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Identification of Candidate Porcine miRNA-302/367 Cluster and Its Function in Somatic Cell Reprogramming

Dong-Chan Son, Jae Yeon Hwang, and Chang-Kyu Lee†

Department of Agricultural Biotechnology, Animal Biotechnology Major, and Research Institute of Agriculture Life Sciences, Seoul National University, Seoul 151-921, Korea

ABSTRACT

MicroRNAs (miRNAs) are approximately 22 nucleotides of small noncoding RNAs that control gene expression at the posttranscriptional level through translational inhibition and destabilization of their target mRNAs. The miRNAs are phylogenetically conserved and have been shown to be instrumental in a wide variety of key biological processes including cell cycle regulation, apoptosis, metabolism, imprinting, and differentiation. Recently, a paper has shown that expression of the miRNA-302/367 cluster expressed abundantly in mouse and human embryonic stem cells (ESCs) can directly reprogram mouse and human somatic cells to induced pluripotent stem cells (iPSCs) efficiently in the absence of any of the four factors, Oct4, Sox2, c-Myc, and Klf4. To apply this efficient method to porcine, we analyzed porcine genomic sequence containing predicted porcine miRNA-302/367 cluster through ENSEMBL database, generated a non-replicative episomal vector system including miRNA-302/367 cluster originated from porcine embryonic fibroblasts (PEF), and tried to make porcine iPSCs by transfection of the miRNA-302/367 cluster. Colonies expressing EGFP and forming compact shape were found, but they were not established as iPSC lines. Our data in this study show that pig miRNA-302/367 cluster could not satisfy requirement of PEF reprogramming conditions for pluripotency. To make pig iPSC lines by miRNA, further studies on the role of miRNAs in pluripotency and new trials of transfection with conventional reprogramming factors are needed.

(Key words : miRNA, Reprogramming, iPSCs, Porcine)

INTRODUCTION

The pluripotency has been known to be acquired directly by transfecting four transcription factors, *Oct4*, *Sox2*, *Klf4*, and *Myc* into the differentiated somatic cell (Takahashi and Yamanaka, 2006). For a decade, after confirming the differentiated cells could be reprogrammed artificially and get pluripotency, the induced pluripotent stem cells (iPSCs) has been suggested to be a good source for stem cell research and patient-specific regenerative therapy without ethical problems. However, although iPSCs are good source for stem cell therapy and its research model, the unstable characters originated by inducing over expression of multiple transcription factors artificially has been pointed out the potential risk for applying iPSCs directly to the stem cell therapy.

To overcome the described limit of iPSCs, the derivation of iPSCs using microRNA (miRNA) was reported recently (Anokye-Danso *et al*., 2011; Miyoshi *et al*., 2011). This non-coding RNA with 20～24 bp length, regulated gene expression by combining with RNA-induced silencing complex (RISC) and binding to target RNA, and resulting in RNA degradation finally (Hannon, 2002). This mechanism known to RNA interference (RNAi), which is observed in various biological processes like proliferation, differentiation, and stem cell maintenance (Bartel, 2004; Ambros, 2004). Over the thousand miRNAs has been annotated in human (1,872) and mouse (1,186). Previous studies revealed that miRNA-302/367 and miRNA-290/371 cluster were expressed embryonic stem cells (ESCs) specifically (Suh and Lee, 2004; Lakshmipathy and Love, 2007; Morin and O'Connor, 2008; Marson and Levine, 2008). Interestingly, one of the ESCs-specific miRNA cluster, miRNA-302/367, could iPSCs without any co-transfection of exogenous transcription factors (Anokye-Danso *et al*., 2011).

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† Corresponding author : Phone: +82-2-880-4805, E-mail: leeck@snu.ac.kr

Although the increasing knowledge about the relation between miRNAs and stem cell research/reprogramming somatic cells in mouse and human, the porcine miRNA information is restricted and their functions in somatic cell reprogramming has not been studied.

