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ORIGINAL ARTICLE

Construction of a doxycycline inducible lentivirus that expresses stem cell-specific miR-302 cluster

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Abstract

Introduction: The polycistronic miR-302 cluster encodes five miRNA genes that have an important role in the regulation of embryonic stem cell function. Studies showed that the miR-302 cluster can reprogram both mouse and human fibroblasts to induced pluripotent stem cells (iPSCs) with high efficiency. The aim of this study was to generate an inducible lentivirus that expresses miR-302 cluster in order to further investigate somatic cell reprogramming by these miRNAs. *Materials and Methods:* The miR-302 cluster was amplified by polymerase chain reaction technique from human genomic DNA and was ligated into pTRIPz, an inducible lentiviral vector. *Results:* MRC5 fibroblasts and HEK293 (human embryonic kidney) cells were infected with pTRIPz-302 cluster lentivirus and the family of 302 miRNAs were strongly expressed in HEK293 cells but lowly expressed in MRC5 fibroblasts. When cultured in hESC conditions, MRC5 cells expressed only low levels of DNMT3B, *Nanog*, *Oct4* and *Lin28* and failed to show stem cell induction. The red fluorescent expression seen in the majority of MRC5 cells, indicated that the rate of infection by lentivirus was efficient. *Discussion:* The efficiency of reprogramming may be improved perhaps by either using a different cell type or a high expression vector with a different type of promoter.

Keywords: lentivirus, miR-302 cluster, induced pluripotent stem cells, microRNA

INTRODUCTION

The miR-302 polycistronic cluster houses 5 precursors of miRNAs, i.e. miR-302b, miR-302c, miR-302a, miR-302d and miR-367 that are abundantly and specifically expressed in human stem cells.[1] This miR-302 cluster is 688 nucleotides (nt) in length and is located in intron 8 of the Larp7 gene on chromosome 4 (Fig. 1). Table 1 lists a collection of sequencing data of members of the miR-302 cluster from 5 different sources, including ours.[1-5] The differences in expression levels of miRNAs such as miR-302a to d and miR-367 that are transcribed from the same promoter is an interesting feature of many miRNA clusters.

Most functional studies have been of miR-302a as this is generally consider as the functional guide strand and has a common seed region with other members of this cluster, namely miR-302b, c and d.[6-8] Inhibition of the miR-302 family in stem cells resulted in the downregulation of pluripotency markers and *vice versa*.[6] Furthermore, global loss of miRNA in DGCR8-deficient stem cells resulted in defects in proliferation and differentiation.[9]

Interestingly, various studies have shown that it is possible to reprogram a differentiated cell back to its unspecialised state, also known as an induced pluripotent stem cell (iPSC). This can be achieved by introducing stemness genes, namely *oct4, sox2, klf4* and *c-myc* (OSKM) transcription factors.[10] Tai *et al*. (2018) reported that iPSC can also be generated by non-integrated method using Sendai virus.[11] Studies described that the miR-302 cluster can reprogram both mouse and human fibroblasts to iPSCs with at a greater efficiency than the OSKM method.[12,13] There is considerable interest in the use of iPSC for regenerative medicine and drug testing and as models for disease such spinal muscular atrophy and LEOPARD syndrome.[14-17] The mechanism by which stem cells can be reprogrammed from somatic cells is also an area of great interest and would seem particularly approachable through the

