

**External pH in culture on somatic cell
reprogramming and cell differentiation
in mouse and chicken cells**

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Table of Contents

Chapter 1: General Introduction.....	3-9
Chapter 2: Immobilized pH in culture reveals an optimal condition for somatic cell reprogramming and differentiation of pluripotent stem cells.....	10-41
2.1 Abstract.....	11
2.2 Introduction	12-13
2.3 Materials and Methods	14-22
2.4 Results	23-38
2.5 Discussion	39-41
Chapter 3: Sensitivity of cells to culture conditions indicates a species-specific pathway for chicken somatic cell reprogramming	42-63
3.1 Abstract	43
3.2 Introduction	44-45
3.3 Materials and Methods	46-51
3.4 Results	52-61
3.5 Discussion	62-63
Chapter 4: General Summary	64-66
References	67-75
Acknowledgements	76

Chapter 1

General Introduction

Cells can be classified into three types based on their differentiation ability. **1.** Cells having ‘totipotency’, which can differentiate into all types of cells including extraembryonic cells and germ cells (e.g., fertilized eggs). **2.** Cells having ‘pluripotency’, which can change their characteristics into other types of cells (e.g., embryonic stem cells, fetal stem cells). **3.** Somatic cells; which cannot change their characteristics by themselves (e.g., fibroblasts, B cells). Pluripotent stem cells are useful for applications such as regenerative medicine, drug discovery, genetic preservation and transgenic animals, because the cells are able to differentiate into several kinds of cells due to the ‘pluripotency.’ Although the cells gradually lose their differentiation ability as their developments progress at the early stage, recent decades, the technologies of somatic cell reprogramming have been developed dramatically and have enabled the somatic cells to be pluripotent state to use for the applications.

Embryonic stem cells (ESCs) have been established first from mouse blastocyst (Evans and Kaufman, 1981) and from human blastocyst subsequently (Thomson et al., 1998). For the high potential of self-renewal and differentiation, ESCs are of use for applications. There are also several reports regarding other kind of animals such as cat, pig, cattle, goat, rabbit, horse and bird (Ezashi et al., 2016, Jean et al., 2012). Although mouse and human ESCs can be maintained long term and are able to be passaged several times, most of other animals cannot be maintained to use for the applications. As additional problems, ESCs can be generated from blastocysts, which are origin of life, therefore, the application to human and other rare livestock is difficult both for the ethical reasons and the rarity of embryos.

From the viewpoints of pluripotency in mouse, there are two types of embryonic pluripotent stem cells so called ‘naïve state’ and ‘primed state’. Mouse ESCs can be established from the inner cell mass (ICM) of embryos at the embryonic day 3.5-4.5 before implantation (Nichols and Smith., 2011). The established ESCs are able to be maintained the pluripotency in culture with leukemia inhibitory factor (LIF) and serum in medium or Mek inhibitor and Gsk3 β inhibitor (2i). The passaged ESCs form doomed

and three-dimensional colonies and can be passaged as single cells. The chimera contribution and individual production using tetraploid embryos prove the totipotency of mouse ESCs (Weinberger et al., 2016). On the other hand, mouse EpiSCs (epiblast stem cells) are derived from the post implanted mouse embryos (Nichols and Smith, 2011), called the state as 'primed state'. For the maintenance, EpiSCs need activin and basic fibroblast growth factor (bFGF). The colonies of EpiSCs form flattened and two-dimensional morphologies. The little contribution of the EpiSCs to chimera indicates the poorer differentiation ability than that of ESCs. The difference between mouse ESCs and EpiSCs indicates pluripotent stem cells in naïve state, which can be contribute to individuals. Indeed, human ESCs derived from human embryos show similar traits with mouse EpiSCs in primed state, and normally are not able to differentiate to germ cells (Weinberger et al., 2016). However, several chemicals or over expressions of some genes have the potential to reprogram the human ESCs into primed or naïve state (Weinberger et al., 2016). Most of mammalian pluripotent stem cells, to date, formed primed state colonies and are difficult to contribute to chimeras, indicating there might be different mechanisms of pluripotent stem cells for derivation and maintenance among animals.

Induced pluripotent stem cells (iPSCs) have been established from mouse somatic cells by forced expression of transcriptional factors (Takahashi and Yamanaka., 2006). The characteristics are similar to ESCs, which can differentiate into three germ layers both in vitro and in vivo and has the ability of self-renewal. Somatic cells, such as fibroblasts, are easy to be obtained and preserved. Consequently, the application to patients or domestic animals have been expected.

Typically, iPSCs can be established by simultaneous exogenous expressions such as Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006) The exogenous transgenes are carried into cells by vectors such as retrovirus, lentivirus and transposon. To prevent a scar to the DNA in donor cells, caused by the random and unknown insertion of exogenous genes, the safer techniques have been also investigated to date regarding the gene transfers (Li et al., 2014). For the difficulties to establish iPSCs which are able

to differentiate into germ cells, on the other hand, the improvement of reprogramming efficiency has been also tried for the elucidation of mechanisms for the somatic cell reprogramming as well as for the application to human, mouse and other animal species.

Cell reprogramming in domestic animals is applicable to cloned and transgenic animals, to induce the reprogrammed cells into germ cells such as oocytes and sperm to obtain the individuals. The technologies to induce cell reprogramming include nuclear transfer, cell fusion and iPSCs (Yamanaka and Blau, 2010). For the first time, the nuclear reprogramming in vertebrates has been found in *Xenopus* cells, transferring a somatic cell nucleus into an enucleated oocyte (SCNT) to produce cloned individuals (Gurdon et al., 1958). The SCNT technology has been subsequently applied to mammals (Wilmut et al., 1997; Wakayama et al., 1998). However, there are over 30 years of SCNT study to be available for mammalian cells from the first reports of cell reprogramming in *Xenopus*, which suggests the difference of cell differentiation system between amphibian and mammals caused difficulties for inducing cell reprogramming. The developmental process of amphibian embryos, in contrast of mammals, progresses outside of the body and amphibian oocytes are much bigger than that of mammals, which simplifies the operation of SCNT. However, due to the differences of fertilization and developmental system, it is difficult for birds and reptiles to be applied in vitro fertilization, SCNT operation itself or the ontogenesis after the SCNT operation.

The technologies of iPSCs are enable to induce nuclear reprogramming much easier than SCNT in some vertebrates. The iPSCs are therefore useful to prevent the extinction of endangered or precious rare domestic animals, but also the production of cloned and transgenic animals through iPSCs is finally more reliable. Although the studies of somatic cell reprogramming have been investigated in various animals, some reports suggest that there are problems in animals other than mouse on cell reprogramming into a pluripotent state contributing germ cells, which seem to be a similar problem for the establishment of ESCs in most of animals (Ezashi et al., 2016).

To produce individual animals, the induction of germ cells from pluripotent cells is

essential. It is known that cell characteristics in each iPSC line is somewhat different in their differentiation ability, and it is believed that a complete nuclear reprogramming, which means throughout the elimination of their original epigenetic memory, is needed to obtain germ cells due to their distinctive epigenetic states.

To produce avian germ cells in vitro, Laval et al (2009) showed the induction of chicken primordial germ cell from chicken ESCs. They showed *CVH*, which is known to express in chicken PGC (Tsunekawa et al., 2000; Laval et al., 2009), had also the possibility to induce germ cells (Laval et al., 2009). The induction was performed by the exogenous expression of chicken vasa homologue (*CVH*) and showed the contribution in the chicken gonad after the injection of the derived PGCs to fertilized eggs.

There are also articles of somatic cell reprogramming in avian species (Lu et al., 2012; Rosselló et al., 2013). To induce avian cell reprogramming, transcription factors derived from mouse or human could reprogram avian cells, suggesting that somewhat shared mechanisms are present among animal species in somatic cell reprogramming. Lu et al (2012) performed the induction of quail somatic cell reprogramming by simultaneous ectopic expression of human 6 factors. The reprogrammed cells differentiate three germ layers and a partial contribution into chimera is observed. But the survival and proliferation of reprogrammed cells depended on the ectopic expressions of transgenes. To establish the stable line of avian pluripotent stem cells, the environmental condition for the avian cells should be essential to be solved the difficulties.

Blastodermal cells (BDCs) derived from freshly laid fertilized avian eggs have been found that the contribution of BDCs to chimera by injecting the cells to recipient embryonic disc at the surface of the yolk (Petitte et al., 1990) and indicating that BDCs have pluripotency. Avian ESCs derived from the BDCs have been studied to be maintained long term in culture (Pain et al., 1996; Etches et al., 1996; Horiuchi et al., 2004). However, reproducible procedures for generating avian pluripotent stem cells have not been established because the characteristics and maintenance of avian ESCs have not been fully investigated.

To evaluate the pluripotency of avian stem cells, the similar markers against mouse and human embryonic stem cells have been used. Pain et al. (1996) have confirmed the pluripotency of avian ESCs by checking their differentiation abilities into three germ layers in vitro, chimera contribution in vivo, alkaline phosphatase activity and protein expression of SSEA-1, EMA-1 and ECMA-7. Regarding gene expressions in avian ESCs, similar in the mouse and human pluripotent stem cells, avian NANOG and OCT4 (POUV) are strongly correlated with the pluripotency of avian stem cells (Lavial et al., 2007). However, the network of each gene expression is still much unclear compared with that of mouse and human.

The efficient induction of somatic cell reprogramming to pluripotent stem cells has been investigated for various kinds of somatic cells (Li et al., 2014). The efficiency of somatic cell reprogramming is varied in the percentage of reprogrammed cells and the range of differentiation ability. These two parameters are often incompatible. Some kind of cells, however, are difficult even to obtain reprogrammed cells. Reprogrammed cells with a high differentiation potential are the required cell characteristics for the pluripotent stem cells.

In most of cases, a growth factor (LIF) and feeder cells are used for the induction of iPSCs and ESCs in medium because they are basically required for the maintenance of pluripotent stem cells. MEK inhibitor and GSK3b inhibitor, which maintain the pluripotent stem cells at high quality, are often added in culture medium for induction of the reprogramming.

Although the switch of somatic cells to pluripotent stem cells achieves under unknown mechanisms, the studies of cell characteristic differences between pluripotent stem cells and somatic cells could be helpful to improve the efficiency of somatic cell reprogramming. In the aspect of epigenetic differences, it is known that the treatment of chemicals, which inhibit several enzymatic activities of histone modification or methylation, enhance the somatic cell reprogramming (Ezashi et al., 2016). Considering environmental condition of pluripotent stem cells in vivo, mammalian embryos are

known to develop in a low oxygen condition in the oviduct (Fischer et al., 1993). The culture system with hypoxia condition supply an appropriate environment for stem cells and induce the reprogramming with high-quality pluripotency through the pathways of hypoxia inducible factors (HIF) (Mathieu et al., 2014). To understand the appropriate condition for the stem cell maintenance would provide useful information to establish pluripotent stem cell lines in various species.

To elucidate the somatic cell reprogramming in animal species, the pH in culture medium was focused. The pH is known to be varied both in vivo and in vitro, however, the effects on the reprogramming have not been explored to date. In the next section, the pH effects on the somatic cell reprogramming and differentiation of ESCs were observed by stabilizing the pH value in culture using mouse cells. Based on the method for maintenance of pH value, chicken embryonic fibroblasts were also checked the effects on the colony formation during somatic cell reprogramming in the following section. In the section of general summary, the founded data in section 2 and 3 were discussed from the aspects of the molecular mechanisms for further study.

Chapter 2

**Immobilized pH in culture reveals an optimal condition for
somatic cell reprogramming and differentiation
of pluripotent stem cells**

2.1 Abstract

One of the parameters that greatly affects homeostasis in the body is pH. Regarding reproductive biology, germ cells such as oocytes or sperm are exposed to severe changes in pH, resulting in dramatic changes in their characteristics. To date, the effect of pH has not been investigated regarding the reprogramming of somatic cells, maintenance and differentiation of pluripotent stem cells. *Methods:* For investigating the effects of pH on cell culture, the method to produce induced pluripotent stem cells (iPSCs) and to differentiate embryonic stem cells (ESCs) into mesendoderm (ME) and neuroectoderm (NE) were carried out in each pH medium of pH 6.6 to pH 7.8. Using the cells of *Oct4*-GFP (green fluorescent protein) carrying mouse, the effects of pH changes were examined on the timing and colony formation at cell reprogramming, and on the cell morphology and direction of differentiation of ESCs. The colony formation rate and timing of the reprogramming of somatic cells were varied depending on the pH of the culture medium. In addition, mesendodermal differentiation of mouse ESCs were enhanced at the high pH (pH 7.8). These results suggest that the pH in culture medium is one of the key factors for the induction of reprogramming of somatic cells and the differentiation of pluripotent stem cells.

2.2 Introduction

pH is one of the important parameters in life, specifying the acidity or basicity of an aqueous solution. pH variation influences every biological process at the cellular, tissue, and whole-body levels (Occhipinti et al., 2015). In reproductive processes, the vaginal pH in women is normally maintained at a pH ranging between 4.0 and 5.0 (Caillouette et al., 1997). The semen in men is maintained normally at a pH above 8.0 (Haugen et al., 1998). After increasing vaginal pH within a few seconds by ejaculation (Fox et al., 1973), pH recovers to being fairly acidic during pregnancy (Hauth et al., 2003). At the cellular level, acrosomal reaction results in the increase of the internal pH of sperm at fertilization (Darszon et al., 1999). Intracellular pH during oogenesis and embryogenesis vary for each developmental stage (Dale et al., 1998; FitzHarris et al., 2009). Thus, germ cells and embryos are exposed to pH fluctuation with dramatic changes in their traits during the development of individuals (Irie et al., 2014). Regarding the processes of cell differentiation and cellular reprogramming, there is little information concerning the influence of pH on these phenomena.

pH affects many molecular mechanisms inside and outside of cells to maintain homeostasis. The proton gradient in cells is maintained by pumps and channels, such as Na^+/H^+ exchangers, $\text{HCO}_3^-/\text{Cl}^-$ exchangers, V-type H^+ pumps and voltage-gated H^+ channel on the plasma membrane (Occhipinti et al., 2015; Casey et al., 2010). Active/passive changes by pH affect cell traits such as motility, enzymatic activity, cell cycle and apoptosis (Occhipinti et al., 2015; McBrien et al., 2013). Cell motility is also caused by the constitution change of the cytoskeleton, which is affected by environmental pH (Damaghi et al., 2013). Recent studies also indicate that actin proteins are essential in transcriptional activation during differentiation and reprogramming of cells (Miyamoto et al., 2013; Guo et al., 2014). However, the phenomena on somatic cell reprogramming caused by pH in culture have not been investigated. Regarding the effects of pH on cell differentiation, mesenchymal stem cells are affected by pH during cell differentiation into

osteogenic and chondrogenic cell lineages (Moghadam et al., 2014). Also, the high pH in murine ESC culture is known to enhance cardiac cell differentiation (Teo et al., 2014). However, the pH effect on differentiation of ESCs have not been well investigated in a wide range of pH value.