Therefore in this study, reprogramming of porcine fibroblast cells was performed inducing porcine specific miRNAs. Because of the absence of annotated porcine specific miRNAs considered to have relation with pluripotency, the region having the same seed sequence of human miRNA-302/367 close to the porcine *LARP7* gene. The selected region was cloned and transfected to the porcine somatic cell. However, sole induction of selected miRNA-302/367 candidate couldn't reprogram the porcine fibroblast fully as in mouse and human.

MATERIAL AND METHODS

Porcine miRNA-302/367 Cluster Search

The sequences of porcine uncharacterized miRNA (ENSEMBL: ENSSSCT00000020547, ENSSSCT00000021153, ENSSSCT00000021456, ENSSSCT00000020039) were used and multiple alignment with human and consensus of miRNA-302/367 cluster (miRBase: MI0000738, MI0000772, MI0000773, MI0000774, MI0000775) were aligned using MultAlin server (http://multalin.toulouse.inra.fr/multalin/multalin.html). Secondary structure of the porcine miRNA was drawn by RNAfold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

MicroRNA Expression Vector Construction

A pig genomic DNA was extracted from pig embryonic fibroblast cells (PEF) using G-DEX IIc genomic DNA Extraction Kit (cell/tissue) (iNtRON, Korea) according to the manufacturer's instructions. A pig genomic DNA fragment comprising miRNA-302/367 of miRNA was amplified by PCR using forward primer (5′-gag aat tcc aag tgc cca aag tcc-3′) and reverse primer (5′-ctg aaat tcc tac tga aga gga gaa gga tac-3′) set. PCR was performed in thermo cycler under the following condition: 94℃ for 5 min, 30 cycles of denaturation at 94℃ for 30s, annealing at 60℃ for 15s, extension at 72℃ for 1min, and a final extension at 72℃ for 10min. The amplified fragment was cloned into pGEM-T easy vector (Promega, USA) and verified by sequencing. Sequencing was performed using an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, USA). The fragment was excised from the pGEM-T easy vector and ligated into EcoRI site of pEGFP-N1 vector (Clontech, USA)) resulting in pEGFP-miRNA-302/367 vector.

Cell Culture and Transfection

PEF cells were cultured in DMEM (high glucose; WELGENE, Korea) containing 10% fetal bovine serum (FBS: collected and processed in the USA), 2 mM glutamax, 0.1 mM β-mercaptoethanol, 1× antibiotic- antimycotic (all from Gibco, USA). Transfection were performed using Lipofectamin2000 (LIFE technology, USA) according to the manufacturer's instructions. To establish the iPSCs, the transfected cells were cultured in porcine embryonic stem cell media (PESM). PESM consisted of 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, low glucose) and Ham's F10 media containing 15% fetal bovine serum (FBS; collected and processed in the USA), 2 mM glutamax, 0.1 mM β-mercaptoethanol, 1X MEM nonessential amino acids, and 1X antibiotic-antimycotic (all from Gibco, USA). To support pluripotency and self-renewal, the iPSCs were cultured in PESM with the following cytokines: 20 ng/ml human recombinant basic fibroblast growth factor (hrbFGF; R&D Systems, USA), and 100 ng/ml heparin sodium salt (Sigma-Aldrich, USA). Media were changed every 24 h and all cells were cultured in humidified conditions with 5% CO_2 at 37℃.

RESULTS

Identification of Porcine miRNA-302/367 Cluster

Because porcine miRNA-302/367 hasn't been annotated, candidate miRNA-302/367 cluster was searched by comparing that of human. As miRNA-302/367 cluster was expressed polycistronically on the intron of *Larp7/LARP7* (NCBI: NM_138593/ NM_001267039) in mouse and human, the presence of the miRNAs or conserved mature miRNA-302/367 sequences were examined in pig *LARP7* locus. As our expectation, the uncharacterized miRNAs were annotated in the intron of porcine *LARP7* gene (NCBI: NM_001243815). The miRNAs present in the eighth intron of the porcine *LARP7* were well aligned to human mature miRNA-302/367 cluster sequence with same seed sequences (Fig. 1A). The uncharacterized porcine miRNAs were possible to form stable secondary structure (Fig. 1B). The results indicate that pig has the candidate miRNA-302/367 cluster orthologous composed to the seed sequence conserved with that of human and mouse miRNA-302/367 cluster.

