Abstract

Apobec2 plays a critical role in self-renewal of mouse ESCs and reprogramming of mouse iPSCs

Jihye You
Department of Molecular Medicine and Biopharmaceutical Sciences
Graduate School of Convergence Science and Technology
The Graduate School Seoul National University

Apolipoprotein B mRNA-editing enzyme catalytic subunit 2 (APOBEC2) is known that highly expressed in heart and muscle tissues and plays an important role in regulating and maintaining muscle development in mammals. Besides regulation of muscle development, Apobec2 expression and function in other cells and tissues are still unknown - especially in pluripotent stem cells.

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the pre-implantation blastocyst. ESCs can be propagated stably in an undifferentiated state *in vitro* (self-renewal) and, under the appropriate

culture conditions, can be induced to differentiate into a variety of cell types (pluripotency).

Proper telomere length is essential for self-renewal and pluripotency of ESCs. Also, telomerase plays a critical role in reprogramming and self-renewal of induced pluripotent stem cells (iPSCs). However, the mechanisms of telomere length regulation during induction and proliferation of iPSCs remain elusive. Here, we reports that Apobec2 is specifically expressed in mouse ESCs and iPSCs, and regulates self-renewal of mouse ESCs as well as reprogramming of mouse iPSCs by regulating expression of Tert mRNA.

Knock down of Apobec2 reduced colonization and proliferation of mouse ESCs. Furthermore, sphere-forming assay showed decreased self-renewal of mouse ESCs by Apobec2 knock down. During reprogramming of mouse iPSCs, the expression level of Apobec2 mRNA was increased in time dependent manner. Next, we performed knock down and overexpression of Apobec2 in prior to mouse iPSC reprogramming, and found that Apobec2 positively regulated reprogramming of mouse iPSCs. In this study, our results suggest that Apobec2 plays an important role in modulating self-renewal of mouse ESCs as well as iPSC reprogramming.

Keywords: Apobec2, mouse ESCs, mouse iPSCs, reprogramming, self-renewal, telomere, Tert

Student Number: 2016-27474

Contents

1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of pre-implantation mammalian embryo, hence, promising donor cell sources for regenerative medicine. ESCs can be propagated stably in an undifferentiated state *in vitro,* and under the appropriate culture conditions, can be induced to differentiate into a variety of cell types. For example, forced transgenic expression of Pdx1 causes ESCs to differentiate into pancreatic cells (endoderm), whereas GATA2 expression promotes leukocyte differentiation (mesoderm), and Mash1 expression induces neuronal differentiation (ectoderm)[1,2]. This plasticity makes ESC culture a useful tool for elucidating the functions of genes involved in early cellular differentiation.

Transcriptional regulation plays an essential role in pluripotency maintenance of ESCs, and a transcriptional regulation network for pluripotency has been characterized[3,4]. The core component of the pluripotency transcriptional regulation network is a feed-forward self-regulating circuitry formed by transcription factors Oct4, Sox2, and Nanog[5,6]. However, other genes involved in ESC pluripotency and self-renewal remain to be identified.

Apolipoprotein B mRNA-editing enzyme catalytic subunit (APOBEC) family members are cytidine deaminases and have diverse roles by

virtue of their ability to edit DNA and/or RNA[7-9]. Apobec2 is one of the oldest members of the APOBEC family, along with the lymphoid-specific activation-induced deaminase, and reported that the expression of Apobec2 is restricted to differentiated cardiac and skeletal muscle in mammals and chicken[10,11]. Unlike other APOBEC family members, the enzymatic activity and substrate of Apobec2 have not been fully demonstrated and its biologic functions remain unknown[12,13]. It has reported that deficiency of Apobec2 leads to a diminished mouse muscle mass and increased myofiber with centrally-located nuclei known as dystrophic phenotypes[14,15]. Recently, it has also reported that Apobec2 negatively regulates myoblast differentiation and fusion in muscle regeneration[16]. There are other studies suggests that Apobec2 might also play important roles in the regulation of left-right axis specification in Xenopus[17] and retinal regeneration of zebrafish glial cells[18,19]. Thus, Apobec2 may have essential roles for body maintenance, not only in muscle but also for normal growth and development in vertebrates, despite its still questioned biochemical activity as a DNA/RNA editing enzyme.

