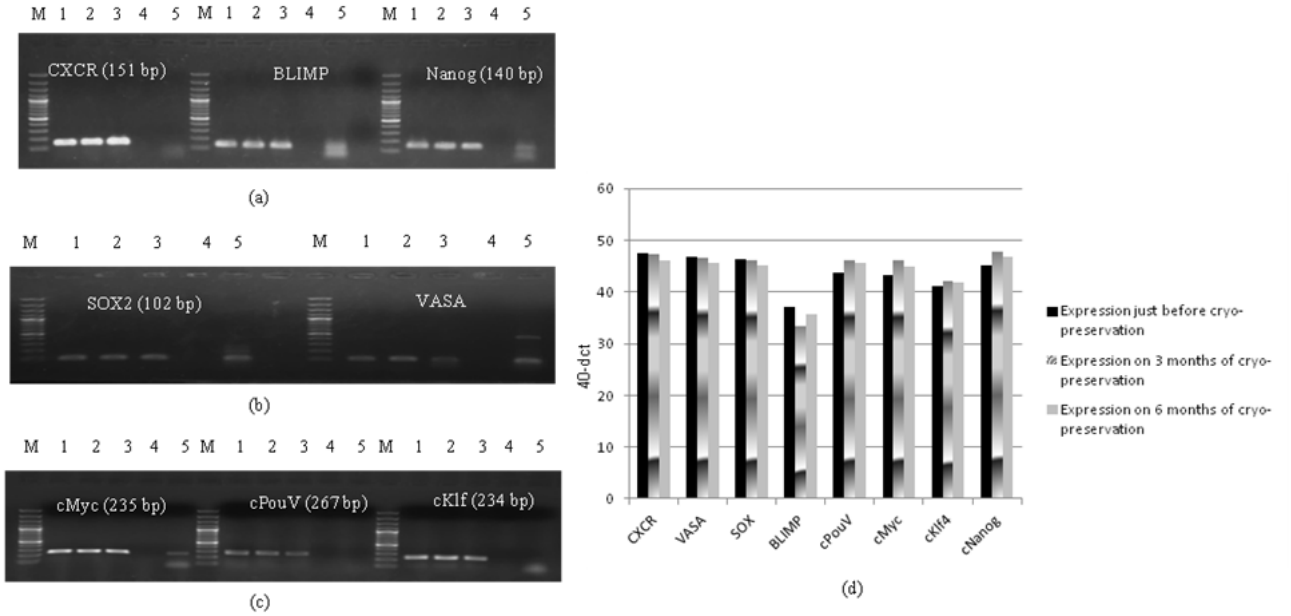

Fig. 2 Expression of different markers in PGCs and other differentiated cells. (a), (b) and (c) are the real time PCR products of marker genes obtained from cDNA synthesized from total RNA isolated from different tissues (PGCs, myoblast cells and liver cells). (d) Expression profile of marker genes in PGCs (Both cryopreserved and uncryopreserved). Lane M= 100 bp ladder marker; Lane 1: PGCs at day 1 (Without cryopreservation); Lane 2: Cryopreserved PGCs at day 90; Lane 3: Cryopreserved PGCs at day 180; Lane 4: Muscle cells (Myoblast cells); Lane 5: Embryonic liver.

Figure 2

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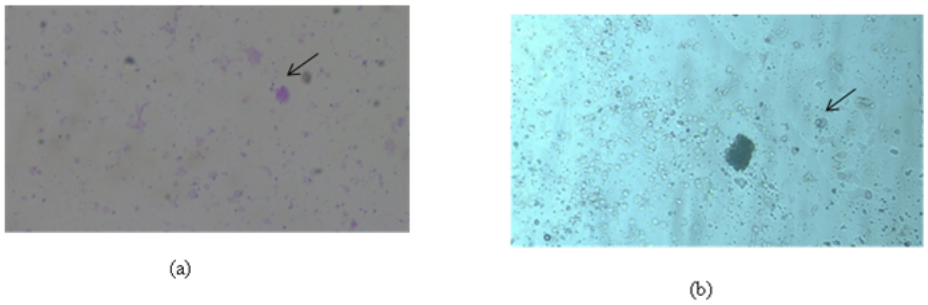

Fig. 3 PGCs stained with PAS (a) and alkaline phosphatase (b). PGCs took pink stain in case of PAS staining while in case of alkaline phosphatase staining, PGCs took blue stain.