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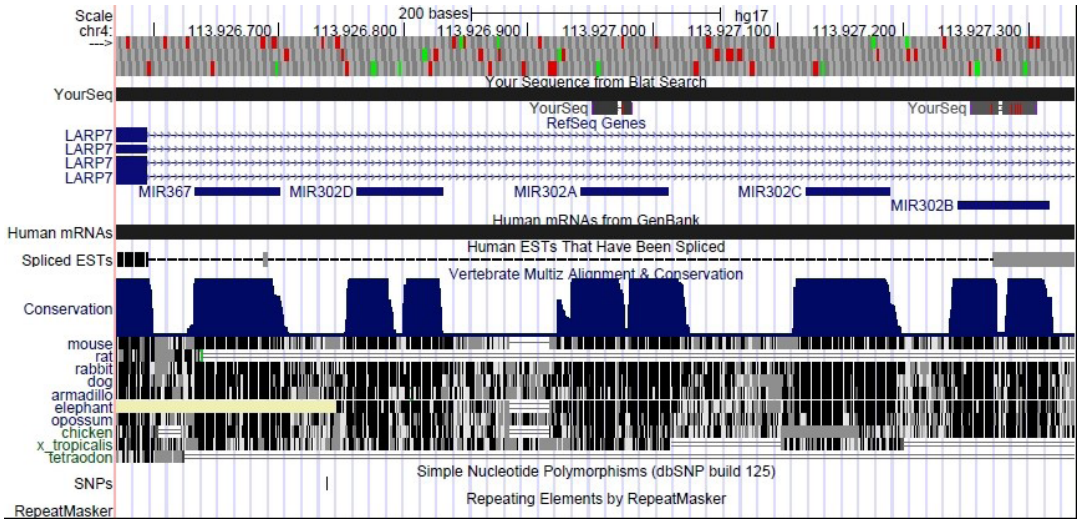

FIG. 1: The polycistronic miR-302 cluster is conserved. Image was taken from UCSC Genome Browser website (http://genome.ucsc.edu/cgi-bin/hgTracks?hgsid=280340015).

use of miRNAs. Here, an doxycycline inducible lentivirus was constructed in order to allow further study on somatic cell reprogramming by the miR-302 cluster.

MATERIALS AND METHODS

General cell culture

All culture dishes (Corning, Costar), flasks (Corning), and serological plugged pipettes (Corning, Costar) were suitable for sterile tissue culture. All cell lines used in the experiment were cultured in D10 media (Dulbecco's Modified Medium (DMEM) (Invitrogen, Gibco) supplemented with 10% (v/v) heat inactivated Foetal Bovine Serum (FBS) (PAA Laboratories), 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 μM glutamine (Invitrogen,

TABLE 1: Comparion of the sequencing results for the miR-302 cluster (302a, 302a*, 302b, 302b*, 302c, 302c*, 302d, 302d*, 367 and 367*) in human embryonic stem cells of selected publications. Numbers that are highlighted in grey denote the most highly sequenced miRNA in the cluster. The numbers are expressed as fractions of the total sequencing reads. 454, Illumina and Solexa represent the sequencing platforms that were used in the deep sequencing

Source	[1]Suh et al., 2004	[2]Bar et al., 2008	[3]Morin et al., 2008	[4]Lipchina et al., 2011		[5]Tan et al., 2014
Species/ Methods	Human	Human 454	Human Illumina	Human 454	Human Solexa	Human Solexa
302a	0.219	0.045	0.519	0.406	0.062	0.037
302a*	0.057	0	0.074	0	0.330	0.838
302b	0.429	0.402	0.214	0.015	0.255	0.041
302b*	0.019	0	0.005	0	0.007	0.002
302c	0.095	0.120	0.055	0.059	0.087	0.024
302c*	0.009	0	0.008	0	0.012	0
302d	0.124	0.433	0.121	0.244	0.080	0.027
302d*	0.048	0	0.004	0	0.002	0.016
367	0	0	0	0.276	0.164	0.015
367*	0	0	0	0	0.001	0

Table 2: Primers for amplification of miR-302 cluster from human genomic DNA

No	Primers		Sequence
1	miR-302cl	Fwd	TACTCGAGATCTTTGGGAACTAGTTCAG
		Rev	TCACGCGTGGATACTGGAGATCTAAAAG

Gibco). Experiments were carried out in sterile condition, in a Class II flow cabinet and all cells were maintained at 37°C in 5% CO_2.

Generally, cells were passaged twice weekly or once they reached approximately 80% confluency. Prior to incubating with 0.25% trypsin (Invitrogen, Gibco) for 5 minutes at 37°C in 5% CO_2, cells were washed in phosphate buffered saline (PBS). Subsequently, the cells were re-suspended in D10 media and centrifuged at 1000 rpm for 5 minutes. Then, the cell pellet was resuspended in D10 and plated to the required density.