Distinctive changes in the molecular activity of cells can be observed in the processes of cell differentiation and reprogramming of somatic cells (Teperek et al., 2013). During the course of the reprogramming of fibroblasts, the appearance of cells changes from mesenchymal to epithelial (Takahashi and Yamanaka, 2006), and reverse phenomena of the differentiation of stem cells with major changes in the epigenetic state also occur (Lamouille et al., 2014). In this article, the effects of pH were examined during cellular reprogramming and differentiation using mouse embryonic fibroblasts (MEFs) and embryonic stem cells (ESCs) from transgenic mice carrying the *Oct4*-GFP (green fluorescent protein) reporter in vitro; these mice are well suited to estimate pluripotency (Stadtfield et al., 2008; Brambrink et al., 2008). Cells cultured in media at various pHs are then observed at the colony formation and timing of reprogramming of MEFs and at the differentiation of ESCs to the mesendoderm (ME) and neuroectoderm (NE).

2.3 Materials and Methods

Chemicals

Unless otherwise noted, all of the chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Animals

MEFs and ESCs obtained from transgenic mice carrying the Oct4-GFP reporter were maintained in RIKEN BioResource Center (Ibaraki, Japan) (Yoshimizu et al., 1999) and were used in experiments for the reprogramming of somatic cells and differentiation. MEFs and ESCs originated from the transgenic mice were obtained and treated as described previously (Tsukiyama et al., 2011).

For the preparation of MEFs, embryos were collected at embryonic days 13.5-15.5 as described previously (Tsukiyama et al., 2011). Isolated embryonic cells were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO, Life Technologies, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 73 iu/ml penicillin (Sigma), and 50 µg/ml streptomycin (Sigma). The fibroblasts with 4 passages or less were used for the reprogramming of somatic cells.

For the maintenance of ESCs, the ESCs were cultured in a medium equally mixed with Neurobasal medium (GIBCO, Life Technologies, Grand Island, NY, USA) and DMEM/F12 (GIBCO, Life Technologies) containing 0.5% (v/v) N2 (GIBCO, Life Technologies), 0.5% (v/v) B27 (GIBCO, Life Technologies), 1% (v/v) L-glutamine, penicillin and streptomycin (GIBCO, Life Technologies), 0.05% (w/v) bovine serum albumin (BSA, Sigma) and 0.15 mmol/l 1-thioglycerol (Sigma) supplemented with leukemia inhibitory factor (LIF) (1:1000), 1 µmol/l MEK/ERK inhibitor (PD0325901; REAGENTS DIRECT, Encinitas, CA, USA) and 3 µmol/l GSK3β inhibitor (CHIR99021; REAGENTS DIRECT) on human plasma fibronectin (Millipore, Darmstadt, Germany)-

coated dishes. As a source of LIF, LIF-conditioned medium (1:1000 dilution) from COS-7 or 293FT cell cultures that had been transduced with a mouse LIF-encoding vector (Niwa et al., 1991; Yoshida-Koide et al., 2004) was used. The medium was changed every day, and the cells were passaged every 2 days using TrypLE (GIBCO, Life Technologies) and reseeded on 35-mm dishes (IWAKI, Tokyo, Japan) at 1×10^6 cells per dish.

Retroviral transfection

To perform retroviral transfection, pMXs-based retroviral vectors (Oct4, Sox2, Klf4, and c-Myc; Addgene plasmid # 13366, # 13367, # 13370 and # 13375) and pCMV-VSV-G vector (Addgene plasmid # 8454) were introduced into Plat-GP cells (Cell Biolabs, Inc., San Diego, CA, USA) (Morita et al., 2000) by using the Lipofectamine LTX transfection reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. The medium was changed the following day, and after another 24 hrs of incubation, virus-containing supernatants derived from the Plat-GP cell cultures were filtered through a 0.45- μ m cellulose acetate filter (Schleicher & Schuell, Dassel, Germany).

The transfection of retroviruses into MEFs was performed on RetroNectin (Takara, Shiga, Japan)-coated dishes according to the manufacturer's recommendations. The virus recovered from the Plat-GP culture was added to the RetroNectin-coated dishes and was incubated at 37 °C. After 6 hrs of incubation, the dish was washed with PBS containing 0.25% (v/v) BSA. MEFs cultured in somatic cell medium were trypsinized and passaged on virus-containing dishes at a density of 2×10^5 cells per well in the fresh somatic cell medium.

Adjusting and monitoring pH in culture medium

Each pH condition in medium was controlled by changing the concentration of NaHCO₃ (Wako, Osaka, Japan) under 5% (v/v) CO₂ according to the Henderson-Hasselbalch equation method applied to tissue culture vessels (Esser. 2010, online). The

fresh ES medium was pre-incubated for 24 hrs in 5% (v/v) CO₂ in air, and the pH of the medium was measured within the standard deviation (SD) $\pm$ 0.1 against the predicted pH. Each pH of the medium before and after medium change was checked by a pH meter (B-212; HORIBA, Kyoto, Japan). The stale medium was immediately changed to the fresh medium after checking pH every day (Figure 2.1, Figure 2.2).

Induction and estimation of reprogramming of MEFs

After 4 days of virus transfection, the transfected cells (approximately 1.6×10^3 - 4.8×10^3 cells/cm²) were reseeded on mitomycin treated STO feeders (approximately 2.5×10^4 cells/cm²) cultured in embryonic stem cell medium (ESM); Glasgow Minimum Essential Medium (GMEM; Sigma) containing 15% (v/v) Knockout Serum Replacement (KSR; GIBCO, Life Technologies), 0.3% (v/v) FBS (SAFC Biosciences), 2 mmol/l L-glutamine (MP Biomedicals, Tokyo, Japan), 1 mmol/l sodium pyruvate (Sigma), 1 $\times$ MEM nonessential amino acids (Invitrogen, Life Technologies), 0.1 mmol/l 2-mercaptoethanol (Wako, Osaka, Japan), 73 iu/ml penicillin (Sigma), and 50 μ g/ml streptomycin (Sigma) supplemented with leukemia inhibitory factor (LIF). Cultured cells were observed using an inverted microscope (DIAPHOT 300; Nikon, Tokyo, Japan), and photos were acquired using a COOLPIX P6000 (Nikon, Tokyo, Japan).

Estimation of ESCs proliferation

The estimation of cell proliferation of ESCs in various pH conditions was performed in the ESM containing 15 % KSR with LIF. The ESCs were passaged on STO feeders or gelatin-coated dish and cultured for 3 days and counted the cell number in each pH of medium.

Alkaline phosphatase activity in pluripotent cell culture and immunofluorescence staining

The cells were fixed with 3.7% paraformaldehyde (PFA; Wako, Osaka, Japan) in PBS for 10 minutes at room temperature. After washing with PBS 3 times, alkaline

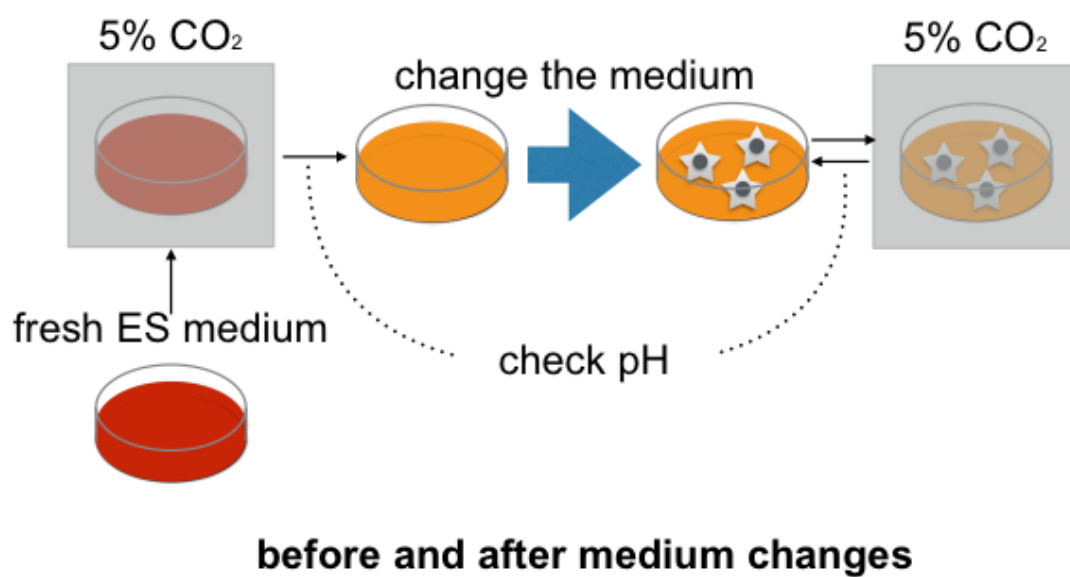


Figure 2.1 Schemes of pH adjustment of the medium. Fresh medium was pre-incubated for approximately 24 hrs at 5% CO₂ to normalize the medium pH. Before changing the medium, the pH before and after medium change was checked to confirm the stability of the pH.

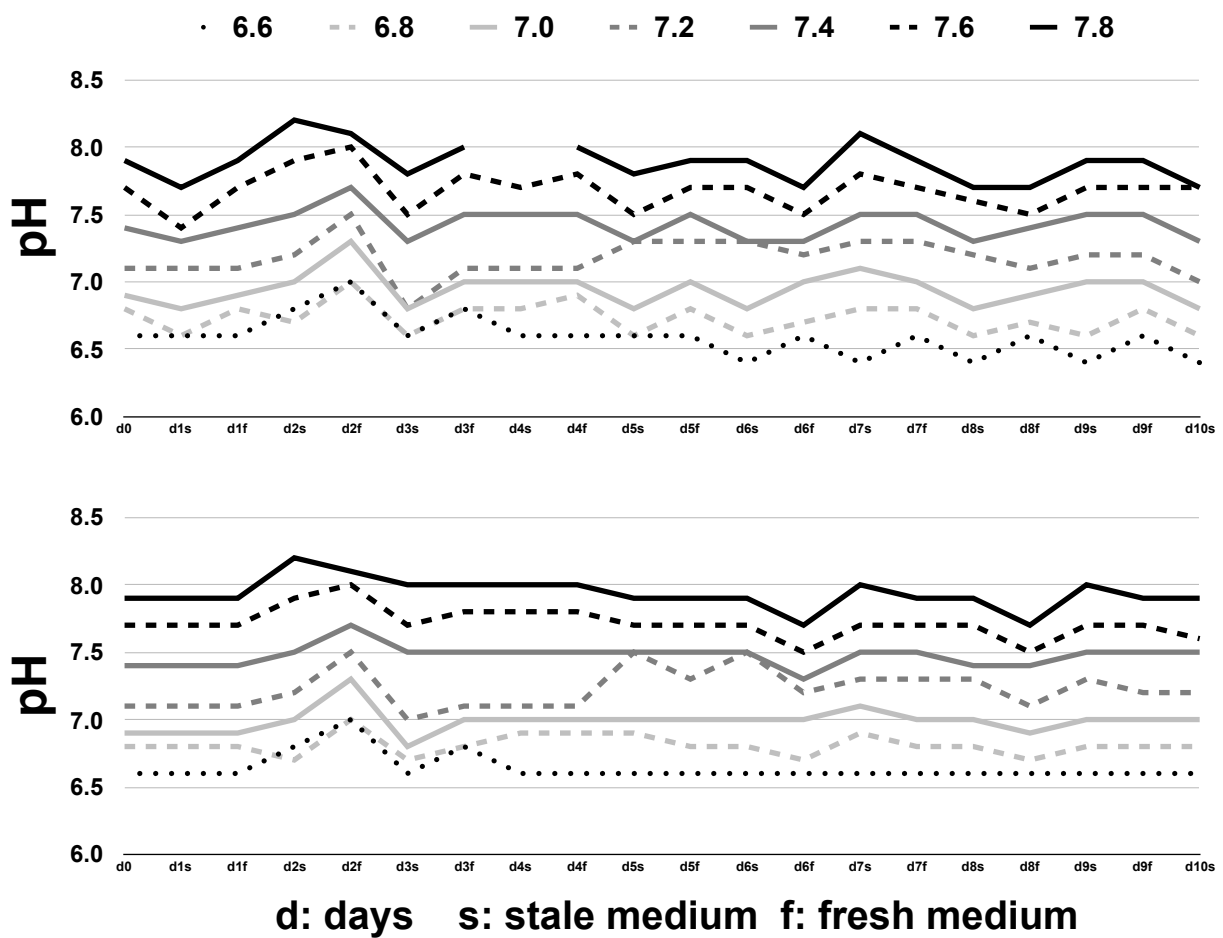


Figure 2.2 The pH fluctuation during the reprogramming of MEFs. Each pH of medium during culture was monitored at both of fresh medium and stale medium as indicated in Figure 2.1.

phosphatase (AP) activity was detected using the Vector Alkaline Phosphatase Substrate kit III (Vector, Burlingame, CA, USA), according to the manufacturer's instructions.

For immunofluorescence analysis, cells were fixed with PBS containing 3.7% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were blocked with PBS containing 5% BSA (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) for 45 min at room temperature, and then incubated overnight at 4°C with primary antibodies against glial fibrillary acidic protein (Brachyury, 1:1000, ab20680; abcam, Tokyo, Japan). Alexa Fluor 594-conjugated goat anti-mouse IgG (1:500; Invitrogen, Life Technologies) was used as secondary antibodies. Nuclei were stained with 1 µg/ml Hoechst 33342 (Sigma-Aldrich).

RT-PCR

Total RNA was prepared using the TRIzol reagent (Ambion, Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions. DNase (Roche, Indianapolis, IN, USA) was added to the preparations to avoid genomic contamination. First-strand cDNA was synthesized using reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) and random primers (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. PCR was performed using ExTaq (Takara, Shiga, Japan) according to the manufacturer's instructions. The transcription levels were normalized by the *Gapdh* expression level.

For quantitative PCR, THUNDEBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) was used according to manufacturer's instruction. The PCR reaction was performed under 2-step cycles; denaturation 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Each expression of genes was normalized by the expression of *Gapdh*. The sequence of primers used for experiments were shown in Table 2.1.