Telomeres are consist of repeated guanine-rich sequences and associated protein complexes known as shelterin that cap the end of chromosomes to maintain genomic stability[20,21]. Telomere length is primarily maintained by the ribonucleoprotein telomerase and the basic components required for telomerase activity. Telomerase reverse

transcriptase (TERT) unit possesses catalytic activity, and the telomerase RNA component (TERC) contains the template for telomere elongation[21]. Reduced telomerase activity in most somatic cells eventually leads to telomere shortening, cellular senescence or replicative aging. In contrast, ESCs can elongate their telomeres and proliferate indefinitely. Thus, telomerase and telomeres are important for proliferation of ESCs and other types of stem cells[22,23]. It has been reported that overexpression of Tert in murine ESCs enhances self-renewal and improves resistance to apoptosis, oxidative stress, and increased proliferation, suggesting that telomerase functions as a survival enzyme in ESCs[24]. Furthermore, recent study suggests that telomerase plays a critical role in reprogramming and self-renewal of iPSCs[25].

In this study, we found that Apobec2 mRNA was specifically expressed in mouse ESCs and iPSCs, and had crucial physiological roles in maintaining the self-renewal of mouse ESCs by regulating Tert mRNA expression. Furthermore, we have demonstrated that Apobec2 is essential for reprogramming and self-renewal of mouse iPSCs.

2. Materials and Methods

Cell culture

Mouse ESC line, E14 cells were cultured on gelatin-coated dishes in 2i medium[26], a serum-free N2B27 medium supplemented with MEK inhibitor (PD0325901, 1 μM) and GSK3β inhibitor (CHIR99021, 3 μM) in the presence of LIF. To regulate knock down or exogenous expression of Apobec2, E14 cells were cultured with puromycin (1ug/ml). 129 mouse dermal fibroblasts (129mDFs) and 293T cells were cultured in DMEM containing 10% fetal bovine serum.

Overexpression and knock down of Apobec2

To construct expression vectors, coding region of Apobec2 was amplified by PCR and cloned into the XhoI and EcoRI sites of pMSCV-puro vector. The primer used for Apobec2 was:
forward, 5' - AAGCTCGAGATGGCTCAGAAGGAAGAG,
reverse, 5' - ACCGAATTCCTACTTCAGGATGTCTGCCA.

pMSCV-puro-Apobec2 expression vector was transfected into 293T cells with retroviral packaging vectors. The retroviruses containing pMSCV-puro-Apobec2 vectors were collected and concentrated before they transducted into 129mDFs or 4F2A MEFs. Cells were seeded at $1X10^5$ cells on 35mm culture dishes and transducted with concentrated retroviruses in 24 hours. Media was completely replaced after 24 hours

of transduction, and the cells were cultured in DMEM containing 10% fetal bovine serum with puromycin (1ug/ml).

pLKO.1 vectors containig shRNAs were purchased from Sigma aldrich. The shRNA vectors were transfected into 293T cells with lentiviral packaging vectors and the lentiviruses containing shRNAs were collected and concentrated before they transducted into E14 cells or 129mDFs. E14 cells were seeded at $2.5X10^4$ cells on gelatin pre-coated 35mm culture dishes and 129mDFs were seeded at $1X10^5$ cells on 35mm culture dishes before they transducted with concentrated lentiviruses. Media was completely replaced after 24 hours, and the E14 cells were cultured in 2i medium containing puromycin (1ug/ml) in the presence of LIF, and 129mDFs were cultured in DMEM containing 10% fetal bovine serum with puromycin (1ug/ml), respectively.

Reprogramming of mouse iPSCs

Cells were seeded at $5X10^4$ or $1X10^5$ cells on 35mm culture dishes before transduction of reprogramming 4 factors - Oct4, Sox2, c-Myc and Klf4. TetO-FUW-OSKM and FUW-M2rtTA vectors were used for reprogramming as previously described[27].

Brifely, TetO-FUW-OSKM and FUW-M2rtTA vectors were transfected into 293T cells with lentiviral packaging vectors. The lentiviruses containing TetO-FUW-OSKM and FUW-M2rtTA vectors were collected and concentrated before they transducted into cells.

Transducted cells were seeded at $5X10^4$ or $1X10^5$ cells on 35mm culture dishes and cultured in DMEM containing 10% fetal bovine serum. To induce exogenic expression of reprogramming 4 factors, cells were treated with doxycyclin (1ug/ml). Media was changed every 24 hours. On Day4, the transfected cells were re-seeded at $5X10^4$ cells on 35mm culture dishes and cultured mouse ESC medium (DMEM containig 15% fetal bovine serum supplemented with 1X NEAA, 1X L-glutamine, 1% penicillin/streptomycin and 1X β-mercaptoethanol) in the presense of LIF until the end of reprogramming process.