Figure 3

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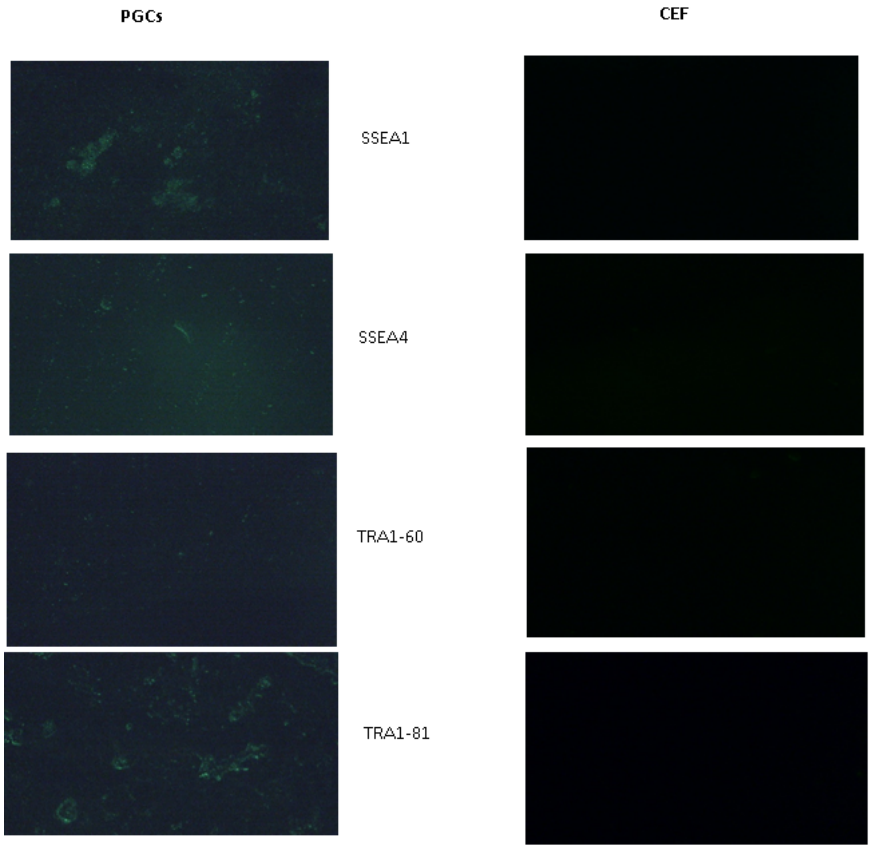

Figure 4

Immuno-cytochemistry of PGCs and chicken embryo fibroblast cells (Differentiated cells) with SSEA1, SSEA4, TRA1-60 and TRA1-81 antibodies. Fluorescence indicates presence of PGCs. Absence of fluorescence indicates differentiated cells viz. CEF. These markers are expressed only on the cell surfaces of PGCs, but not on differentiated fibroblast cells.

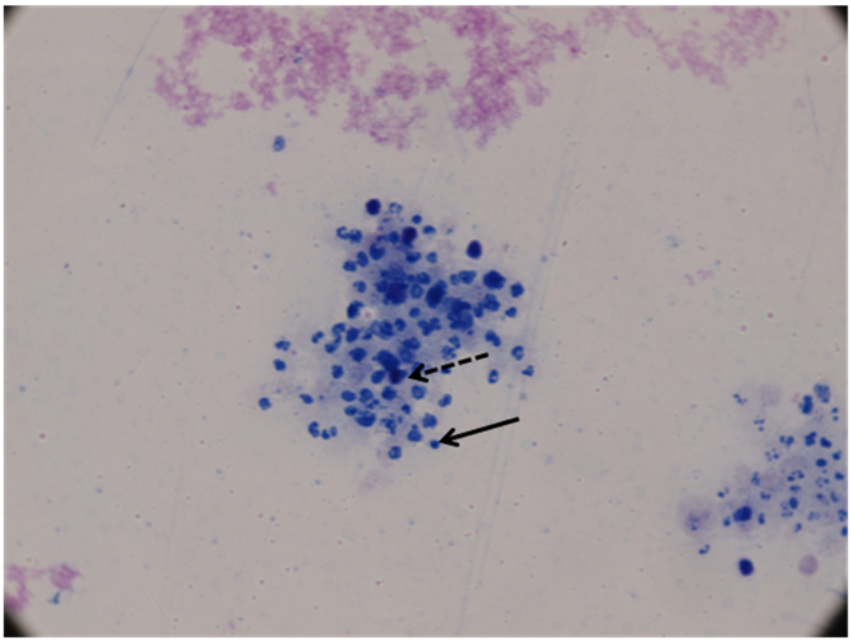

Fig. 5 Karyotyping of chicken PGCs. Arrow indicate chromosomes. Dotted arrow indicates macro-chromosome and normal arrow indicates micro-chromosome.