Induction of the differentiation of ESCs in vitro

To induce the differentiation of ESCs, the cells were passaged onto gelatin-coated 35-mm dishes at a density of 6×10^4 cells per dish in 10 % FBS containing ESM without

LIF, PD0325901 and CHIR99021. Two days after culture, the following chemicals were added to each differentiation medium (Thomson et al., 2011): 500 nmol/l retinoic acid (RA, Sigma) and 5 ng/ml human basic fibroblast growth factor (bFGF; Wako, Osaka, Japan or ReproCELL, Yokohama, Japan) for neuroectodermal differentiation: 3 μ mol/l CHIR99021 (REAGENTS DIRECT, Encinitas, CA, USA) and 10 ng/ml activin A (R&D Systems, Minneapolis, MN, USA) for mesendodermal differentiation (Figure 2.3). At the induction of each germ lineage, the pH of the medium was adjusted to pH 6.8, 7.4 and 7.8.

Statistical analysis

The statistical significance of the difference between the sample means was determined using Student's t test.

Table 2.1. Sequences of primers for RT-PCR.

Gene	Primer Sequence	Anealing degree	Product size (bp)	Sourse
<i>Mixl1</i>	5'-GCACGTCGTTTCAGCTCGGAGC-3' 5'-AGTCATGCTGGGATCCGGAACGTGG-3'	55°C	305bp	Jackson et al., 2010
<i>Nestin</i>	5'- GGAGAGTCGCTTAGAGGTGC-3' 5'-AGGTGCTGGTCCTCTGGTAT-3'	55°C	375bp	NM_016701.3
<i>T</i>	5'-TGCTGCCTGTGAGTCATAAC-3' 5' TCCAGGTGCTATATATTGCC-3'	55°C	948bp	Jackson et al., 2010
<i>Sox2</i>	5'-TAGACTGCACATGGCCCAGCACT-3' 5'-TTGCCTTAAACAAGACCACGAAA-3'	55°C	778bp	NM_011443.3
<i>Oct3/4</i>	5'-AAGTGCCCGAAGCCCTCCCTACAG-3' 5'-CAGAGGGAAAGGCCTCGCCCTCAG-3'	55°C	289bp	NM_001252452.1
<i>Gapdh</i>	5'-ACGGCACAGTCAAGGCAGAG-3' 5'-GTGATGGCGTGGACAGTGGT-3'	55°C	376bp	NM_001289726
For real time				
<i>Mixl1</i>	5'-CAGTTGCTGGAGCTCGTCTT-3' 5'-TCCGGAACGTGGTTCACATC-3'	60°C	266bp	NM_013729.3
<i>Nestin</i>	5'-GGGGCTACAGGAGTGGAAC-3' 5'-GACCTCTAGGGTTCCCGTCT-3'	60°C	213bp	NM_016701.3
<i>T</i>	5'-GGCTGGGAGCTCAGTTCTTT-3' 5'-TGTCCACGAGGCTATGAGGA-3'	60°C	179bp	NM_009309.2
<i>Sox2</i>	5'-GATCAGCATGTACCTCCCCG-3' 5'-TCCTCTTTTGCACCCCTCC-3'	60°C	212bp	NM_011443.3
<i>Oct3/4</i>	5'-CCAATCAGCTTGGGCTAGAG-3' 5'-CCTGGGAAAGGTGTCCCTGT-3'	60°C	130bp	NM_001252452.1
<i>Gapdh</i>	5'-CGTGTTCTTACCCCCAATGTG-3' 5'-TGTCATCATACTTGGCAGGTTTC-3'	60°C	73bp	NM_001289726

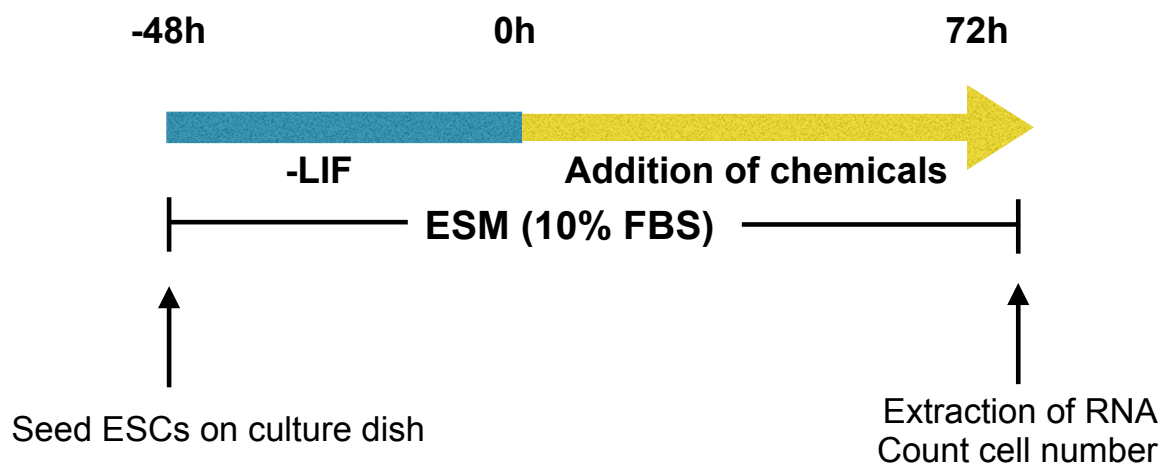


Figure 2.3 Schemes of differentiation of mouse embryonic stem cells (ESCs). Two days after the culture of mouse ESCs in the absence of LIF, chemicals to induce cell differentiation to produce neuroectoderm and mesendoderm were added, respectively.

2.3 Results

Effects of external pH on colony formation during the reprogramming of mouse somatic cells

To investigate the effects of pH during the reprogramming of mouse somatic cells, *Oct4*-GFP-positive colonies were counted in the medium for each pH. The pH was checked every day and was confirmed to be within 0.1 of standard error, indicating little change in the pH throughout the culture (Figure 2.2).

GFP-positive colonies appeared from day 5 to day 10 of culture and were counted at day 17 for each medium pH (Figure 2.4, Figure 2.5, Figure 2.6). The highest number of GFP-positive colonies was obtained at pH 7.4 (Figure 2.4). No colonies, however, were observed at pH 6.6. The maximum difference in the colony number at pH 6.8 was fifteen-fold lower than that at pH 7.4. The average colony number at each medium pH were divided by the colony number at pH 7.4 respectively, and each ratio was found to be significantly different than those at pH 7.4. Interestingly, only a 0.2-point difference of pH caused a significant decrease in the colony formation with the number (Figure 2.4). To examine the effects on established pluripotent stem cells, murine ESCs were cultured at each medium pH from pH 6.8 to 7.8. After 3 days of culture in each pH in culture, the highest number of cells was obtained at pH 7.4 (Figure 2.7) and the lowest number was observed at pH 6.8. Between pH 7.4 and 7.6, however, there were no significant differences at the cell number.

To elucidate the pH effects on somatic cell reprogramming, ESCs were replated as single cell at a low concentration of cell density (Figure 2.8, Figure 2.9). The similar number of colonies among various pH ranges were then obtained.

pH effects on the timing of reprogramming

During the reprogramming of somatic cells at each pH of the medium, the number of GFP positive colonies were counted every day. The fastest appearance of GFP positive

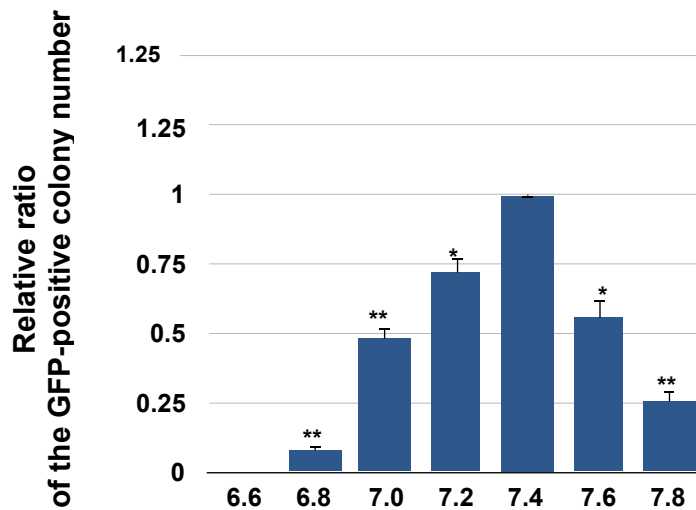


Figure 2.4 Effects of pH on the colony formation of the reprogramming of mouse somatic cells. Relative colony formation rates of the colony number formed under different pH conditions. The GFP-positive colony number was counted after 17 days of the induction of reprogramming. The relative ratio of the colony in each treatment group was obtained by dividing the colony number in each group by the number at pH 7.4. For statistical analysis, each ratio of the treated group was compared with the ratio at pH 7.4. The asterisk indicates significant differences compared with the relative ratio at pH 7.4 ($0.01 < P < 0.05$, $**P < 0.01$; t-test). The data are presented as the means $\pm$ S.E.M. Experiments were repeated three times independently.

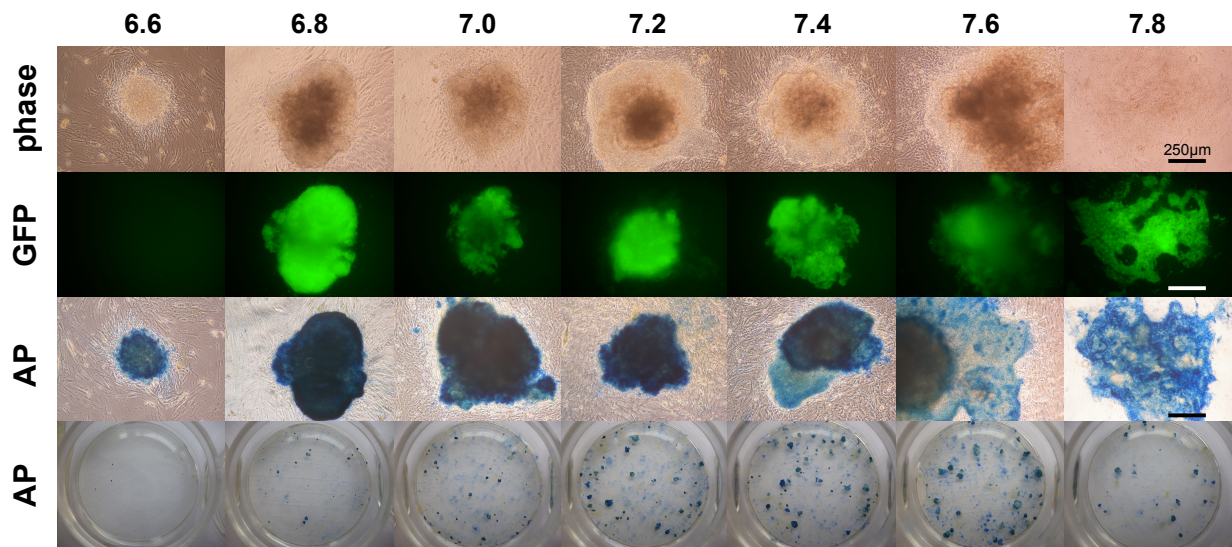


Figure 2.5 Variation in GFP expression and alkaline phosphatase activity on colonies in culture medium with different pH conditions. Phase-contrast (phase), expression of green fluorescence protein (GFP) and alkaline phosphatase (AP) activity in each culture dish were examined in colonies cultured under different pH conditions for 17 days for the induction of somatic cell reprogramming in mice. Scale bar: 250 μm .

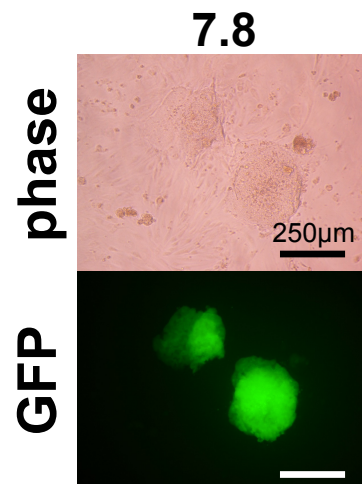


Figure 2.6 Phase contrast images and GFP expressions of the colonies after 17 days of culture at pH 7.8 at the process of somatic cell reprogramming.

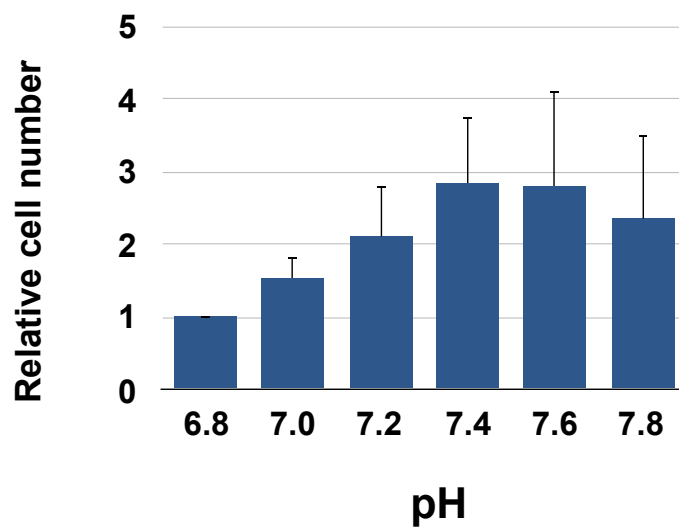


Figure 2.7 Effects of pH on the and proliferation of mouse ESCs. The number of cells of each treatment group obtained after 4 days of culture was divided by the values obtained at pH 6.8. The experiment was independently repeated three times.