RNA isolation, RT-PCR and quantitative real-time PCR

Total RNAs were prepared with TRIzol reagent (Invitrogen), and cDNAs were prepared from 2ug of RNA using ReverTra Ace (Toyobo) according to the manufacturer's instructions. RT-PCRs were performed in non-saturating conditions using Maxime PCR PreMix Kit (i-MAXII) (iNtRON). Quantitative RT-PCR using SYBR green was performed with an Applied biosystem Real-Time PCR System. The expression level of target genes was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot

For Western blot analysis, cells were lysed in cell lysis solution (ELPis) and samples were subjected to Western blot analysis using

anti-Apobec2 (Santacruz, sc-98335), anti-GAPDH (SIGMA, SAB2500451) and anti-Oct4 (Santacruz, sc-5279) primary antibodies followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Santacruz).

ALP staining

For ALP staining, a commercially available reagent (Dako, K0598) was used. Cells were stained following manufacture's instructions.

Immunostaining

E14 cells treated with shApobec2 or control shRNA were stained using anti-Oct4 (Santacruz, sc-5279, 1:200) and anti-Apobec2 (Santacruz, sc-98335, 1:200). DAPI staining was performed to identify nuclei. Cells were observed using a confocal microscope (LSM710, Carl Zeiss).

Cell proliferation assay

The number of viable cells was measured by direct cell count and the WST-1 assay. For the WST-1 assay, E14 cells were treated with shApobec2 or control shRNA, and seeded at $5X10^3$ cells on 24-well cell culture plates. Cells were cultured in 2i medium containing puromycin (1ug/ml) in the presence of LIF. After 2 days of culture, the cells were dissociated into single cells and incubated in 2i medium containing 10% WST-1 (EZ cytox) solution for 3 hours in a humidified atmosphere

(37 ℃, 5% CO_2). The absorbance of the culture medium was measured at wavelengths of 450 nm.

Sphere forming assay

Sphere forming assay on mouse ESCs treated with shApobec2 or control shRNA were performed as previously described[28].

Briefly, cells were cultured in 24-well ultra-low attachment surface dishes at 1000 cells per well. Then the cells were cultured in sphere forming medium, serum-free DMEM/F12 supplemented with N2 supplement, 10ng/ml EGF and 10ng/ml bFGF. Round cell clusters with diameter over 100 μm were judged as spheres.

Telomere length qPCR

The relative telomere length was measured as previously described[29]. Mean T/S ratio of Apobec2 knock down E14 cells was normalized against control.

3. Result

Apobec2 mRNA is specifically expressed in mouse ESCs and iPSCs.

To examine the expression of Apobec family mRNAs in murine cells and tissues, we performed RT-PCR and found that Apobec2 mRNA was specifically expressed in mouse ESCs and iPSCs compared in mouse dermal fibroblasts. The relateve expression level of mRNAs in mouse cells was examined by quantitative real-time PCR and Apobec2 showed the most specific expression in mouse ESCs and iPSCs (Figure 1A). Furthermore, Apobec2 mRNA was highly expressed in heart and muscle tissues as previeously reported[10,11], but not in other tissues. Apobec3 mRNA was also highly expressed in mouse ESCs and iPSCs, but showed ubiquitous expression in varius tissues (Figure 1B). Unlike Apobec2 and Apobec3 mRNAs, Apobec1 mRNA showed low and ubiquitous expression throughout mouse cells and tissues (Figure 1A and 1B). These results indicate that among the Apobec family, only Apobec2 is specifically expressed in mouse ESCs and iPSCs.

Apobec2 is essential for colonization and self-renewal of mouse ESCs but not for pluripotency.

Although Apobec2 mRNA was specifically expressed in mouse ESCs and iPSCs, Apobec1 and 3 mRNAs were also highly expressed in those cells. To determine which one of Apobec family has physiological function in mouse ESCs, we knocked down Apobec1, 2, 3 in the E14 cells (mouse 129 strain ESCs) by using lentiviral shRNAs. When the E14 cells were treated with shApobec1, 2, 3, RT-PCR analysis showed dramatic reduction in each mRNAs compared to control (Figure 2A). Then, we passaged those cells and examined the morphological changes under puromycin selection (1ug/ml). We found that the E14 cells knocked down Apobec2 showed the most significant morphological changes with decreased colonization. More collapsed or smaller colonies were observed in Apobec2 knocked down cells compared to control cells. The E14 cells showed smaller colonies but not collapsed ones by Apobec3 knock down and has no morphological changes by Apobec1 knock down (Figure 2B). According to dramatic morphological changes showed in Apobec2 knocked down E14 cells, we focused on the function of Apobec2 in mouse ESCs, in this study.