Somatic Cell Reprogramming by Porcine miRNA-302/367 Cluster Transfection

To know the identified porcine miRNA-302/367 cluster can reprogram the porcine embryonic fibroblasts (PEFs) as in mouse and human, the porcine miRNA-

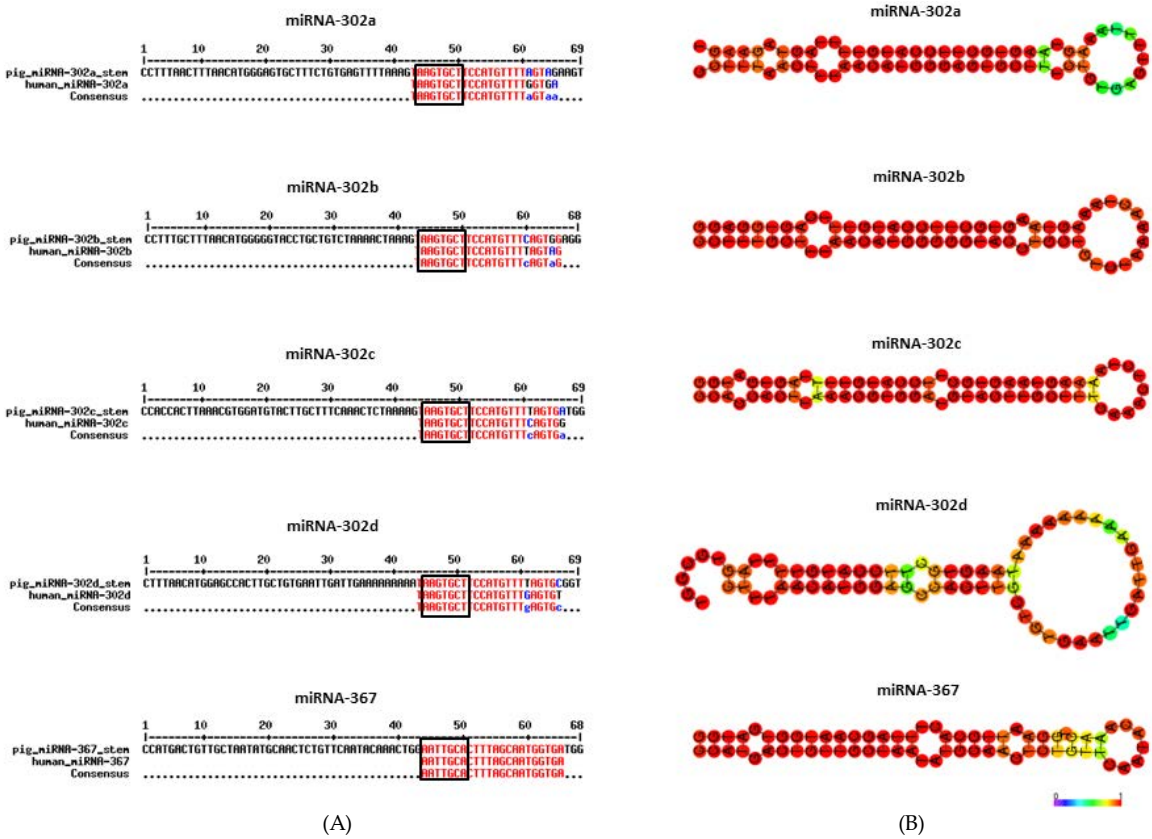

Fig. 1. Putative porcine miRNA-302/367 cluster identification and their secondary structure. (A) Multiple alignment of human miRNA-302/367 cluster to pig miRNA on *LARP7* locus. The porcine miRNAs located on the intron of *LARP7* was aligned to human ESCs- specific miRNA cluster, 302/367 cluster. Mature miRNA sequences were represented with red characters. The sequences in the boxes, AAGTGCT and AATTGCA, mean seed sequence of 302 family and 367, respectively. (B) Predicted secondary structure of identified porcine miRNA-302/368 cluster. Colors of each sequence indicate expected structure stability (0~1). The structure was predicted by RNAfold web server (http://rna.tbi. univie. ac.at/cgi-bin/RNAfold.cgi).