Construction of inducible pTRIPz miR-302 cluster lentivirus

Human genomic DNA (RP11-148B6; chromosome 4) comprising the miR-302 cluster, accompanied by 120 bp upstream and 150 bp downstream to the cluster was amplified by polymerase chain reaction (PCR) technique (Table 2). The amplified product is 975 bp in length. The amplified fragment was ligated into the pGEM-T easy vector and verified by sequencing. Subsequently, it was excised and ligated into XhoI and MluI restriction sites, at position 3806 and 4064 respectively of the pTRIPz inducible lentiviral vector (Fig. 2).

pTRIPz vector has an inducible red fluorescent protein (RFP) marker. It was tested by infecting HEK293 (human embryonic kidney) and MRC5 (human lung fibroblasts) cells to observe for RFP. Doxycycline induced pTRIPz infected-HEK293 and -MRC5 cells expressed RFP (Fig. 3A). In addition, Northern blots of total RNAs collected from the infected HEK293 and MRC5 cells showed miRNA expressions from members of the miR-302c (Fig. 3B).

The HEK293 cell line (human embryonic kidney cells; ATCC) was used to determine the efficiency of miR-302 cluster expression. The MRC5 cell line (human lung fibroblasts; ATCC) was used to test the ability of the miR-302 cluster to reprogramme fibroblasts.

Production of lentivirus

One day prior to transfection, $1.5x10^6$ HEK293 cells were seeded in a 10 cm tissue culture plate containing 15mls of D10 (DMEM with glutaMAX and HEPES (Invitrogen), P/S and 10% heat inactivated FBS). The next morning, the medium was replaced with 12 mls of fresh D10 and transfection was performed in the afternoon. A plasmid DNAs mix of 15 µg lentivector, 10µg pCMVΔ8.91 packaging construct and 5µg VSV-g envelope construct was prepared in a ratio of 3:2:1 (Lentivector: Packaging: Envelope). Calcium phosphate transfection kit (Invitrogen) was used in the transfection.

Then, sterile water and calcium phosphate were added to a total volume of 500 µl. Followed by, 500 µl of 2x HBS was added drop-wise into the plasmid/ calcium phosphate mix, while mixing them rigorously and incubated at room temperature for 30 minutes. 25µM chloroquine

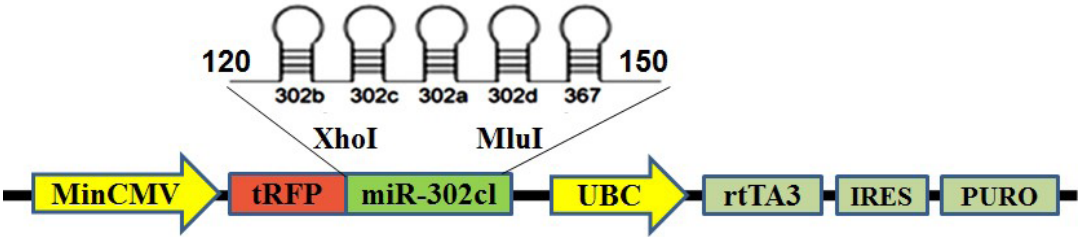

FIG. 2: MiR-302 cluster in the pTRIPz-miR-302 cluster lentiviral vector is driven by minimal CMV with tetracycline response element. Its expression can be monitored by turbo red fluorescent protein expression. 120 and 150 represent the length of nts extended upstream and downstream from the miR-302 cluster gene. MinCMV – Minimal cytomegalovirus promoter; tRFP – turbo red fluorescent protein; miR-302cl – miR-302 cluster; UBC – Ubiquitin promoter; rtTA3 – Reverse transactivator; IRES – Internal ribosome entry site; PURO – Puromycin.