Because more collapsed or smaller colonies were observed in Apobec2 knocked down E14 cells, we hypothesized that Apobec2 might regulate proliferation and self-renewal of mouse ESCs. To examine

whether Apobec2 regulates proliferation and cell viability of mouse ESCs, we performed cell counting and WST-1 assay. The proliferation and viability of E14 cells were reduced by Apobec2 knock down (Figure 2C). We have also performed sphere-forming assay to explore whether Apobec2 modulates self-renewal of mouse ESCs and found that the number of spheres in Apobec2 knocked down E14 cells was decreased compared to control (Figure 2D). Taken together, these data indicate that knock down of Apobec2 decreases colonization, proliferation and self-renewal of mouse ESCs.

Next, we have investigated whether Apobec2 controls pluripotency of mouse ESCs. We knocked down Apobec2 in E14 cells and checked Alkaline Phosphatase (ALP) activity. The ALP activity in Apobec2 knocked down E14 cells was comparable to control (Figure 2E). The mRNA and protein expression of pluripotency markers were also similar between E14 cells treated with shApobec2 and control shRNA (Figure 2F-H). These results suggest that Apobec2 has an important role in colonization and self-renewal of mouse ESCs but not in pluripotency.

Apobec2 regulates transcription of Tert and cell adhesion molecules.

Since Apobec2 regulates colonization and self-renewal of mouse ESCs, we examined mRNA expression of cell adhesion molecules and Tert in Apobec2 knocked down E14 cells. It is well known that the MET (Mesenchymal to Epithelial Transition) markers – E-cadherin, Epcam and Cloudin6 – are highly expressed in mouse ESCs and have important roles in colonization of mouse ESCs. Also, the maintenance of telomere length is required for long-term self-renewal of mouse ESCs[30]. It has been reported that the upregulation of Tert and increased telomerase activity enhances the proliferation and colony-forming ability of human ESCs[31].

We knocked down Apobec2 in the E14 cells using letiviral shRNA and cultured the cells in 2i medium containing puromycin (1ug/ml). On day7 of culture, the relative expression of cell adhesion molecules and Tert mRNAs was examined by quantitative real-time PCR and we found that the mRNA expression of E-cadherin, Epcam, Cloudin6 and Tert was reduced in Apobec2 knocked down E14 cells (Figure 3A). The mRNA expression of EMT (Epithelial to Mesenchymal Transition) markers – Twist, Snail, Slug and N-cadherin – was not changed (data not shown). Next, we analyzed the relative telomere length by quantitative real-time PCR and found that telomere length in Apobec2 knocked down E14 cells

was shortened compared to control (Figure 3B). Taken together, these data indicate that Apobec2 modulates telomere length via regulating Tert mRNA expression that is essential for telomerase activity.

To estimate whether Apobec2 overexpression increases cell adhesion molecules and Tert mRNAs in somatic cells, we performed quantitative real-time PCR. When the exogenic expression of Apobec2 was induced in 129 mouse dermal fibroblasts (129mDFs), however, the expression level of cell adhesion molecules and Tert mRNAs was not increased compared to control. The representitive data was shown in Figure 3C.

Apobec2 plays an important role during reprogramming of mouse iPSCs.

We have shown that Apobec2 is essential for self-renewal of mouse ESCs and regualtes the expression of cell adhesion molecules and Tert mRNAs. Since it has been reported that telomerase plays a critical role in reprogramming and self-renewal of iPSCs[25], we hypothesized Apobec2 might modulate reprogramming of mouse iPSCs by regulating Tert mRNA expression.

First, we examined the mRNA expression level of Apobec2 during mouse iPSC reprogramming. The schematic figure in Figure 4A shows the reprogramming process of mouse iPSCs we performed. On reprogramming day4, the number of cells under MET was reached peak and on reprogramming day8, the cells became smaller and more compact, and started to form few primary iPSC colonies. On reprogramming day12, there were number of compact and shiny true iPSC colonies throughout the culture dish. To establish 129 mouse iPSCs, we picked one of the colonies and dissociated into single cells before the cells were cultured on gelatin pre-coated cell culture dish. We cultured 129 mouse iPSCs under feeder-free culture condition likewise the E14 cells (Figure 4B). We harvested cells on reprogramming day4, 8 and 12 and performed RT-PCR to examine Apobec2 mRNA expression. The mRNA expression level of endogenous Oct4 was shown as a positive control.

During reprogramming process, we found that Apobec2 mRNA was increased in time dependent manner likewise endogenous Oct4 mRNA and reached peak on reprogramming day12. Furthermore, the expression of Tert mRNA was also increased in time dependent manner, but later than Apobec2 mRNA. The relative mRNA expression of Apobec2, endogenous Oct4 and Tert was examined by quantitative real-time PCR and we found that Apobec2 mRNA expression in 129 mouse iPSCs was comparable to E14 cells (Figure 4C).

