Research Note: Development of a chicken experimental model platform for induced pluripotent stem cells by using CRISPR/Cas9-mediated NANOG knock-in reporter DF1 cells

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ABSTRACT NANOG, as a transcription factor, plays a key role in maintaining pluripotency in higher vertebrates. Thus, NANOG gene expression is a critical index for the transition from somatic cells to the pluripotent stage. Here, we established chicken knock-in DF1 cells in which the red fluorescent protein (**RFP**) gene was specifically inserted into the transcriptional start site of the NANOG gene through the CRISPR–Cas9 (clustered regularly interspaced short palindromic repeat-CRISPR associated protein 9) technical platform. Subsequently, 4 transcription factors (Pou5f3, Sox2, Nanog, and Lin28A) were introduced into the NANOG-RFP DF1 cells, and finally, the induced pluripotent cells were established and examined by endogenous NANOG promoter-controlled RFP gene expression. The development of induced pluripotent stem cells (**iPSC**s) in avians would be useful for practical applications in the field of avian biotechnology, including biobanking genetic materials and restoring endangered species. In this study, a reporter cell line system was established to efficiently identify the induced pluripotent stage, and it will facilitate potential use for various purposes in the field of avian experimental models.

Key words: chicken, NANOG, genome editing, reporter system, pluripotency

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INTRODUCTION

Since induced pluripotent stem cells (**iPSC**s) were established by Yamanaka factors (Takahashi and Yamanaka, 2006), numerous articles and reviews have demonstrated that iPS cells have great potential and provide a powerful tool for practical applications in therapeutic approaches as well as basic bioscience research.

In avian species, Pain et al. established chicken embryonic stem (**ES**) cells derived from blastodermal cells of Stage X chick embryos. However, chicken ES cells from balstodermal cells showed low germline transmission (Pain et al., 1996). Additionally, early studies have reported the generation of iPSCs from embryonic fibroblasts, feather follicle cells and muscle fibroblasts from quail and chicken with the limitations of partial reprogramming to be overcome (Su et al., 2020). Alternatively, Katayama et al., (2018) used the 6 defined reprogramming factors and derived chicken iPS cells from fibroblast somatic cells. They investigated the differentiation ability into 3-germ-layer derived tissues and analyzed differential gene expression profiles between fibroblast and iPS cells, although the capacity of germline transmission of chicken iPS cells was not examined (Katayama et al., 2018).

The development of iPSCs in avians would be useful for practical applications in the field of avian biotechnology, including biobanking genetic materials and restoring endangered species. In particular, Fuet et al. discovered that NANOG is required for fully reprogrammed cells with similar properties to those of chicken ES cells (Fuet et al., 2018). However, the intricate regulatory mechanism(s) regarding the induction of pluripotency and many key factors that control stemness and germness in chickens have yet to be investigated in detail. Most recently, induced primordial germ cells (iPGCs) were successfully derived from iPS cells that were transdifferentiated from chick somatic fibroblasts. Subsequently, the transplanted iPGCs produced offspring by mating the recipient chickens (Zhao et al., 2021). Interestingly, Lu et al. demonstrated that induced pluripotency in chickens results in a germ cell fate with the expression of several PGC marker genes (Lu et al., 2014), suggesting that the induction of

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pluripotency in avians is different from that in mammals. Thus, the development of a precise reporter system that can trace pluripotent acquisition and maintenance in chickens is needed.

In this study, we developed a chicken experimental model platform for induced pluripotent stem cells through CRISPR/Cas9-mediated NANOG knock-in reporter DF1 cells. The experimental model platform can be used to design research to efficiently generate chicken iPS cells and provide a genetic module system for investigating regulatory pathways and mechanisms of pluripotency in avian species.

MATERIALS AND METHODS

Culture of Chicken DF1

Chicken DF1 fibroblast cells (CRL-12203; ATCC, Manassas, VA) were maintained and subpassaged in DMEM (Invitrogen, Waltham, MA) supplemented with 10% FBS and 1x antibiotic−antimycotic solution. Cells were cultured in an incubator at 37°C with 5% CO_2 and 60 to 70% relative humidity.