Figure 5

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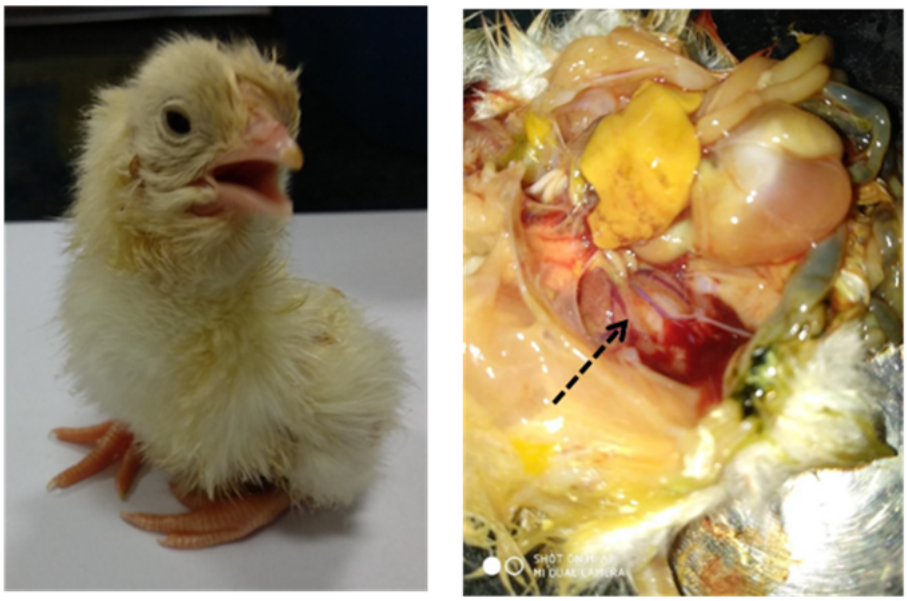

Fig. 6 Chimeric chick and its gonads. (a) Chimeric chick. Gonad is indicated through dotted arrow in (b).

Figure 6

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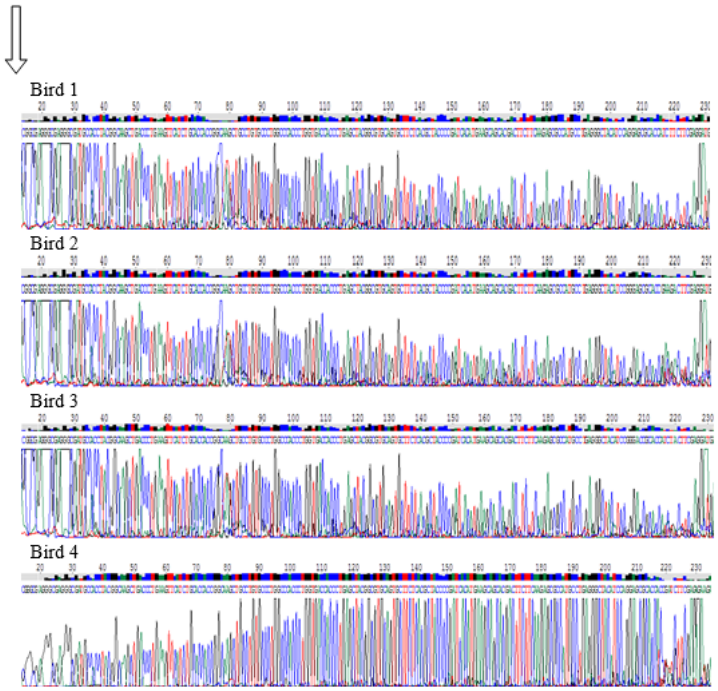

Figure 7

Chromatogram of chimeric birds showing presence of GFP gene fragment. The primer sequences are in bold letters.

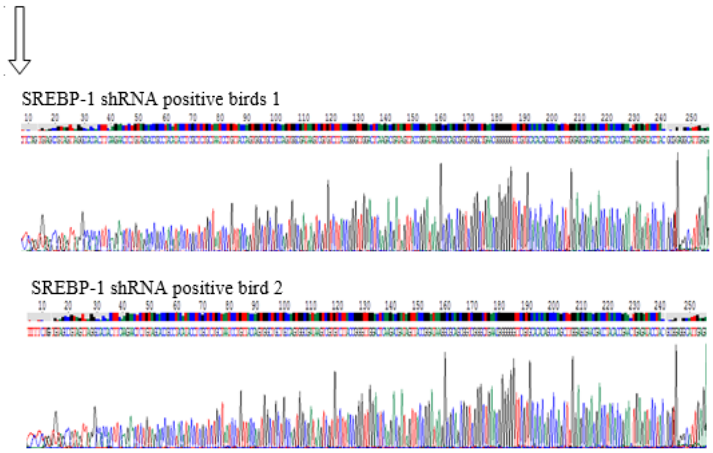

Figure 8

Chromatogram of chimeric chicks showing presence of fragment of pENTRU6-shRNA construct. The primer sequences are in bold letters.

Supplementary Files

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