302/367 cluster was transfected into the porcine PEF. First, the genomic region coding candidate porcine miRNA-302/367 cluster was amplified by PCR and cloned into the pEGFP-N1 vector (Fig. 2A). About 1 Kb region harboring candidate porcine miRNA-302/367 cluster was passed into the somatic cells by serial transfection (Fig. 2B). After 20 days from the first transfection, the GFP positive cells with changed morphology were detected only porcine miRNA-302/367 cluster transfected PEFs. The cells were formed the compact colony. This result showed that identified porcine miRNA-302/367 cluster could induce reprogram of PEFs (Fig. 2C). However, the colonized cell populations were failed to maintain on the feeder cells.

DISCUSSION

Somatic cells are possible to be reprogrammed and induced to pluripotent stem cells which could be differentiated to various types of cells like ESCs. Although the iPSCs have the potential as a research model and regenerative therapy, the accurate regulation of genetic and epigenetic change is required to derive stable status of pluripotent stem cell (Barroso-del and Lucena-Aguilar, 2000). The abnormalities of iPSCs are observed in various studies after first derivation report (Pera, 2011). Laurent *et al*. (2011) reported that high number of copy number variation (CNV) was detected in the differentiated cells compared to the iPSCs by single nucleotide polymorphism (SNP) genotyping. This abnormality on CNV number was detected in the pluripotent gene relatively and considered to be occurred by *in vitro* culture condition (Hussein and Batada, 2011). Not just on the changes of short genome range, the iPSCs showed differences on the chromosome abnormality compared to ESCs (Mayshar, 2010). Epigenetic abnormality also was reported by Lister *et al*. (2011). They found a

thousand differentially methylated region (DMRs) between iPSC and ESC lines. Importantly a core set of DMRs which failed epigenomic reprogramming were found. These reports clearly demonstrate the genetic and epigenetic abnormalities on iPSCs and suggest the suitable iPSCs derivation with reprogramming of somatic cells properly.

The deriving methods of iPSCs are various following the exogenous delivering system. Viral vectors, like retro- and lenti-virus, application has been firstly used for deriving iPSCs from fibroblast. The viral vector induction showed efficient reprogramming and stable expression of exogenous gene, but infected transgenes weren't turned-off after iPSCs derivation (Hotta and Ellis, 2008) because of exogenous gene integration. This was pointed as a serious problem because the continued expression of the transgenes could induce abnormality on differentiation of iPSCs and cancer caused by abnormal cell cycle (Wong and Segal, 2008). To solve this restriction, various vector systems like adenovirus, which has no exogenous gene integration mechanism, and Piggybac transposon system, which could efficiently remove the transgenes from the genome, were applied (Woltjen and Michael, 2009). However, these applications showed limit because of very low iPSCs derivation efficiency. Other method for deriving the iPSCs are also tried like serial transfection of non-replicating vectors and direct delivering of protein into the cells without exogenous DNA delivering, but these methods also showed lower iPSCs derivation efficiency.