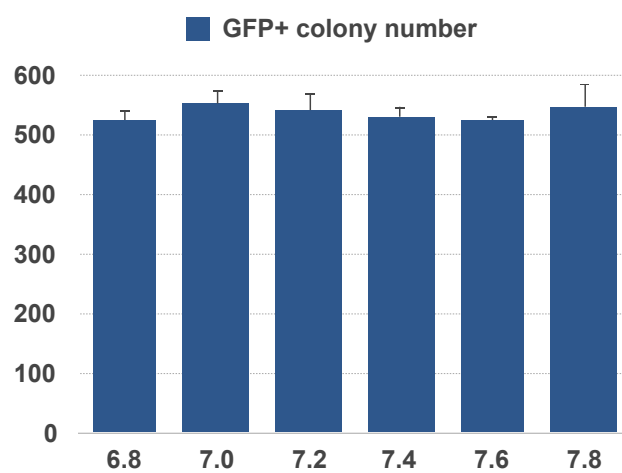
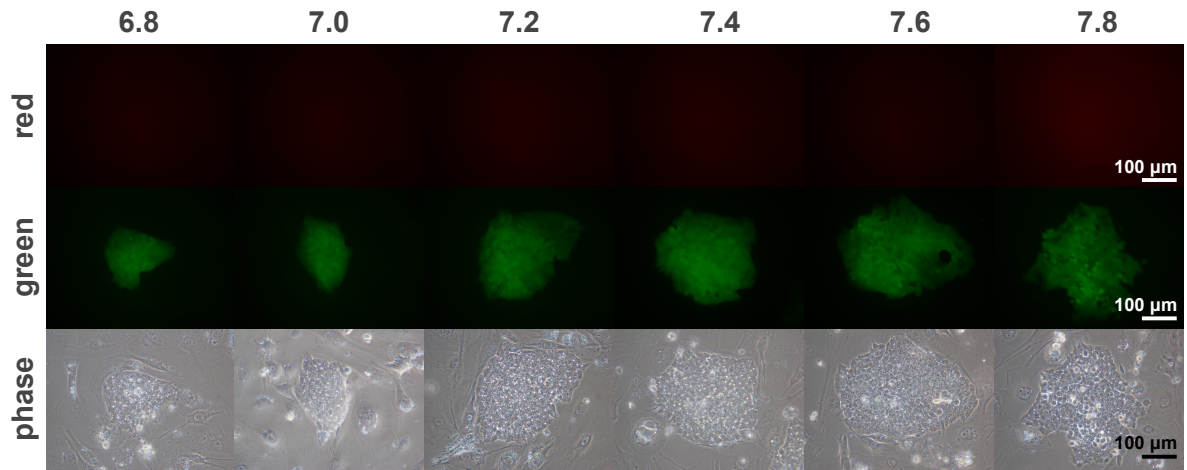


Figure 2.8 The number of Oct4-GFP positive ESC colonies formed at each pH of medium. The experiments were performed independently for three times.

(a)



(b)

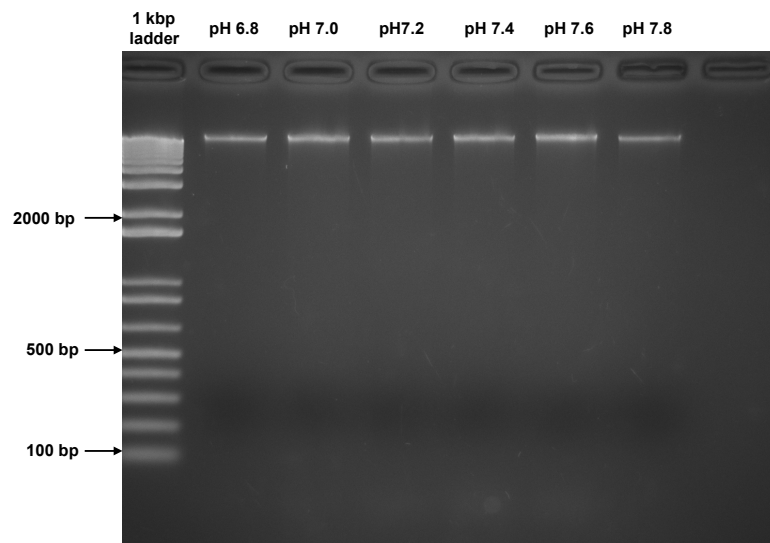


Figure 2.9 (a) The micrographs of ESC colonies formed. The ESCs were reseeded as single cells at a low density in each pH of medium and cultured for 3 days. **(b)** DNA ladder assay of ESCs. ESCs were formed colonies at each pH of medium, even they were passaged as single cell. For three days of culture at each pH of medium, the DNA of ESC colonies in each pH of medium were extracted and confirmed the DNA fragmentations were not observed.

Table 2.2. Effect of pH on the timing of reprogramming of somatic cells

Trial 1												
pH/days	1	2	3	4	5	6	7	8	9	10	11	12
6.6	0	0	0	0	0	0	0	0	0	0	0	0
6.8	0	0	0	0	0	0	0	0	0	0	2	2
7.0	0	0	0	0	0	0	0	0	4	7	9	9
7.2	0	0	0	0	0	0	2	5	8	10	12	15
7.4	0	0	0	0	0	2	3	6	8	10	16	18
7.6	0	0	0	0	1	1	2	3	3	4	4	6
7.8	0	0	0	0	0	0	0	0	0	0	0	0

Trial 2												
pH/days	1	2	3	4	5	6	7	8	9	10		
6.6	0	0	0	0	0	0	0	0	0	0		
6.8	0	0	0	0	0	0	0	0	0	0		
7.0	0	0	0	0	0	0	0	0	4	7		
7.2	0	0	0	0	0	0	2	5	8	10		
7.4	0	0	0	0	0	2	3	6	8	10		
7.6	0	0	0	0	1	1	2	3	3	4		
7.8	0	0	0	0	0	0	0	0	0	0		

Trial 3												
pH/days	1	2	3	4	5	6	7	8	9	10	11	12
6.6	0	0	0	0	0	0	0	0	0	0	0	0
6.8	0	0	0	0	0	1	0	0	0	0	0	1
7.0	0	0	0	0	0	1	2	2	4	4	6	5
7.2	0	0	0	0	0	1	1	8	6	8	7	8
7.4	0	0	0	0	2	1	4	5	4	5	6	10
7.6	0	0	0	0	1	1	3	5	2	4	5	6
7.8	0	0	0	0	1	3	2	1	1	2	2	1

Oc3/4-GFP positive colonies were counted in each pH condition every day during the culture of the transfected MEFs on the reprogramming. Gray columns indicate the number of GFP positive colonies.

colonies was observed at 5 days of culture at pH 7.8, but the latest appearance of colonies was at 11 days of culture at pH 6.8 (Table 2.2).

pH effects on the colony morphology of iPSCs and ESCs

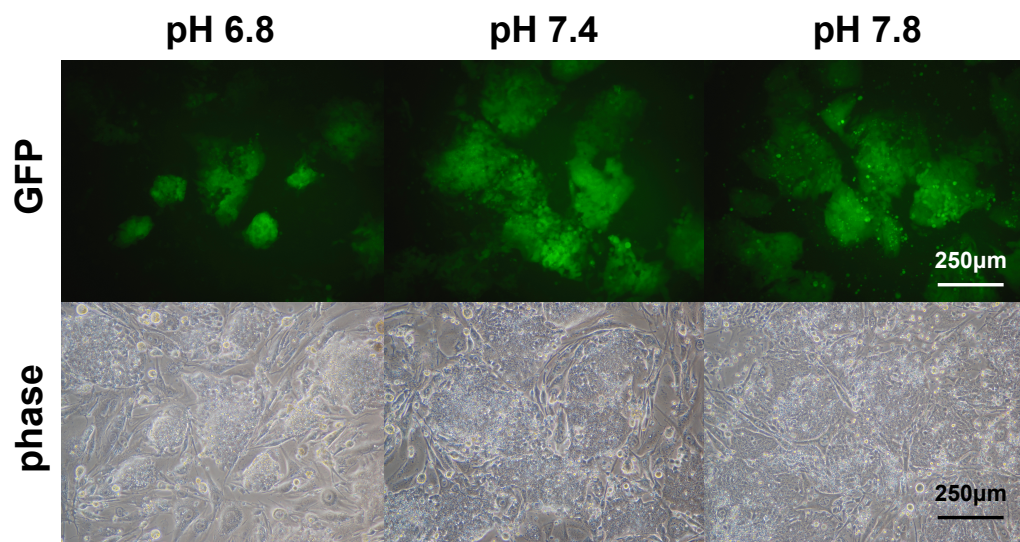
During somatic cell reprogramming, the morphology of iPSC colonies was differed depending on the pH (Figure 2.5, Figure 2.6, Figure 2.10). The colonies obtained between pH 7.0 and 7.4 had compact morphologies with strong GFP expression and AP activity (Figure 2.5, Figure 2.10). On the other hand, the colonies obtained at pH 6.6 had a compact morphology with weak GFP expression and AP activity. The colonies obtained at pH 7.6 and pH 7.8 were dispersed and flat in appearance compared with the colonies obtained in other treatment groups of pH.

When ESCs were cultured at various pH values, morphological differences in the colonies were observed at each pH (Figure 2.11, Figure 2.9). The cell distance among neighbor ESCs in a colony was different depending on the pH. At pH 6.8, the cells were located close to each other, and the cell boundary was not clear. On the other hand, at pH 7.8, the cells were more dispersed and could be observed as single cells.

Effects of pH on the pluripotency and differentiation of ESCs

The ESCs were cultured in the differentiation-inducing medium, leading to mesendoderm in culture medium at pH 6.8, 7.4 and 7.8. For three days of culture in the presence of CHIR and activin A, the endodermal markers of *Mixl1* and *T* was expressed in the cells at pH 7.4 and 7.8 but not at pH 6.8 (Figure 2.12). *Nestin* expression, which is a neural marker, was slightly expressed at pH 6.8 but not observed at pH 7.4 and 7.8. To confirm the further effects of pH on ESCs differentiation, the cells were cultured in neural differentiation medium in the presence of bFGF and retinoic acid. For three days of the culture, *Nestin* was expressed at pH 6.8 and pH 7.4 but not expressed at pH 7.8 in the cells (Figure 2.12). Under the mesendodermal differentiation medium, the qPCR analysis also showed the high expression of mesendodermal differentiation genes in the high pH

(a)



(b)

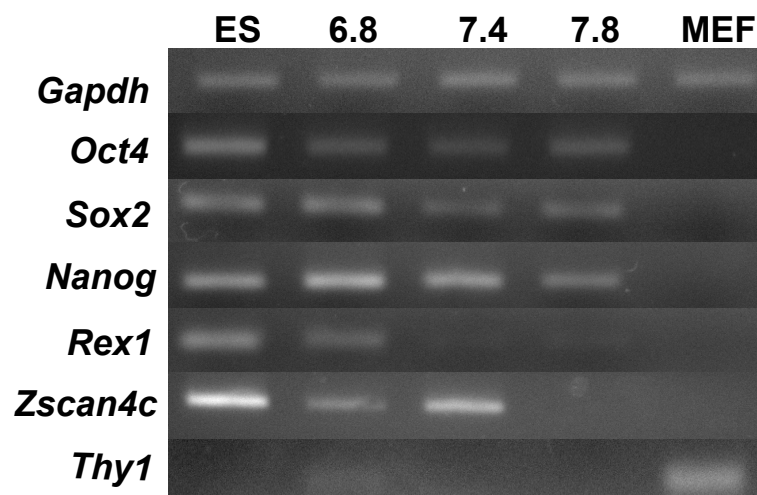


Figure 2.10 (a) Phase contrast images and GFP expressions of iPS cell lines established at pH 6.8, 7.4 and 7.8. Reprogrammed colonies obtained at each pH medium were passaged and cultured throughout at the same pH of medium. **(b)** Pluripotent gene expressions in iPS cells lines established at each pH of medium. MEF=mouse embryonic fibroblasts.

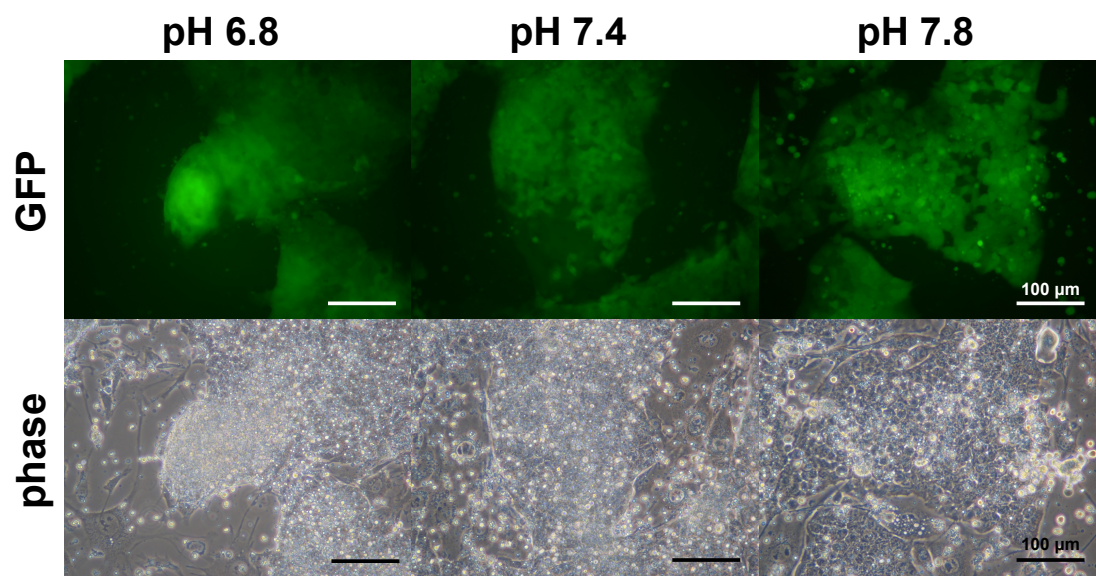


Figure 2.11 Morphology of the colony formation of mouse ESCs at different conditions of pH. Green fluorescence signals of the Oct4 reporter and phase-contrast images were examined in ES cells cultured at pH 6.8, 7.4 and 7.8 in the presence of LIF. Scale bar: 100 μm.

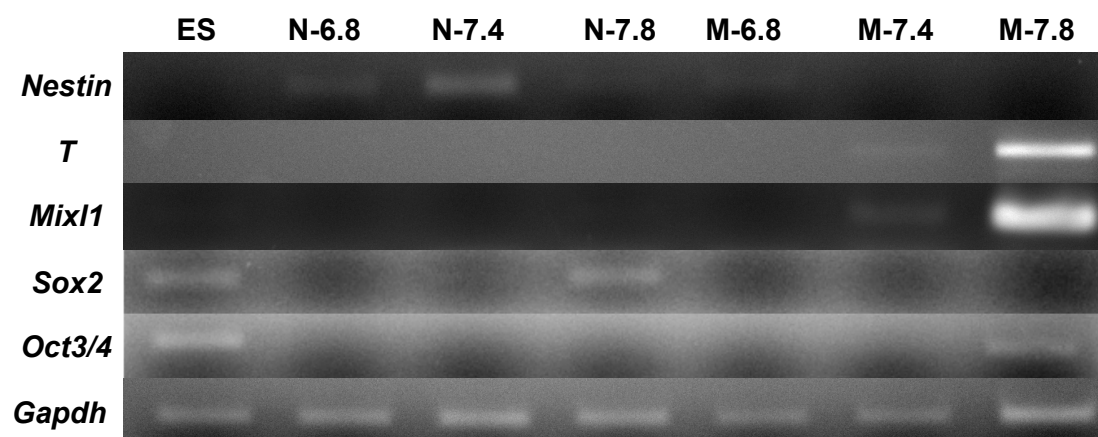


Figure 2.12 PCR analysis of neuroectodermal and mesendodermal gene expressions in differentiated ESCs under different pH conditions. N indicates the ESCs cultured under the neural differentiation condition for 3 days as well as M indicates mesendodermal differentiation condition.

condition (Figure 2.13).