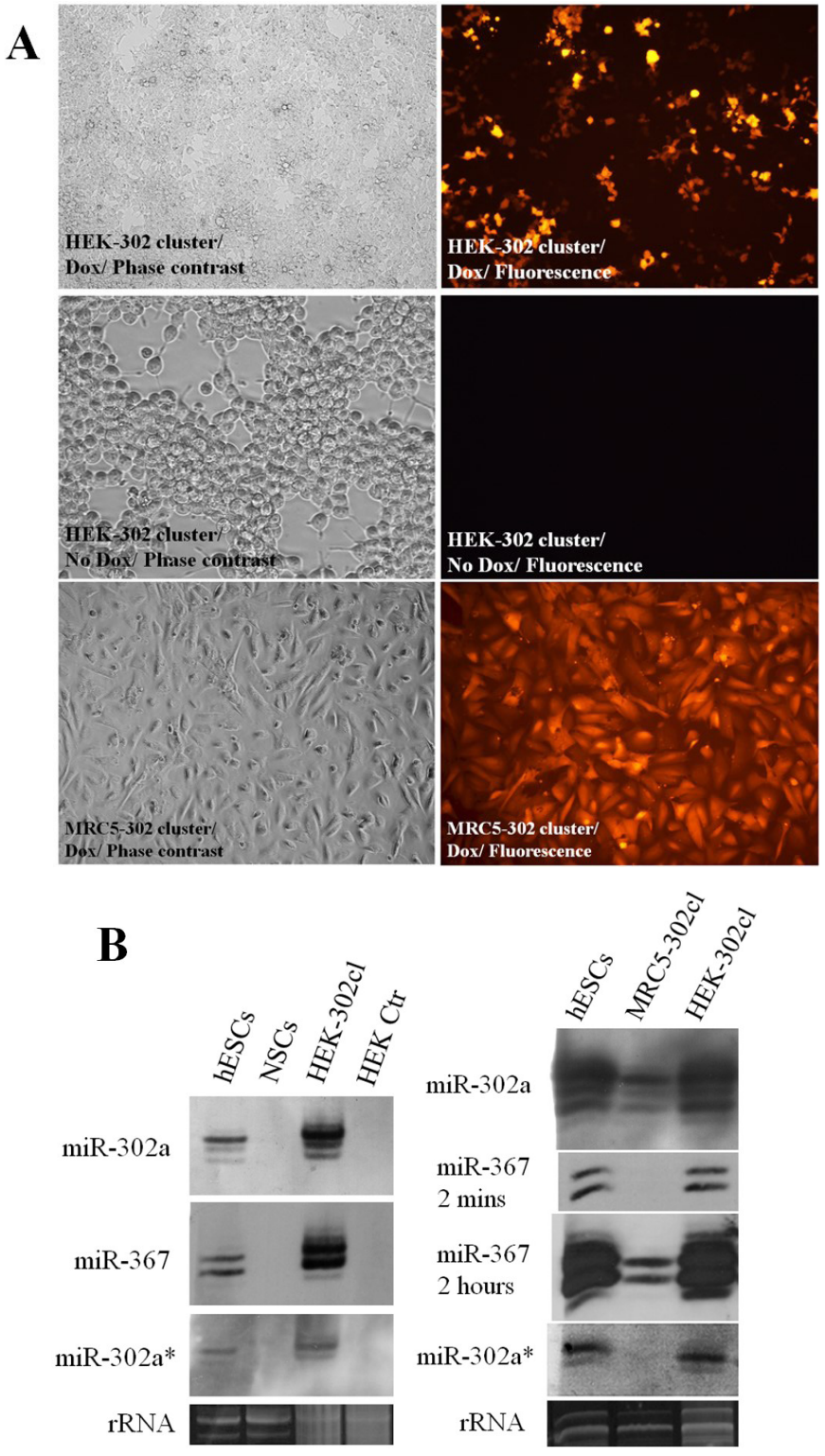

FIG. 3: **MiR-302 cluster expression in HEK293 and MRC5 cells**
(A) HEK293 and MRC5-infected cells expressed RFP after induction with doxycycline.
(B) Northern blots of infected HEK293 and MRC5 cells were probed for miR-302a, miR-367 and miR-302a*. rRNA stained with ethidium bromide was used as loading control. hESCs- human embryonic stem cells; NSCs- neural stem cells; HEK- human embryonic kidney cells; 302cl- miR-302 cluster; Ctr-Control.