Next, to estimate whether Apobec2 modulates reprogramming of iPSCs, we knocked down Apobec2 in 129mDFs in prior to reprogramming process (Figure 4D). We treated 129mDFs with shApobec2 or control shRNA and cultured the cells with puromycin (1ug/ml). Then the cells were re-seeded before transduction of reprogramming 4 factors - Oct4, Sox2, c-Myc and Klf4. The MET was obseved in both of 129mDFs treated with shApobec2 or control shRNA on reprogramming day4 (data not shown), however, the cells knocked down Apobec2 was failed further colonization (Figure 4E). RT-PCR analysis showed that Tert mRNA expression was reduced in Apobec2 knocked down cells compared to control. The relative expression of endogenous Oct4 mRNA was shown as a control (Figure 4F). These data indicate that Apobec2 is essential for reprogramming of mouse iPSCs and regulates Tert mRNA expression during the process.

Finally, we have investigated whether overexpression of Apobec2 increases mouse iPSC reprogramming efficiency. We overexpressed Apobec2 in mouse embryonic fibroblasts (MEFs) before transduction of reprogramming 4 factors – Oct4, Sox2, c-Myc and Klf4. We used the 4F2A MEFs for Apobec2 overexpression reprogramming to reduce variables and analyze accurate reprogramming efficiency[32]. The procedure of Apobec2 overexpression reprogremming was shown in Figure 5A. When the 4F2A MEFs was induced exogeneous Apobec2 expression, RT-PCR analysis showed dramatic increase of Apobec2 mRNA expression compared to control (Figure 5B). On reprogramming day18, the 4F2A MEFs with exogenic Apobec2 expression showed more iPSC colonies compared to control (Figure 5C). The relative number of ALP positive iPSC colonies was doubled by Apobec2 overexpression (Figure 5D). The ALP staining showed in Figure 5E. Taken together, these data indicate that Apobec2 plays an important role in reprogramming of mouse iPSCs.

4. Discussion

Embryonic stem cells (ESCs) can self-renew indefinitely *in vitro* and differentiate into all germ layers[33]. The core transcription factors of the pluripotency – Oct4, Sox2 Noanog – and their transcriptional network are well characterized. However, other genes involved in ESC pluripotency and self-renewal remain to be identified.

Apobec2 is one of the oldest members of the Apolipoprotein B mRNA-editing enzyme catalytic subunit (APOBEC) family. The expression of Apobec2 has known to be restricted to differentiated cardiac and skeletal muscle in mammals and chicken[10,11] and the exprssion and physiological function of Apobec2 in mouse ESCs and iPSCs have not been reported. In this study, we have found that Apobec2 is highly expressed in mouse ESCs and iPSCs, and has a critical role in self-renewal of those cells. When Apobec2 was knocked down in mouse ESCs, the proliferation and colonization of cells were reduced compared to contol.

Telomerase and telomeres are important for proliferation of ESCs and other types of stem cells[22,23]. Recent study suggests that telomerase plays a critical role in reprogramming and self-renewal of iPSCs[25]. Here, we have shown that knock down of Apobec2 reduces the proliferation and colonization of mouse ESCs compared to contol. The mRNA

expression of MET genes and Tert mRNAs was decreased in Apobec2 knocked down mouse ESCs, but pluripotency related genes had not been changed. These data suggest that self-renewal and pluripotency may have independent transcriptional network and Apobec2 modulates self-renewal of mouse ESCs via regulating Tert mRNA expression.

The mRNA expression of Apobec2 was increased during reprogramming of mouse iPSCs in time dependent manner. We have performed Apobec2 knock down reprogramming found that Apobec2 is essential for reprograming. Although Apobec2 overexpression had no significant changes in mouse dermal fibroblasts, in reprogramming process, however, Apobec2 overexpression enhanced reprogramming efficiency in double. Taken together, we conclude that Apobec2 has a critical role in reprogramming of mouse iPSCs.

In this study, we have suggested that Apobec2 is essential for self-renewal of mouse ESCs and iPSCs as well as in reprogramming of mouse iPSCs. Unlike knock down of Apobec2 in mouse ESCs, the overexpression of Apobec2 in mouse dermal fibroblasts showed no significant changes. However, during reprogramming process, both of Apobec2 knock down and overexpression regulated reprogramming of mouse iPSCs in positive way. These data suggest possibilities that Apobec2 does not act as a transcription factor and may interact with other proteins. The mechanisms of Apobec2 function on self-renewal

of mouse ESCs and reprogramming of mouse iPSCs need to be further studied.

The schematic result of this study was shown in Figure 6.