At the same time, the cell number was estimated; the lowest number was observed at pH 6.8 in the mesendodermal differentiation medium, which was less than one-third at pH 7.4 (Figure 2.14). On the other hand, in the neural differentiation medium, there were no major differences in the number at pH 6.8 and 7.4. The cell number at pH 7.8 was the lowest both in the mesendodermal and neuroectodermal differentiation media (Figure 2.14). In the pH 6.8 condition, each cells were scattered and *Oct4*-GFP expressions in cells were weak for three days after the treatment of chemicals for mesendodermal differentiation (Figure 2.15). On the other hand, Oct4-GFP expression was still remained at pH 7.8 (Figure 2.15). Also, *T* protein, which is the early mesendodermal marker, was highly expressed at pH 7.8 (Figure 2.16)

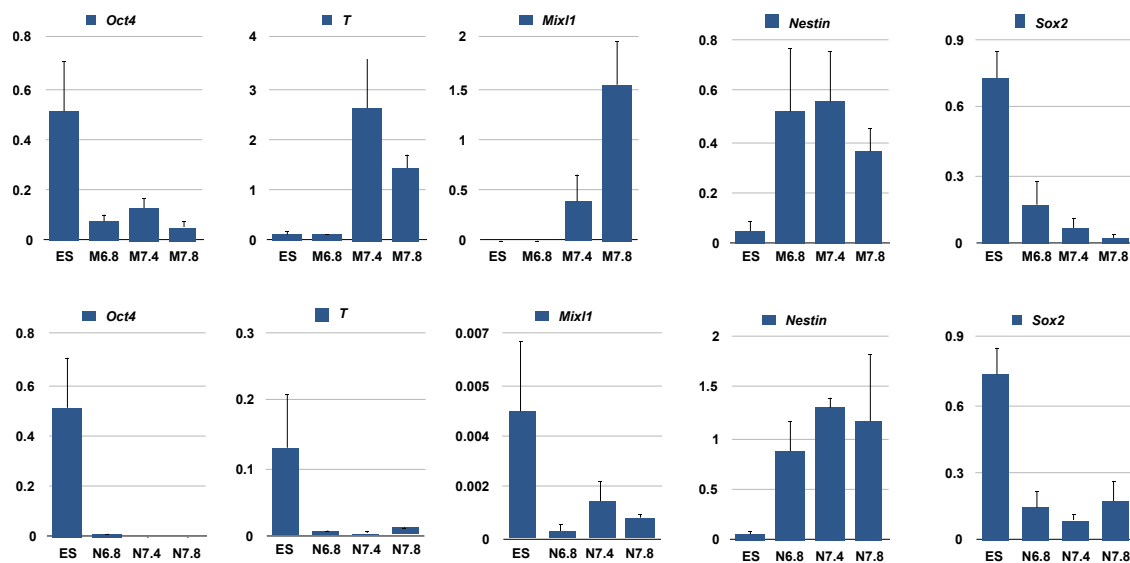


Figure 2.13 Quantitative real-time PCR analysis of neuroectodermal and mesendodermal gene expressions in differentiated ESCs under different pH conditions. Cells were cultured at a pH of 6.8, 7.4 and 7.8. Each expressed gene was normalized by the expression of Gapdh. N: Induction medium for neuroectodermal differentiation. M: Induction medium for mesendodermal differentiation. The experiments were independently performed three times.

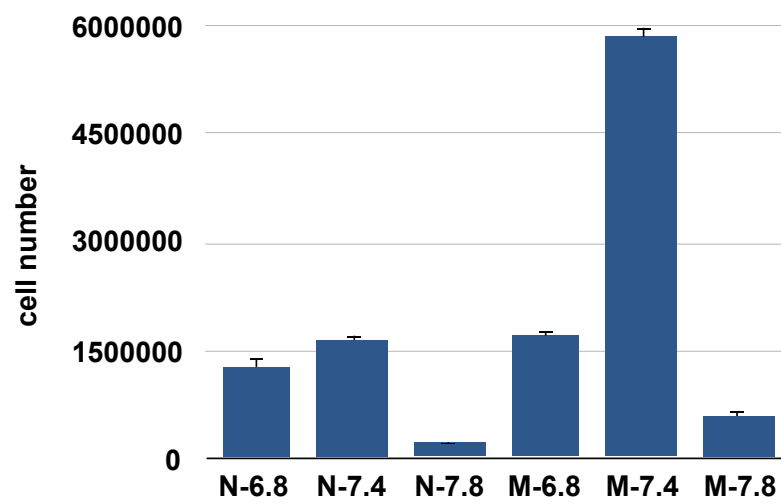


Figure 2.14 Number of cells in each treatment group at day 3 in each differentiation induction medium. n=3

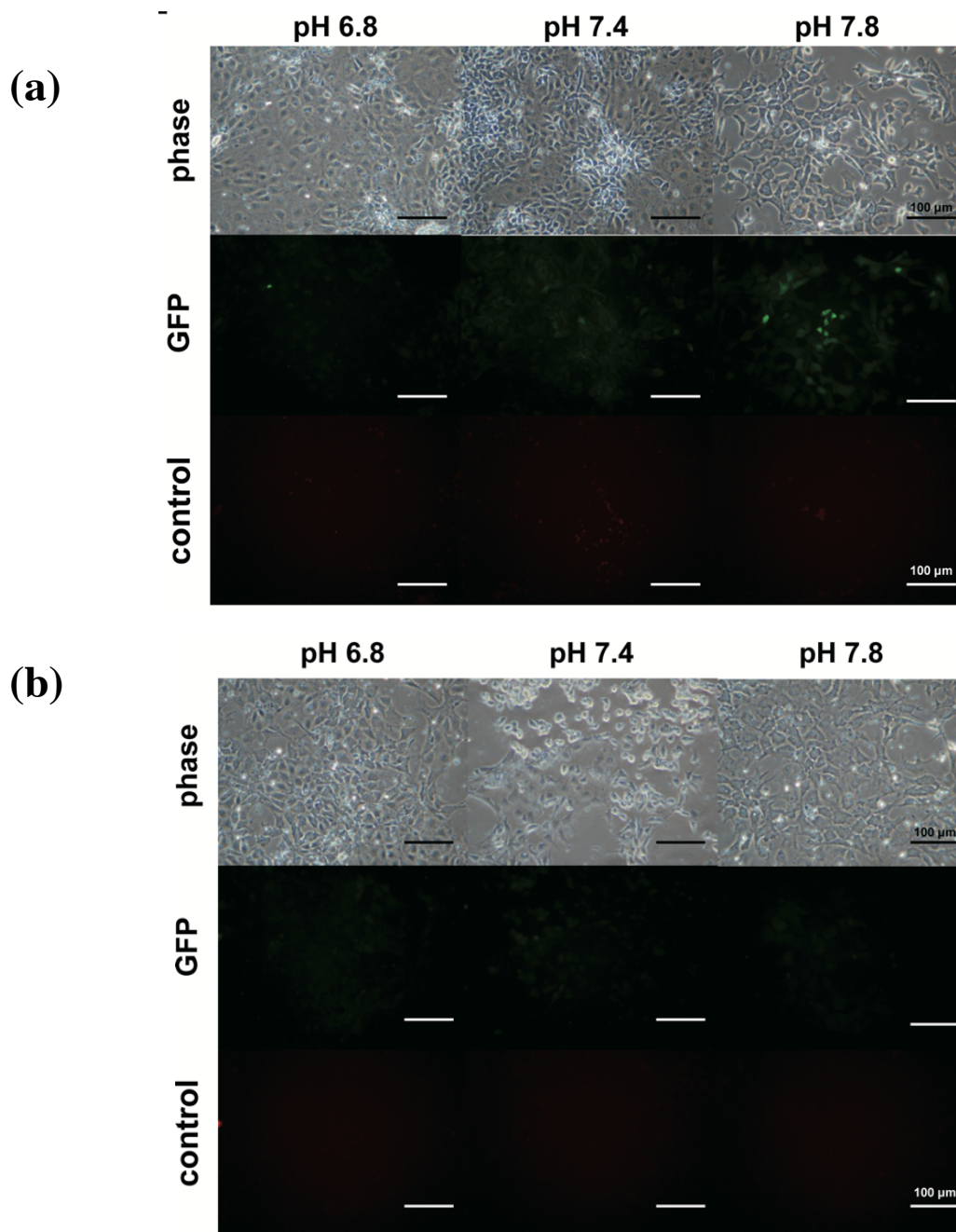


Figure 2.15 (a) Phase-contrast (phase) and GFP expression (GFP) of microscope images of ESCs cultured for 3 days in mesendodermal differentiation induction medium at pH 6.8, 7.4 and 7.8. Control means the micrographs observed by red filter to eliminate the possibility of self-fluorescence. Scale bar: 100 μm . (b) Phase-contrast (phase) and GFP expression (GFP) of microscope images of ESCs cultured for 3 days in neuroectodermal differentiation induction medium at pH 6.8, 7.4 and 7.8.

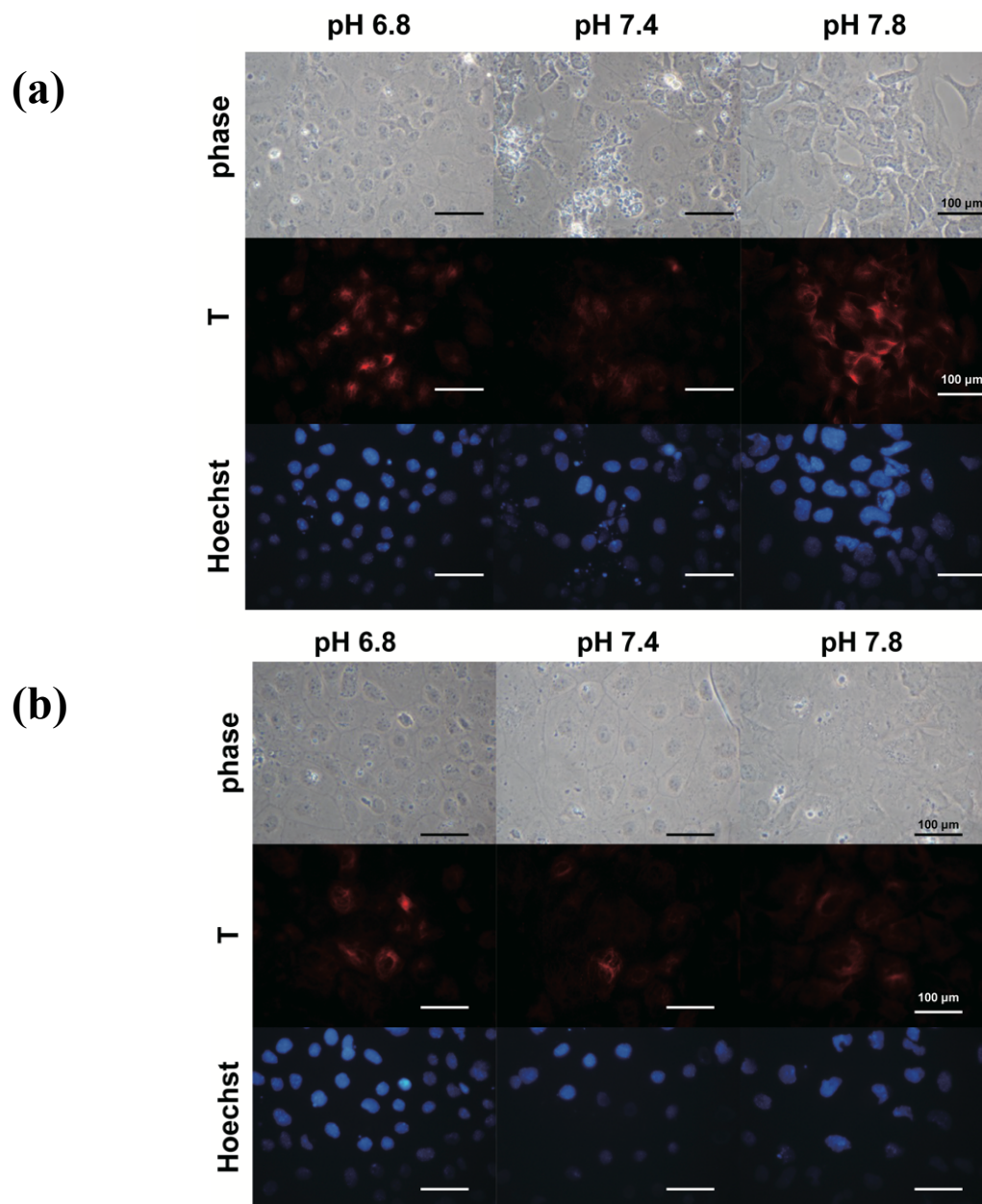


Figure 2.16 (a) Immunofluorescent staining of *T* protein (T) and DNA (Hoechst) of ESCs at pH 6.8, 7.4 and 7.8 for three days in mesendodermal differentiation condition. (b) Immunofluorescent staining of *T* protein (T) and DNA (Hoechst) of ESCs at pH 6.8, 7.4 and 7.8 cultured for 3 days in neuroectodermal differentiation condition.

2.4 Discussion

pH is well known to fluctuate significantly in cells and tissues and affects many biological phenomena. In this study, we have found that the fluctuation of pH in culture medium has an effect on the processes that occur during the somatic cell reprogramming and differentiation of ESCs.

The medium containing sodium bicarbonate stored at 4 °C in air usually indicates a higher pH with a 0.03% CO₂ concentration in air than the predicted pH under a 5 % CO₂ in the incubator at chemical equilibrium. The culture of pluripotent stem cells with high metabolic activity dependent on glycolysis, results in an immediate decrease in pH due to supplying lactic acid to the culture. The fresh medium was then pre-incubated, and the culture medium was changed every day in the process of somatic cell reprogramming to maintain the stable pHs in culture.

An acidic pH is known to suppress the cell cycle (Taylor et al., 1984). The proliferation of ESCs at lower pH (6.8-7.2) was decreased compared with that at pH 7.4, indicating that a lower pH inhibits the cell proliferation of ESCs (Figure 2.7). During somatic cell reprogramming, another 4 to 8 days were needed at pH 6.8 and 7.0 for the appearance of GFP-positive cells (Table 2.2). A high proliferation rate of cells is necessary for the induction of cell reprogramming and the maintenance of pluripotent stem cells (Ruiz et al., 2011). A finding was considered to be caused by the acidic pH of the medium. These results indicate that the delay in the appearance of colonies and the lower number of colonies at acidic pH may be caused by the inhibition of the cell cycle, resulting in a slow or inefficient induction of cell reprogramming.