Generation of the CRISPR/Cas9-mediated NANOG knock-in Reporter Cell Line

NANOG gRNA and RFP-2A-puroR donor template were synthesized by Bionics Co. (Seoul, Korea) and co-transfected by Lipofectamine 3000 reagent (Invitrogen) with Cas9 nuclease expression plasmid. Briefly, 7.5 μL of Lipofectamine 3000 reagent was diluted in 250 μL of OPTI-MEM (Invitrogen), and each Cas9-GFP coexpression plasmid, NANOG gRNA and RFP-2A-puroR donor template were mixed with Lipofectamine P3000 reagent in 250 μL of OPTI-MEM at room temperature. After incubation for 5 min, the mixtures were combined and incubated for an additional 5 min. The mixture was gently pipetted and dropped onto cultured chicken DF1 cells. One day after lipofection, GFP-expressing cells were sorted using a FACSAria III cell sorter (Becton, Dickinson and Co., Franklin Lakes, NJ). After harvesting, chicken DF1 cells were resuspended in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (**BSA**) and strained through a cell strainer for FACS separation (40 μm; BD Falcon; Becton, Dickinson and Co.). After sorting, the cells were regrown with complete DMEM. To develop NANOG knock-in RFP DF1 cells, single cells were picked up and regrown. Subsequently, single cell-derived NANOG knock-in RFP DF1 sublines were identified by genomic PCR analysis and sequencing with chNANOG KI primers (chNANOG KI F: 5'-tgt gat gca gac acc atc ct-3', chNANOG KI R: 5'-ggg tcc tcc ttt tgt gac ct-3').

Induction of Pluripotency

The coding sequences of chicken NANOG (NM_001146142), Pou5f3 (NM_001110178), SOX2 (NM_205188), and LIN28A (NM_001031774) were synthesized and cloned into each expression vector at downstream of the CAG promoter region. Chicken NANOG knock-in reporter DF1 cells were harvested by treatment with 0.05% (v/v) trypsin supplemented with 0.53 mM EDTA and electroporated with each of the 4 reprogramming vectors using a Basic Fibroblast Nucleofector kit (Lonza VPI-1002, Basel, Switzerland) according to the manufacturer's instructions. Briefly, each vector was mixed with 80 μL of basic nucleofector solution and 16 μL of supplement 1. The mixture was carefully transferred into a Nucleocuvette Vessel and electroporated with the T-023 program (Lonza). Transfected cells were plated onto a 6-well plate in DMEM (Invitrogen) supplemented with 10% FBS and 1x antibiotic−antimycotic solution. After 2 d of transfection, the medium was removed, and transfected cells were replated onto a Matrigel-coated 6-well plate with mTeSR1 medium (Stem Cell Technology, Vancouver, Canada). The medium was changed every 2 d. The cells were monitored for the expression of red fluorescent protein (**RFP**). At 7 d of transfection, colonies were transferred onto a Matrigel-coated 6-well plate with mTeSR1 medium.

Periodic Acid-Schiff (PAS) and Alkaline Phosphatase (AP) Staining

ciPSCs at P5 were collected and fixed in 50 mM phosphate buffer containing 2% (v/v) glutaraldehyde, 2% formaldehyde, and 2 mM MgCl2 for 10 min at room temperature as previously described (Park et al., 2003). After rinsing in PBS, the cells were immersed in periodic acid solution (Sigma–Aldrich, St. Louis, MO) for 15 min. The stained ciPSCs were observed under an inverted microscope. Alkaline phosphatase activity was detected by an alkaline phosphatase chromogen kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA from prepared samples, including Eyal-Giladi and Kochav (**EGK**) stage X embryos and chicken induced pluripotent stem cells (**ciPSC**s), was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) as described previously (Lee et al., 2020), and quantitative RT–PCR was performed to assess the expression of pluripotent markers, as summarized in Table 1. The PCR mixture was prepared by adding 2 μL of 10 pmol of each forward and reverse primer, 7 μL of nuclease-free water, 10 μL of SYBR Green qPCR Mater Mix, and 1 μL of cDNA to a final volume of 20 μL. The relative gene expression was determined using

Table 1. Primers used for this study for the gene expression analysis of ciPSCs.

No.	Gene name	Forward	Reverse
1	GAPDH	CCTCTCTGGCAAAGTCCAAG	CATCTGCCCATTTGATGTTG
2	NANOG	ACCTTCAGGCTGTGACCAGT	GGTGCTCTGGAAGC TGTAGG
3	Pou5f3	GAGGACCTCAACCT GGACAA	TTGTGGAAAGGTG GCATGTA
4	SOX2	GATGGAAACCGAGC TGAAAC	TTGCTGATCTCCG AGTTGTG

the StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA) and determined with a housekeeping gene (GAPDH) using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Development of CRISPR/Cas9-mediated NANOG Knock-in RFP DF1 Cells