5. References

1 Kitajima, K., Masuhara, M., Era, T., Enver, T. & Nakano, T. GATA-2 and GATA-2/ER display opposing activities in the development and differentiation of blood progenitors. *EMBO J* **21**, 3060-3069, doi:10.1093/emboj/cdf301 (2002).

2 Kanda, S., Tamada, Y., Yoshidome, A., Hayashi, I. & Nishiyama, T. Over-expression of bHLH genes facilitate neural formation of mouse embryonic stem (ES) cells in vitro. *Int J Dev Neurosci* **22**, 149-156, doi:10.1016/j.ijdevneu.2004.01.002 (2004).

3 Jaenisch, R. & Young, R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* **132**, 567-582, doi:10.1016/j.cell.2008.01.015 (2008).

4 Young, R. A. Control of the embryonic stem cell state. *Cell* **144**, 940-954, doi:10.1016/j.cell.2011.01.032 (2011).

5 Boyer, L. A. *et al.* Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122**, 947-956, doi:10.1016/j.cell.2005.08.020 (2005).

6 Loh, Y. H. *et al.* The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* **38**, 431-440, doi:10.1038/ng1760 (2006).

7 Teng, B., Burant, C. F. & Davidson, N. O. Molecular cloning of an apolipoprotein B messenger RNA editing protein. *Science* **260**,

1816-1819 (1993).

8 Navaratnam, N. *et al.* The p27 catalytic subunit of the apolipoprotein B mRNA editing enzyme is a cytidine deaminase. *J Biol Chem* **268**, 20709-20712 (1993).

9 Navaratnam, N. & Sarwar, R. An overview of cytidine deaminases. *Int J Hematol* **83**, 195-200, doi:10.1532/IJH97.06032 (2006).

10 Liao, W. *et al.* APOBEC-2, a cardiac- and skeletal muscle-specific member of the cytidine deaminase supergene family. *Biochem Biophys Res Commun* **260**, 398-404, doi:10.1006/bbrc.1999.0925 (1999).

11 Li, J. *et al.* APOBEC2 mRNA and protein is predominantly expressed in skeletal and cardiac muscles of chickens. *Gene* **539**, 263-269, doi:10.1016/j.gene.2014.01.003 (2014).

12 Mikl, M. C. *et al.* Mice deficient in APOBEC2 and APOBEC3. *Mol Cell Biol* **25**, 7270-7277, doi:10.1128/MCB.25.16.7270-7277.2005 (2005).

13 Lada, A. G. *et al.* Mutator effects and mutation signatures of editing deaminases produced in bacteria and yeast. *Biochemistry (Mosc)* **76**, 131-146 (2011).

14 Sato, Y. *et al.* Differential expression of sarcoplasmic and myofibrillar proteins of rat soleus muscle during denervation atrophy. *Biosci Biotechnol Biochem* **73**, 1748-1756, doi:10.1271/bbb.90085 (2009).

15 Sato, Y. *et al.* Deficiency in APOBEC2 leads to a shift in muscle fiber type, diminished body mass, and myopathy. *J Biol Chem* **285**, 7111–7118, doi:10.1074/jbc.M109.052977 (2010).

16 Ohtsubo, H. *et al.* APOBEC2 negatively regulates myoblast differentiation in muscle regeneration. *Int J Biochem Cell Biol* **85**, 91–101, doi:10.1016/j.biocel.2017.02.005 (2017).

17 Vonica, A., Rosa, A., Arduini, B. L. & Brivanlou, A. H. APOBEC2, a selective inhibitor of TGFbeta signaling, regulates left–right axis specification during early embryogenesis. *Dev Biol* **350**, 13–23, doi:10.1016/j.ydbio.2010.09.016 (2011).

18 Powell, C., Cornblath, E. & Goldman, D. Zinc–binding domain–dependent, deaminase–independent actions of apolipoprotein B mRNA–editing enzyme, catalytic polypeptide 2 (Apobec2), mediate its effect on zebrafish retina regeneration. *J Biol Chem* **289**, 28924–28941, doi:10.1074/jbc.M114.603043 (2014).

19 Powell, C., Elsaeidi, F. & Goldman, D. Injury–dependent Muller glia and ganglion cell reprogramming during tissue regeneration requires Apobec2a and Apobec2b. *J Neurosci* **32**, 1096–1109, doi:10.1523/JNEUROSCI.5603–11.2012 (2012).

20 Blackburn, E. H. Switching and signaling at the telomere. *Cell* **106**, 661–673 (2001).

21 Palm, W. & de Lange, T. How shelterin protects mammalian telomeres. *Annu Rev Genet* **42**, 301–334,

doi:10.1146/annurev.genet.41.110306.130350 (2008).