Morphological differences in colonies at different pH values were observed during the induction of cell reprogramming and maintenance of ESCs, in which cells were dispersed in morphology at a high pH (pH 7.6-7.8) and compacted at a low pH (pH 6.6-7.2) (Figure 2.5, Figure 2.6, Figure 2.9, Figure 2.10, Figure 2.11). In oligodendrocyte precursor cells, acidic pH has effects on the cell migration (Jagielska et al., 2013).

Additionally, in cancer cells, migration is affected by pH, but vesicle trafficking, contraction, invasion, and metastasis are also affected (Damaghi et al., 2013). The compacted morphology of colonies in acidic pH might be caused by the inhibition of cell migration.

The variation of pH in the medium affected early cell differentiation into ME, which is the precursor of mesoderm and endoderm, and into NE. *Mixl1* and *Brachyury* (*T*) are observed in the primitive streak of embryos at the gastrula stage, which allow cells differentiate into mesoderm and endoderm (Ng et al., 2005; Tada et al., 2005; Kubo et al., 2004; Yasunaga et al., 2005). It has been known that Oct4 and Sox2 genes are inducers for mesendodermal and neuroectodermal cell differentiation at early ESCs differentiation, respectively (Thomson et al., 2011). In this experiment, Oct4-GFP expression at pH 7.8 also indicates the direction of cell differentiation to mesendodermal cells (Figure 2.12, Figure 2.13). Previously, Teo et al. showed the high pH in culture effects on the cardiac differentiation at pH 7.1 and 7.4 rather than pH 6.8 (Teo et al., 2014). In this study, cells showed much higher expression of *Mixl1* at pH 7.8. In addition, under the mesendodermal differentiation condition, *Sox2* and *Nestin* expression were relatively higher at lower pH. However, under the neuroectodermal differentiation condition, the expression of *Nestin* was not inhibited in any pH range compared to mesendodermal differentiation (Figure 2.13). These results indicate that the broad range of pH in culture affects cell differentiation and the specific inhibitory effects of low pH on ESCs of differentiation into mesendoderm. Cells are determined the differentiation direction by their environment to differentiate into the progenitors of ME or NE (Thomson et al., 2011). These results suggest that pluripotent stem cells define the direction of cell differentiation in culture, and environmental pH is one of the cues to determine the directional property.

The present study indicates that extracellular pH effects cell reprogramming and cell differentiation. There are many considerable pathways in which the pH affects the processes. For example, a low pH down-regulates cell proliferation by inducing p53 activation and p53-dependent cell cycle inhibition (Taylor et al., 1984; Williams et al.,

1999). Furthermore, the inhibition of p53 supports the establishment of iPSCs (Hong et al., 2009). Previous papers (Taylor et al., 1984; Williams et al., 1999; Hong et al., 2009), thus, have indicated that there is a close relationship between the pH effects on cell reprogramming and the cell cycle. In addition, it should also be considered that the effects of intracellular pH on cell physiology might act through organelles such as the nucleus, mitochondria, and endoplasmic reticulum as well as through internal epigenetic regulation (Casey et al., 2010). Although we examined the effects of pH in vitro, the fluctuation of pH is also considered to affect cells in vivo.

Chapter 3

Sensitivity of cells to culture conditions indicates a species-specific pathway for chicken somatic cell reprogramming

3.1 Abstract

Nuclear reprogramming is a useful technique that can produce transgenic and cloned animals, as well as assist in basic research. To date, this technique is still difficult in many kinds of animals, indicating there is an unknown mechanism that is interfering with reprogramming. Optimal pH is a fundamental factor that differs between cell lines and animal species, but its effects on somatic cell reprogramming have been little studied. Chicken embryonic fibroblasts (CEFs) were reprogrammed into induced pluripotent stem cells (iPSCs) under different pH conditions. A pH of 7.0 produced the most reprogrammed colonies within the tested range of 6.6 to 7.8. A pH difference of only 0.2 had a large effect on colony formation, with a pH of 6.8 producing only 10% of the number of colonies at pH 7.0. The optimal pH for growing CEFs also differed. Before reprogramming, proliferation was higher at a pH of 7.4 than at a pH 7.0, but during reprogramming, a pH of 7.0 was preferred. In addition, the pluripotency-related genes *NANOG* and *OCT4* had increased RNA expression at pH 7.0 during reprogramming. This study indicates that there is an optimal, chicken-specific method for reprogramming.

3.2 Introduction

For decades, methods that can produce transgenic and cloned animals have been investigated for their potential utility in the genetic modification of livestock, the preservation of endangered species, and their use both in the clinic and in basic research. These methods include nuclear transfer, cell fusion, and making induced pluripotent stem cells (iPSCs) (Stadtfelt et al., 2010; Yamanaka and Blau., 2010). In all of these approaches, the use of germ cells, such as oocytes and sperm, are required to produce individuals. Somatic cell reprogramming has been reported for many kinds of animals (Ezashi et al., 2012; Kumar et al., 2015), but there are still few successful reports that use iPSCs to produce individuals in species other than mouse, even when using methods known to improve reprogramming efficiency in mouse or human. To apply these techniques effectively, the optimal reprogramming conditions for each animal need to be revealed.

Avian embryonic stem cell (ESC) lines have been established from the blastodermal cells (BDCs) of freshly laid, fertilized eggs (Etches et al., 1996; Pain et al., 1996; Petite et al., 2004; Jean et al., 2012). Due to the mode of avian development, it is difficult to use oocytes for production of transgenic and cloned birds via methods such as nuclear transfer. Consequently, avian pluripotent stem cells like ESCs are a superior model. Chicken ESCs have several characteristics similar to mouse and human ESCs: high alkaline phosphatase activity, endogenous expression of *NANOG* and *OCT4*, and the ability to differentiate into three germ layers (Pain et al., 1996; Laval and Pain, 2010). However, the optimal conditions for working with chicken ESCs have not been fully explored.

The strategy of inducing iPSCs to form germ cells is an ideal technique for producing cloned and transgenic birds, especially for endangered and rare species for which it is difficult to obtain fertilized eggs (Laval et al., 2009; Nakamura et al., 2013). Several reports indicate that non-avian transgenes have the ability to partially reprogram avian somatic cells to a pluripotent state (Rosselló et al., 2013; Lu et al., 2012). In addition,

part of the homologous region of *Nanog* shared in *Xenopus*, chicken, and mouse induces reprogramming in murine cells, which suggests that reprogramming mechanisms are somewhat common in vertebrates (Theunissen et al., 2011; Tapia et al., 2012). However, the optimal culture conditions for avian pluripotent stem cells have not been fully revealed and do not match those of murine or human cells, which can result in poor differentiation ability and short-term viability, suggesting there might be avian-specific culture requirements. Knowing these avian-specific conditions is required for efficient application of nuclear reprogramming technology.

One of the fundamental factors that fluctuate *in vivo* is pH. During development, the pH of amniotic fluid is different between human and pig (Lorenzen et al., 2015), and in avian species, the pH of blood vessels fluctuates at each developmental stage (Kuwana et al., 1996). pH thus differs among species and across development. In this study, the effect of the culture medium's pH on the reprogramming of chicken embryonic fibroblasts (CEFs) into iPSCs was investigated and the expression of chicken pluripotency-related genes was examined.

3.3 Materials and Methods

Preparation of chicken embryonic fibroblasts

Chicken cells were obtained from the White Leghorn (WL), Barred Plymouth Rock (BPR), and Rhode Island Red (RIR) breeds (Freshney, 2011) and used in the experiments. Fertilized eggs were obtained from the National Livestock Breeding Center, Okazaki station (Nagoya, Japan). After 8 days of incubating the fertilized eggs at 38.5°C and 70% humidity, ensuring the embryos were between developmental stage 25–27 according to the Hamburger and Hamilton staging series (1951), the heads and viscera were removed with fine surgical scissors in phosphate buffered saline (PBS). The bodies of the embryos were immersed in TrypLE (GIBCO, Life Technologies, Grand Island, NY, USA) in a 15-ml tube overnight at 4°C. After removing the extra TrypLE, the embryos were incubated at 37°C for 30 minutes, and 3 ml of Dulbecco's modified Eagle's medium (DMEM, GIBCO, Life Technologies) containing 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) was added. The embryos were then dispersed by pipetting; 500 µl of this supernatant was centrifuged, and the pellets were washed three times with PBS. The cells were then replated on gelatin-coated dishes with DMEM containing 10% (v/v) FBS. The CEFs were used within 3 passages.

Retroviral transfection and induction of CEF reprogramming

Retroviral transfection to CEFs were performed as described in the section 2.3. Induction of CEF reprogramming was also performed as described in section 2.3; Cells transfected with retrovirus were replated on mitotically inactivated STO feeders (approximately 2.5×10^4 cells/cm²) (Figure 3.1). The cells were then cultured in embryonic stem cell medium (ESM) supplemented with leukemia inhibitory factor (LIF) (see the section 2.3).

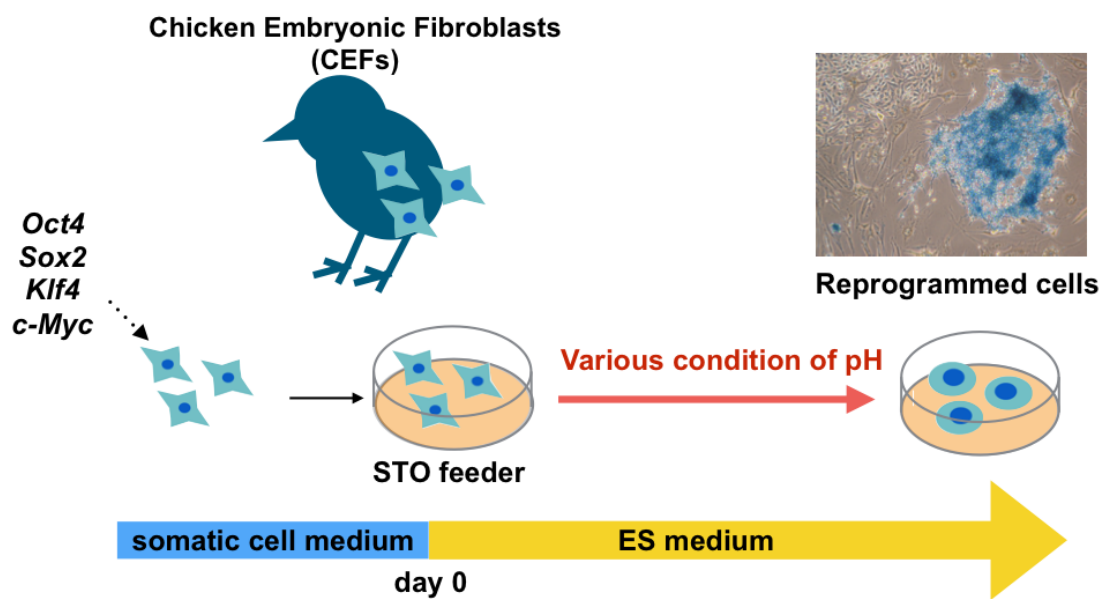


Figure 3.1 A scheme of reprogramming of CEFs. After CEFs were infected by the retrovirus, the cells were passaged onto feeders, which defined day 0 as the start of reprogramming. At that time, the pH of the culture medium was adjusted using sodium bicarbonate. To normalize the medium pH, fresh medium was pre-incubated for approximately 24 hrs under 5% CO₂. The micrograph shows the alkaline phosphatase staining of a colony 5 days after the induction of reprogramming.

Maintenance of constant pH in the culture medium

The pHs of the culture medium were adjusted as described in the section 2.3. The pH values were confirmed within 0.1 of standard error (Figure 3.2). The culture medium was monitored using a pH meter (B-212, HORIBA, Kyoto, Japan), and both stale and fresh media were checked.

Alkaline phosphatase activity in reprogrammed CEFs

The detection of alkaline phosphatase activity was carried out as described in the section 2.3.

RT-PCR

Total RNA was extracted from the cells using TRIzol (Ambion, Life Technologies, Foster City, CA, USA). To avoid genomic contamination, DNase (Roche, Indianapolis, IN, USA) was added. cDNA was synthesized using reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) and Random Primer (Invitrogen, Life Technologies). PCR was done using ExTaq (Takara). Transcription levels were normalized to the expression of chicken *GAPDH* or *ACTB*. All primer sequences are shown in Table 3.1.

Embryoid body (EB) formation and immunofluorescence

Reprogrammed colonies were picked and passaged on STO feeders. The hanging drop method was used to form EBs (Kurosawa, 2007). Briefly, medium with approximately 1000 cells per 150 μ l was plated on the bottom of the dish lid, allowing EBs to form at the bottom of the droplets. The medium used was Iscove's modified Dulbecco's medium (IMDM, Invitrogen) containing 10% (v/v) FBS (Sigma-Aldrich), 22-mercaptoethanol (Wako), 73 unit/ml penicillin (Sigma-Aldrich), and 50 μ g/ml streptomycin (Sigma-Aldrich). After 3 days of culture, floating cell masses were transferred onto gelatin-coated dishes and cultured in EB medium for another 3 days. The resulting cell culture was analyzed using immunocytochemistry.