NANOG is a key factor for the induction of pluripotency to successfully reprogram avian somatic cells (Fuet et al., 2018). Here, we report the development of a NANOG-RFP reporter DF1 cell line using a CRISPR/Cas9-mediated knock-in (**KI**) system. In this study, we used chicken DF1 cells (Figure 1A). First, we designed and applied guide RNA (gRNA) targeting the first exon of the chicken NANOG gene (Figure 1B). The homology-directed repair (**HDR**) knock-in donor template was designed for promoter-less RFP-2A-puroR (Figure 1B). During the double-strand breakage and repair process induced by the Cas9-gRNA complex, the promoter-less RFP-2A-puroR donor template could be inserted in frame at the start of exon 1 in the NANOG gene. Next, single cell-derived NANOG knock-in RFP DF1 sublines were generated and identified by genomic PCR analysis and sequencing with chNANOG KI primers. A total of potential 13 single cell-derived NANOG knock-in RFP DF1 sublines were analyzed, and only 9 single cell-derived NANOG knock-in RFP DF1 were finally confirmed. As a result, the promoter-less donor RFP gene was specifically inserted into the transcriptional start site of the NANOG gene in NANOG knock-in RFP DF1 cells and used for further study regarding the induction of pluripotency. Experimental procedures regarding the development of CRISPR/Cas9-mediated NANOG knock-in RFP DF1 cells are shown in Figure 1C.

NANOG Promoter-derived RFP Expression After the Delivery of 4 TFs into chNANOG-RFP KI DF1 Cells

We introduced 4 transcription factors (Pou5f3, Sox2, Nanog, and Lin28A) to reprogram chicken NANOG knock-in reporter DF1 cells and generated induced

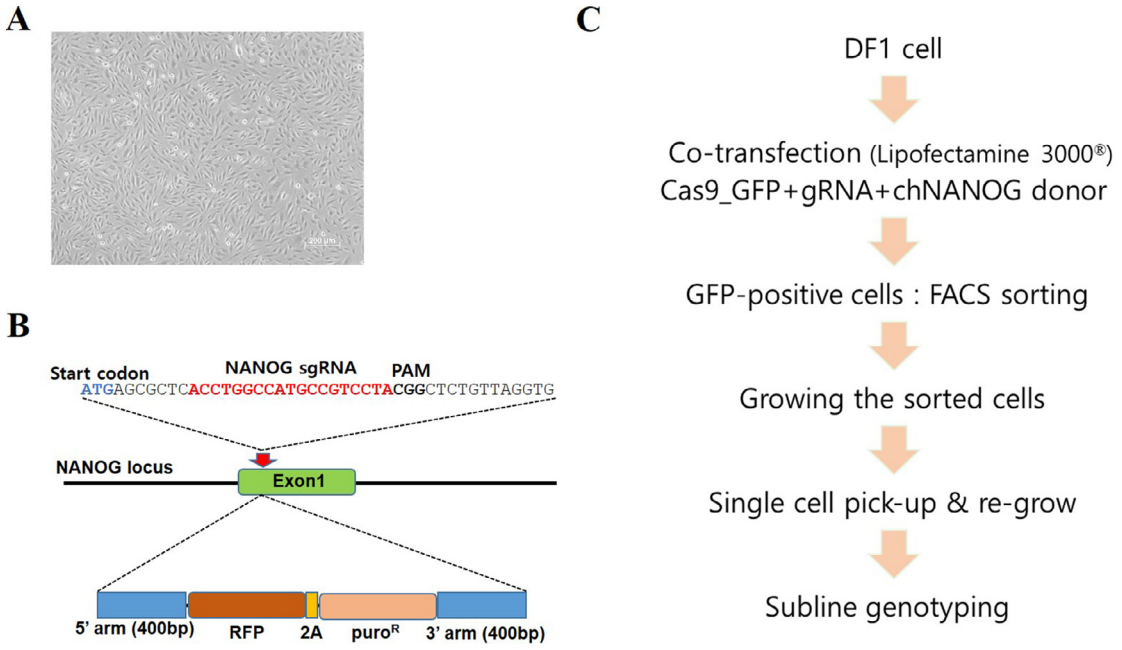

Figure 1. Development of chNANOG-RFP KI DF1 cells using a CRISPR/Cas9-mediated knock-in (KI) system. (A) Chicken DF1 fibroblast cells. Scale bar: 200 μm. (B) Targeting strategy to insert promoter-less RFP-2A-puroR into the first exon of the chicken NANOG gene. (C) Experimental procedures regarding the development of CRISPR/Cas9-mediated NANOG knock-in RFP DF1 cells.