In this study, we tried somatic cell reprogramming by inducing miRNA to solve the limit of iPSCs derivation revealed after first iPSCs derivation. To reduce the genetic modification, we tried inducing reprogram by non-replicating vector delivery with serial transfection. Although the transfected PEF cells were showed morphological change, like colony forming (Fig. 2(C)), the cells were not maintained in the feeder cells. The failure of iPSCs derivation would be caused by following two reasons. Firstly, because the efficiency of transient transfection is different among the cell types and species (Yu and Hu, 2009), the vector delivery method used in this study was not proper in pig. Secondly, identified porcine miRNA-302/367 cluster would not possible to reprogram cells independently. Contrary to the previous reports in human and mouse, which cou-

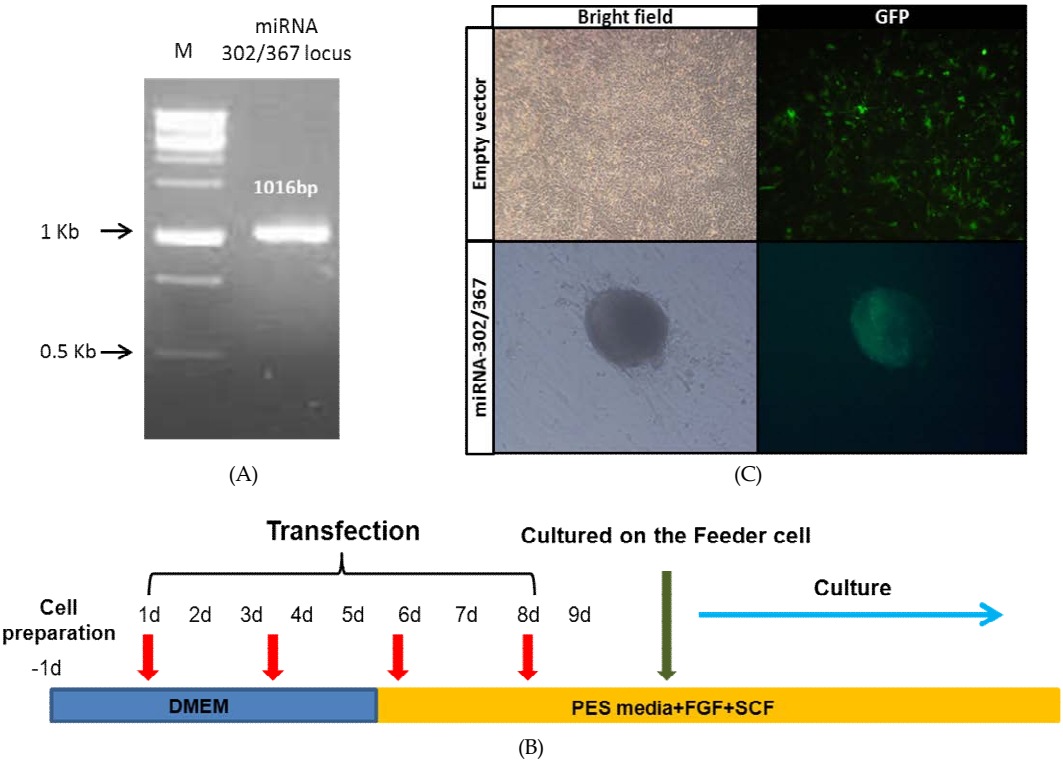

Fig. 2. Reprogramming of porcine embryonic fibroblasts by transfection of candidate porcine miRNA-302/367 cluster. (A) Amplification of genomic region containing porcine miRNA-302/367 cluster. About 1 kb range harboring whole candidate miRNA-302/367 cluster was amplified. M indicates DNA-ladder. (B) Diagram of serial transfection process. Transfection was performed four times for 8 days (red arrow). The culture medium was changed at the sixth date from the first transfection. (C) Morphogenic change of porcine miRNA-302/367 cluster transfected porcine embryonic fibroblast. The image was magnified 40X. Empty vector was used for negative control.

ld successfully induced iPSCs only using miRNA-302/367 cluster (Warren and Manos, 2010), our result suggest the reprogram in pig need other factors, not just through miRNA-302/367. And the porcine miRNA-302/367 cluster applied in this study could be less relation with pluripotency and having differential role in pig. So, further studies for relation between porcine miRNA-302/367 cluster and pluripotency in pig, and transfection with porcine miRNA-302/367 cluster or other candidate miRNAs and conventional reprogramming factor combination will be tried to solve the limit reprogramming of somatic cells in pig.

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