For immunofluorescence analysis, cells were fixed with PBS containing 3.7%

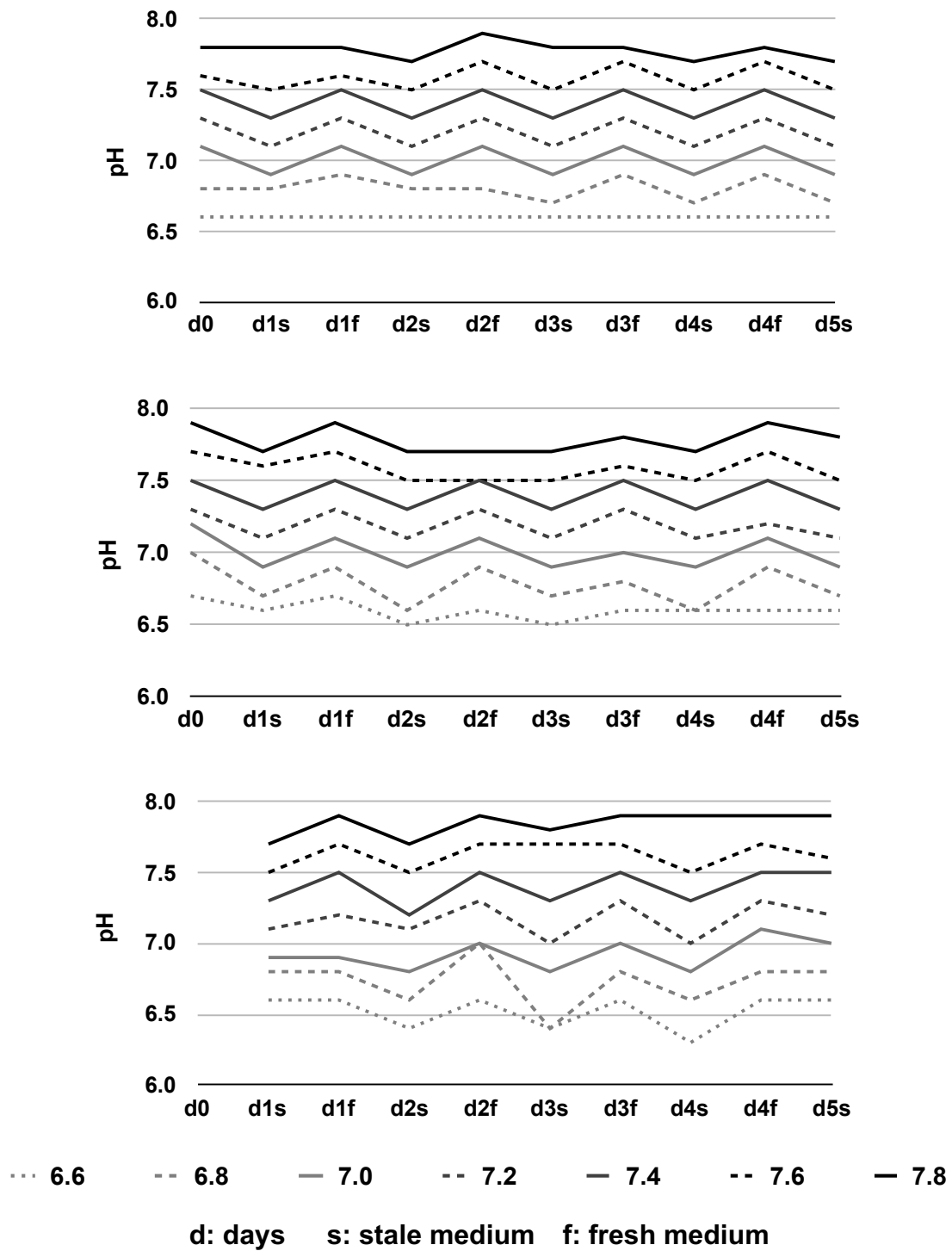


Figure 3.2 The pH fluctuation during the reprogramming of CEFs.

Table 3.1. Sequences of primers for RT-PCR

Gene	Forward Primer (5' to 3')		Reverse Primer (5' to 3')		Source
Transgene	<i>pMXs-Sox2</i>	GGTTACCTCTTCCCTCCCACTCCAG	TTATCGTTCGACCACTGTGCTGCTG	#13367*	
	<i>pMXs-cMyc</i>	CAGAGGAGGAACGAGCTGAAGCGC	TTATCGTTCGACCACTGTGCTGCTG	#13375*	
	<i>pMXs-Klf4</i>	GCGAACTCACACAGGCGAGAAACC	TTATCGTTCGACCACTGTGCTGCTG	#13370*	
	<i>pMXs-Oct4</i>	TTGGGCTAGAGAAGGATGTGGTTC	TTATCGTTCGACCACTGTGCTGCTG	#13366*	
Endogenous	<i>OCT4</i>	GTTGTCCGGGTCTGGTTCT	GTGGAAGGTGGCATGTAGAC		Lavial et al., 2007
	<i>NANOG</i>	CAGCAGACCCTCTCCTTGACC	TTCCTTGTCCCACTCTCACC		Lavial et al., 2007
	<i>GAPDH</i>	CTTGATCAATGGGCACGC	ACTTCCCCACAGCCTTAGC		NM_204305
	<i>ACTB</i>	CACCTTCCAGCAGATGTGGAT	AGTCCGGTTTAGAAGCATTTGC		NM_205518.1

* Addgene plasmid number

paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were blocked with PBS containing 5% BSA (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) for 45 min at room temperature, and then incubated overnight at 4°C with primary antibodies against glial fibrillary acidic protein (GFAP, 1:100, Z0334; DAKO, Glostrup, Denmark), actin smooth muscle (ASM, 1:1000, MS-113-P0; Thermo, Yokohama, Japan), or alpha-fetoprotein (AFP, 1:100, MAB1368; R&D Systems, Minneapolis, MN, USA). Alexa Fluor 594–conjugated goat anti-mouse IgG (1:500; Invitrogen, Life Technologies) was used as secondary antibodies. Nuclei were stained with 1 µg/ml Hoechst 33342 (Sigma-Aldrich).

Statistical analysis

Student's t test was applied to confirm the statistical significance of the difference between the sample means.

3.4 Results

Induction of reprogramming on CEF

CEFs from BPR, WL, and RIR were reprogrammed into iPSCs using a retrovirus (Takahashi and Yamanaka, 2006). Two days after the infected cells were replated on feeders, the cell morphology changed from mesenchymal cells to epithelial-like cells, signaling the start of reprogramming (Li et al., 2010). Reprogrammed colonies were compact and had a three-dimensional morphology at 4 days of culture (Figure 3.3). Several colonies had high alkaline-phosphatase activity and high expression of endogenous *NANOG* and *OCT4*. Almost all colonies expressed the exogenous genes from the pMXs vectors (Figure 3.4). Several colonies were picked, and the cells could be passaged approximately four times until they gradually lost the ability to proliferate. Among the three chicken strains, the colonies obtained from BPR chickens could be passaged 4 to 5 times over 20 days, whereas half the colony lines from WL chickens could not be maintained for 20 days (Table 3.2). While the cells were proliferating, embryoid bodies (EBs) could be formed (Figure 3.5). After further culture of EBs on gelatin-coated dishes for 3 days, the cells differentiated into three germ layers (Figure 3.5).

Effects of pH on colony formation during the reprogramming of CEFs

The pH of the culture media was varied during the reprogramming of CEFs, and the colony formation rate was estimated for each pH. Colonies having positive alkaline-phosphatase (AP) activity were counted across a range of pH values from 6.6 to 7.8. After 5 days of culture, the number of AP-positive colonies was the highest at a pH of 7.0 (Figure 3.6). The number of colonies at pH 6.8 and 7.4 were almost one-tenth the number at 7.0. There were no colonies obtained for pH 6.6 or 7.8. Across the range of pH, colony morphology differed. The lower pH values (6.8–7.2) yielded colonies that were compact and stacked, but the colonies were dispersed at higher pH (7.4–7.6) (Figure 3.7). The pH effects on colony formation were examined using WL, BPR, and RIR chickens, and all

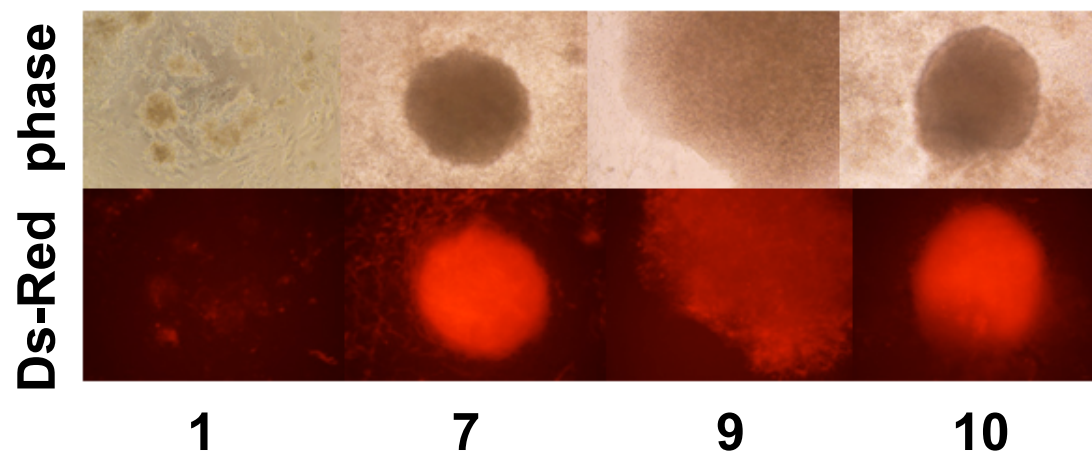


Figure 3.3 Phase-contrast images and pMXs-Ds-Red (red color) expression of the primary colony (7, 9, 10) and passaged colony (1).

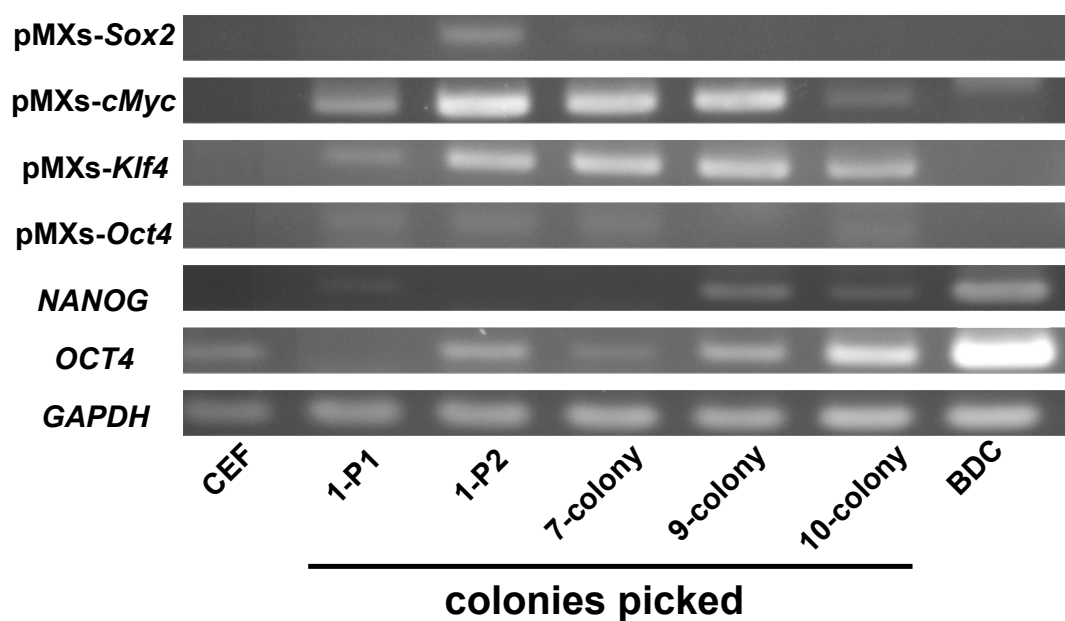


Figure 3.4 RNA expressions of the original CEFs, BDCs, and the colonies of reprogrammed cells. (P: number of passage, colony: primary colony) The expression of the transgenes from the pMXs vectors and the endogenous *NANOG*, *OCT4*, and *GAPDH* was examined.

Table 3.2. Length of culture for each reprogrammed colony.

Colonies/Days	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Day14	Day15	Day16	Day 7	Day18	Day19	Day20	Day21	Day22	Day23
WL #1	PU	MC	MC	Passage		MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage
WL #2	PU	MC	MC	Passage		MC	MC	stop													
WL #3			PU	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	stop	
WL #4			PU	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	stop	MC	MC	MC	MC
WL #5	PU	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	MC	stop				
WL #6	PU	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	MC	Passage	MC	MC	stop	
WL #7			PU	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	stop				
WL #8			PU	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	stop				
WL #9			PU	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	stop				
WL #10			PU	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC
WL #11			PU	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	stop	
WL #12			PU	MC	Passage		MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	stop				
BPR #1	PU	MC	MC	Passage		MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	stop	
BPR #2	PU	MC	MC	Passage		MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	MC	MC
BPR #3	PU	MC	MC	Passage		MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	stop	
BPR #4	PU	MC	MC	Passage		MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage
BPR #5		PU	PU	MC	MC	MC	MC	Passage	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage
BPR #6			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage
BPR #7			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	stop	
BPR #8			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC
BPR #9			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC
BPR #10		PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	STO	MC	MC	MC	MC	Passage
BPR #11			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC	MC	Passage	MC
BPR #12			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC
BPR #13			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage	MC
BPR #14			PU	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	MC	Passage

Compact and packed colonies were picked and cultured on feeder cells. 'Day' indicates the time since the transfectected cells were replated on feeders. The cells were maintained until they stopped proliferating. WL: White Leghorn, BPR: Barred Plymouth Rock, PU: date of picking, MC: date of medium change, Passage: date of passage, Stop: date of stopping the cell culture.

had the most colonies at a pH of 7.0.

pH effects on CEF proliferation and endogenous expression of pluripotency-related genes during reprogramming.

The proliferation rate and gene expression of the CEFs were examined at pH 7.0 and 7.4 (Figure 3.8). The proliferation rate of CEFs before infection by the retroviral vectors was lower at pH 7.0 than at pH 7.4 (Figure 3.8a). On the other hand, the proliferation rate of CEFs during reprogramming was higher at pH 7.0 than at pH 7.4 (Figure 3.8b).

The whole cells during reprogramming were collected after 2 and 4 days of culture, and the endogenous expression of *NANOG* and *OCT4* was examined. At pH 7.0, *NANOG* and *OCT4* were up-regulated, but not at pH 7.4 (Figure 3.9).

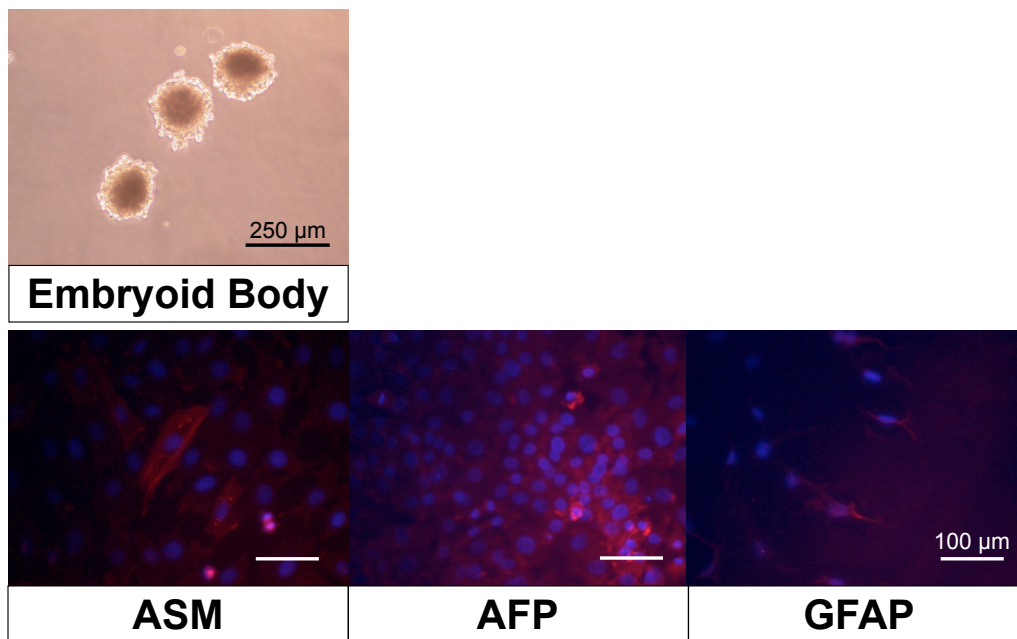


Figure 3.5 Embryoid bodies (EBs) of the reprogrammed cells (un upper micrograph) and their differentiation into three germ layers (lower micrographs). EBs derived from iPSCs were grown for 3 days in hanging drops under the dish lid. Immunocytochemical staining for markers of three germ layers were performed in differentiated cells derived from the EBs. Red color in lower figures indicate α -fetoprotein (ASM, endoderm), actin smooth muscle (AFP, mesoderm), and glial fibrillary acidic protein (GFAP, ectoderm), respectively. Blue indicates nuclear stained by Hoechst.