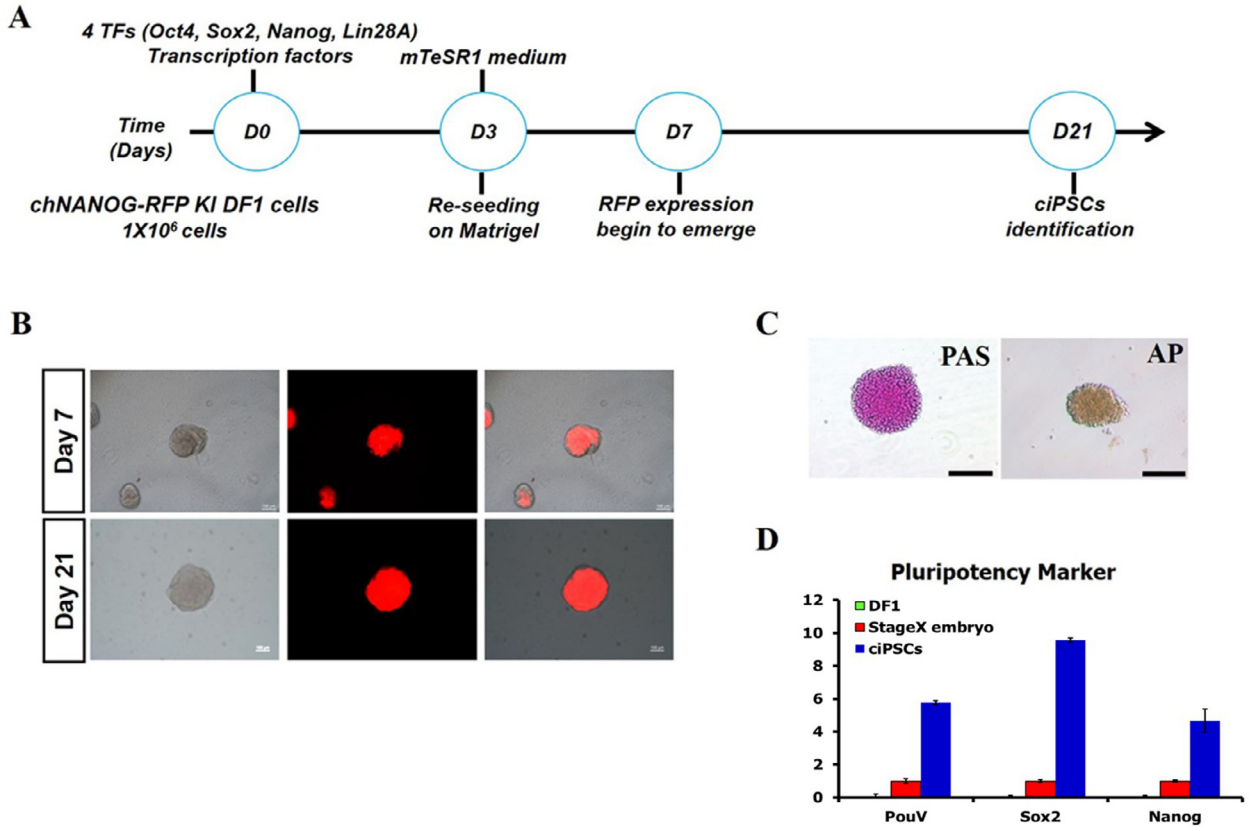

Figure 2. chNANOG-RFP KI DF1 cells can be reprogrammed into ciPSCs using 4 chicken transcription factors. (A) Experimental procedures regarding the generation of ciPSCs after the delivery of 4 chicken transcription factors (Pou5f3, Sox2, Nanog, and Lin28A) into chNANOG-RFP KI DF1 cells. (B) chNANOG-RFP KI DF1 cells precisely reflected endogenous NANOG promoter-controlled RFP gene expression upon the induction of pluripotency. Scale bar: 100 μm. (C) ciPSCs at passage 5 (P5) showed stem cell morphology and positive staining of key stem cell markers, including alkaline phosphatase and periodic acid-Schiff. Scale bar: 100 μm. (D) The endogenous Pou5f3, Sox2, and Nanog genes in ciPSCs showed higher expression than those in undifferentiated stage X embryos and DF1 cell. All data are expressed as the mean ± standard error from 3 independent experiments.

pluripotent stem cells (iPSCs), as shown in Figure 2A, which was reported by Zhao et al. (2021). Chicken-induced pluripotent cells (ciPSCs) exhibited the specific expression of red fluorescent protein (RFP) during several passages in pluripotent culture conditions (Figure 2B), indicating that this reporter system precisely reflected endogenous NANOG promoter-controlled RFP gene expression upon the induction of pluripotency. Furthermore, ciPSCs at passage 5 (P5) had stem cell morphology, such as clear boundaries and spherical shapes, and key stem cell markers, including alkaline phosphatase and periodic acid-Schiff, were examined (Figure 2C). Analysis of pluripotent gene expression showed that the endogenous Pou5f3, Sox2, and Nanog genes in ciPSCs were more highly expressed than those in undifferentiated stage X embryos and DF1 cells (Figure 2D).

CONCLUSION

Chicken NANOG knock-in reporter DF1 cells could be used to monitor the induction of pluripotency during the generation of ciPSCs and iPGCs. This reporter system will be a valuable tool for investigating the regulatory pathways and mechanisms of pluripotency as well as germ cell fate decisions in avian species.

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DISCLOSURES

The authors declare that they have no competing interests.

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