22 Wang, Y. *et al.* An increase in telomere sister chromatid exchange in murine embryonic stem cells possessing critically shortened telomeres. *Proc Natl Acad Sci U S A* **102**, 10256-10260, doi:10.1073/pnas.0504635102 (2005).

23 Hiyama, E. & Hiyama, K. Telomere and telomerase in stem cells. *Br J Cancer* **96**, 1020-1024, doi:10.1038/sj.bjc.6603671 (2007).

24 Armstrong, L. *et al.* Overexpression of telomerase confers growth advantage, stress resistance, and enhanced differentiation of ESCs toward the hematopoietic lineage. *Stem Cells* **23**, 516-529, doi:10.1634/stemcells.2004-0269 (2005).

25 Wang, F. *et al.* Molecular insights into the heterogeneity of telomere reprogramming in induced pluripotent stem cells. *Cell Res* **22**, 757-768, doi:10.1038/cr.2011.201 (2012).

26 Tong, C., Huang, G., Ashton, C., Li, P. & Ying, Q. L. Generating gene knockout rats by homologous recombination in embryonic stem cells. *Nat Protoc* **6**, 827-844, doi:10.1038/nprot.2011.338 (2011).

27 Carey, B. W. *et al.* Reprogramming of murine and human somatic cells using a single polycistronic vector. *Proc Natl Acad Sci U S A* **106**, 157-162, doi:10.1073/pnas.0811426106 (2009).

28 Yata, K. *et al.* MicroRNA expression profiles of cancer stem cells in head and neck squamous cell carcinoma. *Int J Oncol* **47**, 1249-

1256, doi:10.3892/ijo.2015.3145 (2015).

29 Cawthon, R. M. Telomere measurement by quantitative PCR. *Nucleic Acids Res* **30**, e47 (2002).

30 Zhang, Q. *et al.* Tcstv1 and Tcstv3 elongate telomeres of mouse ESCs. *Sci Rep* **6**, 19852, doi:10.1038/srep19852 (2016).

31 Yang, C. *et al.* A key role for telomerase reverse transcriptase unit in modulating human embryonic stem cell proliferation, cell cycle dynamics, and in vitro differentiation. *Stem Cells* **26**, 850-863, doi:10.1634/stemcells.2007-0677 (2008).

32 Wernig, M. *et al.* A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat Biotechnol* **26**, 916-924, doi:10.1038/nbt1483 (2008).

33 Evans, M. Discovering pluripotency: 30 years of mouse embryonic stem cells. *Nat Rev Mol Cell Biol* **12**, 680-686, doi:10.1038/nrm3190 (2011).

6. Figures

Figure 1.