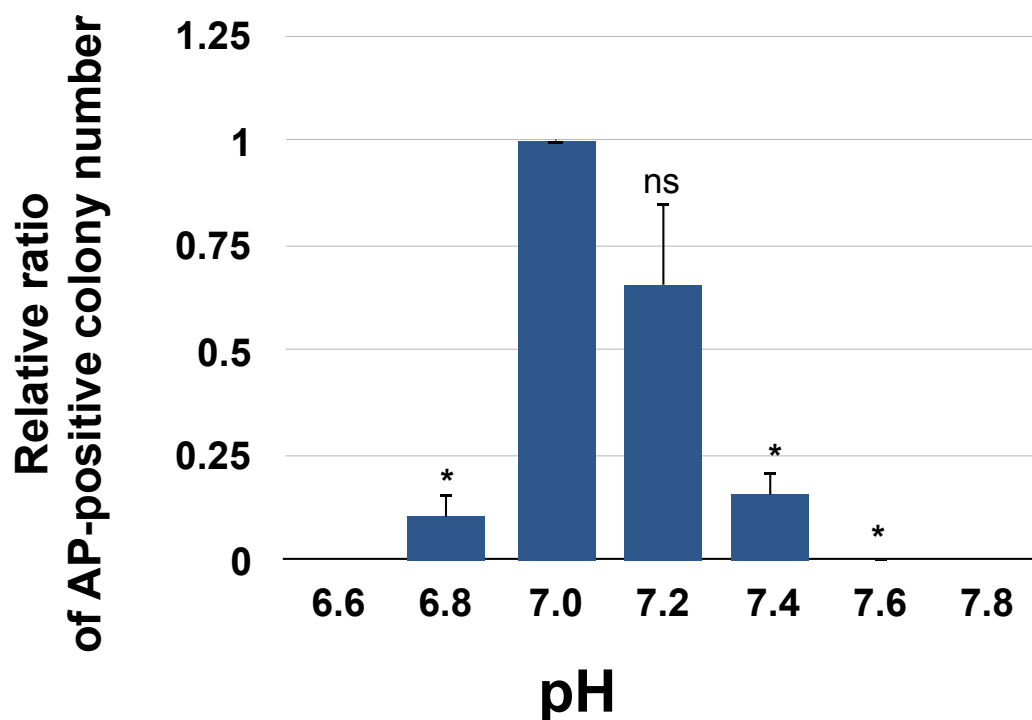


Figure 3.6 The number of colonies formed under different pH conditions during the reprogramming of CEFs. The relative ratio of colonies in each treatment group was the number of colonies divided by the number of colonies at pH 7.0. Experiments were repeated three times independently. For statistical analysis, each ratio of the treated group was compared to the ratio at pH 7.0. The asterisk indicates significant differences when compared to pH 7.0 (* $P < 0.01$; t-test). The data are presented as the means $\pm$ S.E.M.

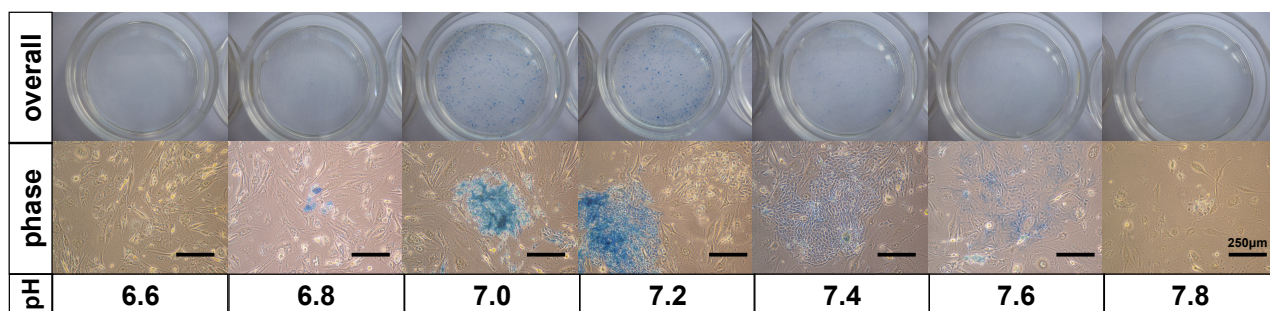


Figure 3.7 Phase-contrast images of AP-positive colonies 5 days after the induction of reprogramming.

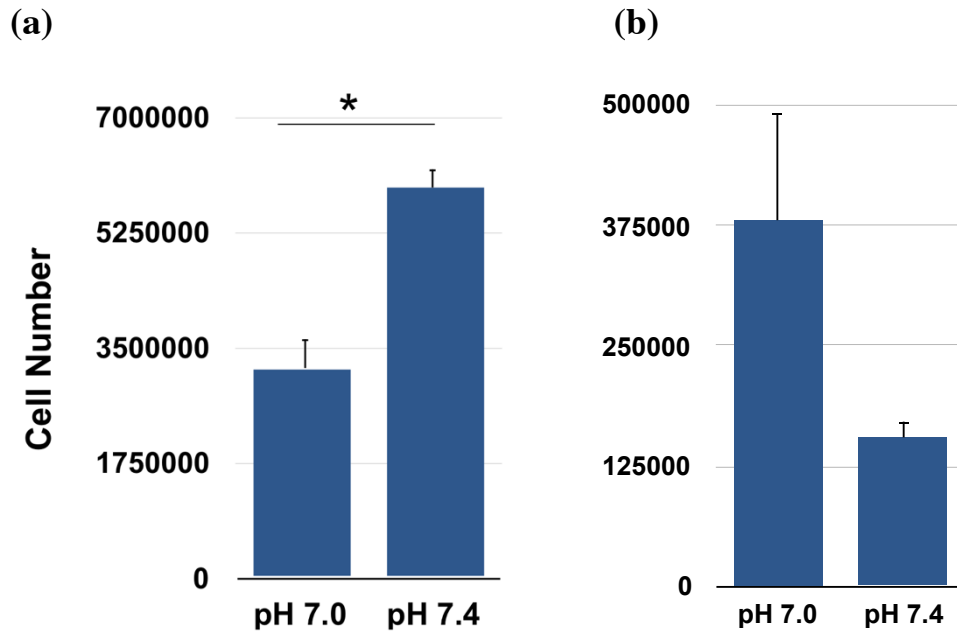


Figure 3.9 The comparison of the CEFs cultured at pH 7.0 and pH 7.4. **(a)** The average number of original CEFs cultured at pH 7.0 or pH 7.4 for 4 days. The asterisk indicates a significant difference between pH 7.0 and pH 7.4 (* $P < 0.01$; t-test). The error bars indicate S.E.M. n=3 **(b)** The average number of CEFs 5 days after the induction of reprogramming at pH 7.0 and pH 7.4. n=3.

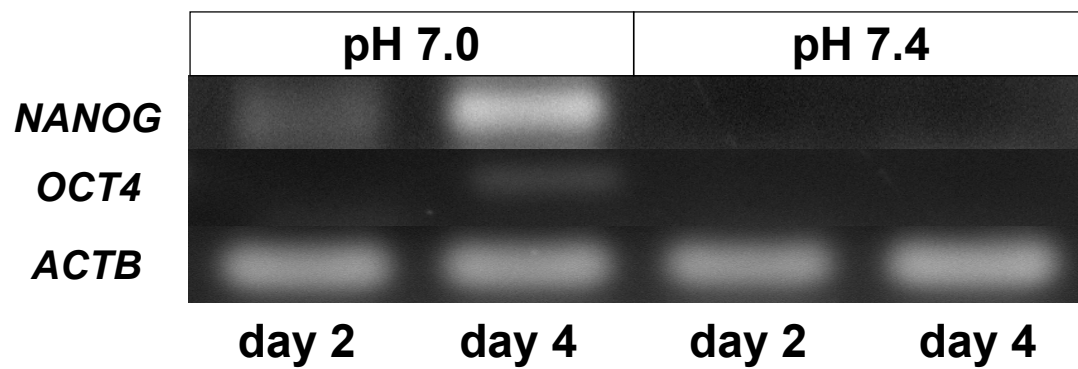


Figure 3.8 PCR analysis of CEFs at pH 7.0 and pH 7.4 at two and four days on the course of the induction of reprogramming.

3.5 Discussion

pH is one of the basic environmental factors of culture medium, and *in vivo*, affects every cell in the body. This study found that pH affects the efficiency of reprogramming somatic chicken cells into iPSCs.

Mouse strains are known to be one of the factors which cause the difference of the reprogramming efficiency in the nuclear reprogramming (Meissner et al., 2007; Ogura et al., 2013). Embryonic fibroblasts from three strains of chicken, BPR, WL, and RIR, were reprogrammed and there was a difference in the maintenance of reprogrammed colonies (Table 3.2). Although the reprogrammed colonies obtained from the BPR strain could be maintained longer than those of the WL strain, the effect of pH on colony formation rate had a uniform trend: the highest colony formation rate was observed at pH 7.0, and the reprogrammed chicken cells could not be maintained over 1 month at any pH. However, the reprogramming and maintenance of cells should be considered separate challenges, and the pH effect on reprogramming might be shared across the chicken species, rather than having strain-specific effects. Additionally, an opposing effect of pH was found on the proliferation rate of CEFs before and during reprogramming. These results indicate that the pH of the medium affects chicken somatic cell reprogramming in a species-specific manner.

There was a morphological difference in the reprogrammed colonies, which were more compact at pH 7.0 than at pH 7.4 (Figure 3.6). There are several molecular pathways in cells affected by environmental pH (Damaghi et al., 2013). Acidic pH inhibits the motility of several kinds of cells including cancer cells and oligodendrocyte precursors (Jagielska et al., 2013; Damaghi et al., 2013). The closeness of the cells in a low pH environment might result in a high concentration of paracrine cytokines around each cell. As the chicken cells were cultured in culture medium meant for mouse embryonic cells, there might be crucial factors needed for reprogramming that were among the cytokines secreted by the chicken fibroblasts themselves.

When the pH was varied during somatic cell reprogramming in mice, the highest number of colonies were obtained in medium with a pH of 7.4 (Chapter 2). However, the optimal pH for chicken was 7.0. In addition, reprogrammed colonies could be obtained across a pH range of 6.8 to 7.6 for chicken, but in mouse, the range was 6.6 to 7.8 (Figure 3.5) (Chapter 2). The change in colony number between a pH of 7.0 and 7.4 was two-fold in mouse, but ten-fold in chicken, indicating that the chicken cells are more sensitive to environmental conditions (Figure 3.5) (Chapter 2). This difference between mouse and chicken indicates that there are optimal culture conditions for reprogramming somatic cells for each animal species.

Revealing the different mechanisms operating during reprogramming and maintenance of pluripotency across different animal species is necessary, especially when using rare animal strains and those for which pluripotent stem cells cannot currently be maintained (Manoli et al., 2012; Verma et al., 2012). This study found a significant difference in the efficiency of chicken reprogramming due to the pH of the media. In addition, the sensitivity of chicken cells to environmental conditions will be a useful tool for investigating phenomena against which mouse reprogramming is robust. pH, a basic environmental factor, affected chicken somatic cell reprogramming, indicating that it is affecting an unknown, species-specific pathway.

Chapter 4

General Summary

Pluripotent stem cells are expected to be applied in various species for transgenic animals and cloned animals as well as for regenerative medicine and basic research. Although the generation of pluripotent stem cells is indeed useful to livestock production and the preservation of endangered and rare animals, there are difficulties in the generation of stem cell lines in various animal species other than mouse and human. As one of the solutions, the exploration of an optimal culture condition for generation and maintenance of pluripotent stem cells in animal species is necessary. In this study, the effects of pH in culture medium for the induction of cell reprogramming and differentiation were examined on mouse and chicken cells.

Despite being one of the most fundamental factors in medium, the effects of pH on pluripotent stem cells have not been fully investigated even using mouse cells. The transgenic mouse carrying the Oct4-GFP, which is enhanced by the endogenous expression of pluripotency-related gene (Oct4) with the green fluorescence protein (GFP), were used for the experiments regarding the induction of induced pluripotent stem cells (iPSCs) and differentiation of embryonic stem cells (ESCs). For the iPSC generation, the pH fluctuation in culture medium (pH 6.6 - pH 7.8) affected the efficiency and timing of cell reprogramming; the colony formation rate of mouse iPSCs was the highest at the pH 7.4 and the delayed cell reprogramming was observed at the lower pH. For cell differentiation of ESCs, the culture of ESCs at high pH in medium (pH 7.8) enhanced the mesendodermal differentiation rather than neuroectodermal differentiation, indicating the pH in culture affected not only the somatic cell reprogramming but also the destination of ESCs differentiation.

In the case of the chicken, the method for the induction of somatic cell reprogramming has not been established and the mechanisms on reprogramming are not fully understood. The most effective pH in culture at chicken somatic cell reprogramming was pH 7.0 despite the appropriate pH was pH 7.4 in mouse cells. In addition, the pH range that could obtain the reprogrammed colonies was more narrow in chicken, which was pH 6.8 to pH 7.6, compared with the mouse ranging between pH 6.6 and 7.8. These

results indicated that there are more sensitive characteristics of chicken cells to the environmental condition during cell reprogramming than those in mouse.

There was an optimal condition for the somatic cell reprogramming and differentiation of pluripotent stem cells. In addition, there was a different optimal pH condition for somatic cell reprogramming between mouse and chicken, indicating there might be a species-specific condition for the induction of somatic cell reprogramming. The elucidation of these phenomena would be expected to eliminate the interferences to establish pluripotent stem cells in various animals for bioindustrial applications.

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