(Sigma) was added to the media just before the addition of plasmid/ calcium phosphate mix, gently drop-wise. The cells were incubated overnight at 37°C with 5% CO_2. After 18 hours post-transfection, the cells were washed twice with D10 and replaced with 8mls fresh media. The media containing lentivirus was harvested continuously for 2 days, concentrated and stored at -80°C.

RESULTS

Both HEK293 (human embryonic kidney) and MRC5 cells (human lung fibroblasts) expressed RFP after they were infected with pTRIPz-302 and treated with doxycycline (Fig. 3A). Subsequently, northern blots of total RNAs collected from the infected HEK293 and MRC5 cells showed miRNA expressions from members of the miR-302 cluster, i.e., miR-302a, miR-302a* and miR-367 (Fig. 3B), confirming that pTRIPz-302 expressed the miR-302 cluster.

At day 5 post-transduction, MRC5 cells that were infected with the pTRIPz-302 cluster expressed RFP (Fig. 4) and formed colony-like cluster (Fig. 4). Total RNA extracted was analysed by PCR and showed expression of low levels of DNMT3B and *Nanog*, equivocal levels of *Oct4* and *Lin28*, but did not express *Sox2* (Fig. 5).

DISCUSSION

To date, few groups have successfully reprogrammed somatic cells to a pluripotent state by using miRNA alone.[12,18,19] Other groups have shown that miRNAs can enhance reprogramming by OSKM factors (*Oct4*, *Sox2*, *Klf4* and *Myc*).[20,21] Anokye-Danso *et al*., (2011)[12] reported that they have achieved a reprogramming efficiency with miR-302 cluster alone that was 2 orders of magnitude higher than conventional reprogramming using OSKM factors. In addition, somatic cell reprogramming was successfully performed using mature miRNA by simple transfection.[19]

Our data shows that pTRIPz-302 cluster did not fully reprogram the human lung fibroblasts cell line MRC5 to a pluripotent state, probably due to low miRNA expression. Lin *et al*., (2011)[22] showed that reprogramming of human hair follicle cells would only take place above a certain level of miRNA expression, approximately 1.5-fold of the miRNA expression in hESCs. Although we achieved a high level of expression of the miR-302 cluster in HEK293 cells, expression in MRC5 cells was relatively low.

Relatively little is known about the regulation of pluripotency and differentiation of stem cells by the miR-302a cluster. Rosa *et al*. (2011)[7]

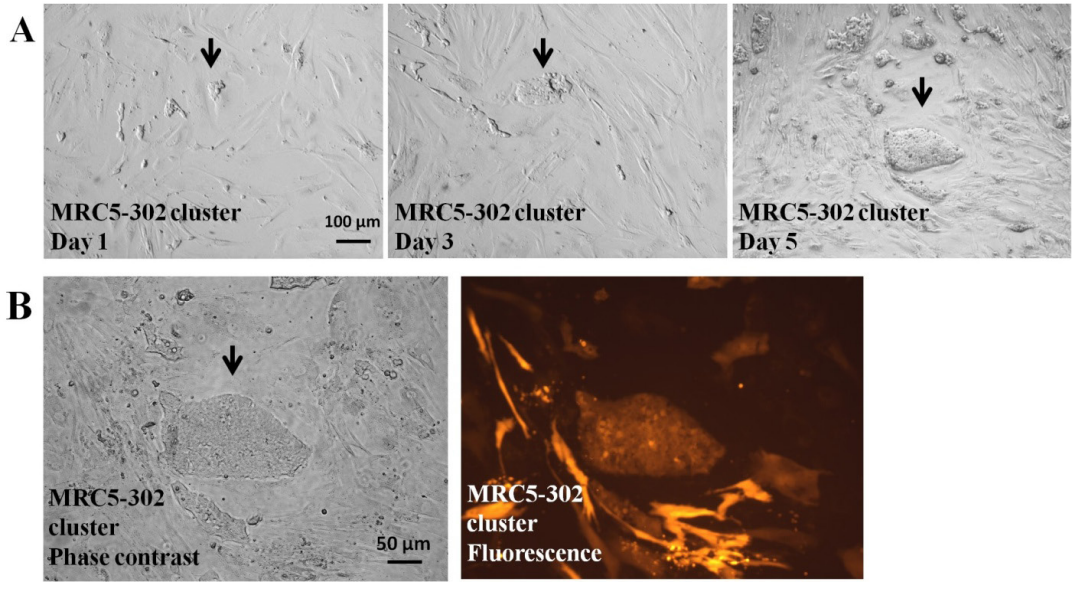

FIG. 4: **pTRIPz-302 cluster lentivirus infection in MRC5 cells**
MRC5 cells were infected with pTRIPz-302 cluster lentivirus, cultured in hESCs condition. Cell colonies appeared approximately 6-8 weeks after infection. A) Cell colonies at day 1, day 3 and day 5 from the day of appearance. Black arrows show cells forming colony. B) These cells expressed red fluorescent protein indicates that they were infected.

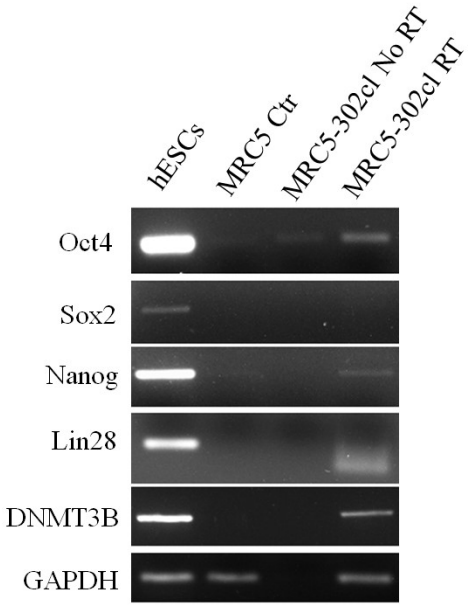

FIG. 5: **Comparison of pluripotency gene expressions between hESCs and infected MRC5**
Total RNAs were extracted from wild type/ control and pTRIPz-302 cluster virus infected MRC5 cells with and without reverse transcription. The pluripotency genes were compared between the MRC5 cells and hESCs. Ctr- Control; 302cl- 302 cluster; RT- reverse transcription.

reported that NR2F2, an inhibitor of Oct4 was a target of miR-302a. They showed that both Oct4 and miR-302a directly repress NR2F2, and regulate the maintenance of pluripotency and differentiation of embryonic stem cells. Notably, NR2F2 was a predicted target of miR-302a as well as miR-302a* by TargetScan Human. SP3 is a possible inhibitor of Oct-4 and Nanog transcription factors.[23,24]

Lin *et al*. (2011)[22] attributed reprogramming by the miR-302a cluster to global demethylation due to the repression of epigenetic regulators such as AOF1, AOF2, MECP1-p66 and MECP2. They found that expression of the miR-302 cluster repressed the above-mentioned proteins, accompanied by the appearance of pluripotency associated proteins and global demethylation. Other targets of the miR-302 cluster that might be involved in the maintenance of pluripotency and differentiation include lefty1 and lefty2,[7] CDKN1A, p21, Cyclin D1, BTG1, BTG2 and BTG3,[25,26] RHOC and TGFb,[21] and PTEN.[4,27]

In conclusion, the low level of expression of key stem cell genes and lack of expression of Sox 2 (Fig. 5) suggests that the MRC5 cells were not fully reprogrammed. This might be due to the low level of miR-302 cluster expression by pTRIPz-302 cluster in MRC5 cells. Red fluorescent expression is seen in majority of the MRC5 cells, however, miR-302 cluster expression in lentivirus infected-MRC5 cells was lower compared to HEK293 cells. Furthermore, the red fluorescent appeared less bright if compared to the HEK293 cells. This indicates the lentivirus infection is efficient but the expression is not as efficient. The efficiency of reprogramming might be overcome perhaps by either using a different cell type or a high expression vector with different type of promoter.

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