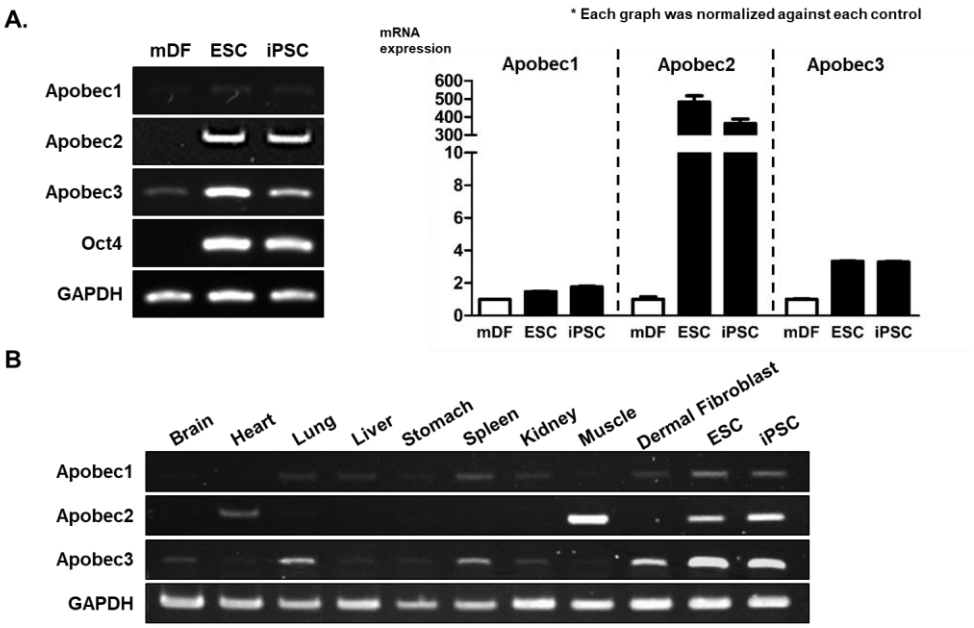
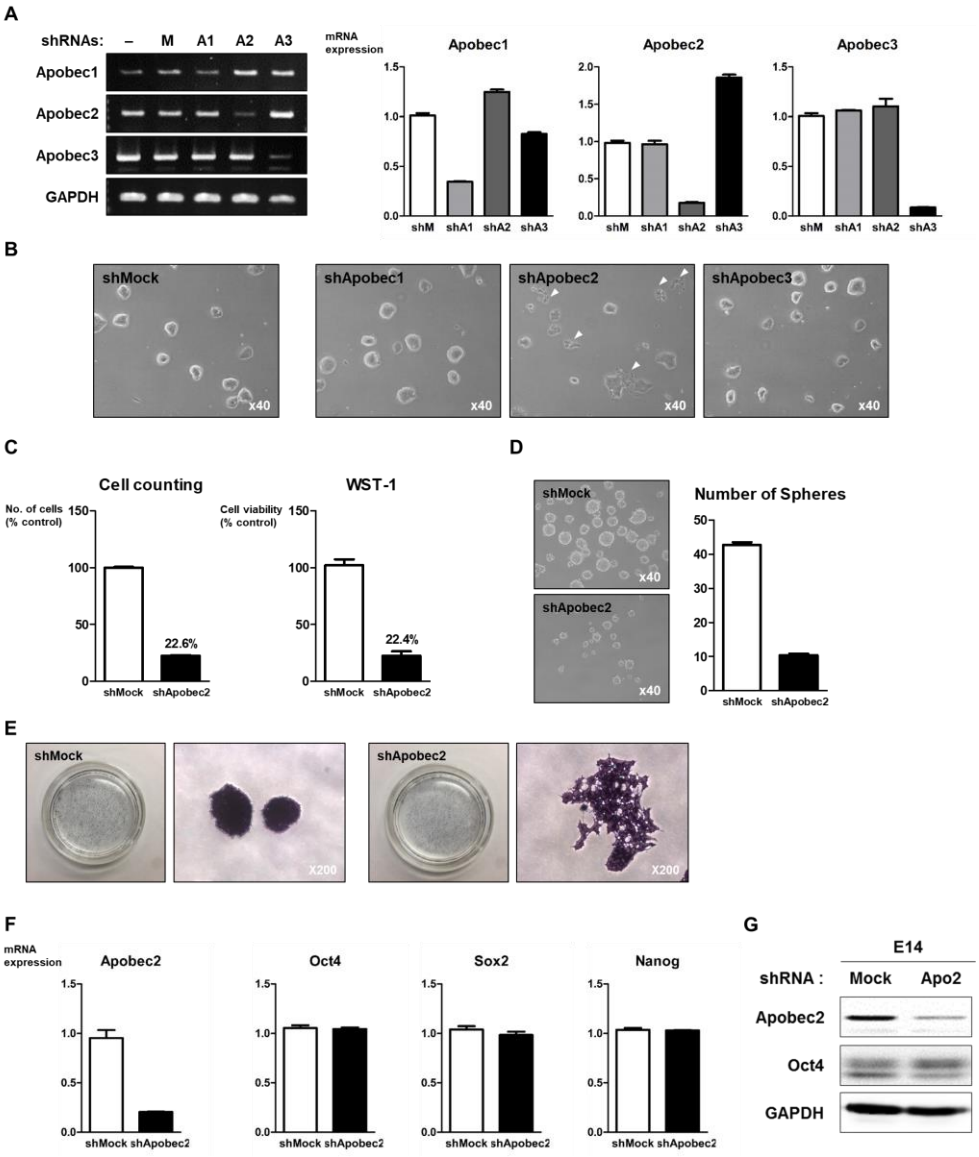


Figure 1. Expression profile of Apobec family mRNAs in mouse cells and tissues.

The expression level of Apobec family mRNAs in (A) mouse ESCs, iPSCs and mDFs (mouse dermal fibroblasts) and (B) various tissues was examined by RT-PCR. The relative expression level of Apobec family mRNAs was normalized against each control.

Figure 2.


A
shRNAs: – M A1 A2 A3
Apobec1
Apobec2
Apobec3
GAPDH
mRNA expression
Apobec1
Apobec2
Apobec3
shM shA1 shA2 shA3
B
shMock
shApobec1
shApobec2
shApobec3
x40
C
Cell counting
No. of cells (% control)
22.6%
WST-1
Cell viability (% control)
22.4%
shMock shApobec2
D
shMock
shApobec2
x40
Number of Spheres
E
shMock
shApobec2
X200
F
mRNA expression
Apobec2
Oct4
Sox2
Nanog
shMock shApobec2
G
E14
shRNA : Mock Apo2
Apobec2
Oct4
GAPDH

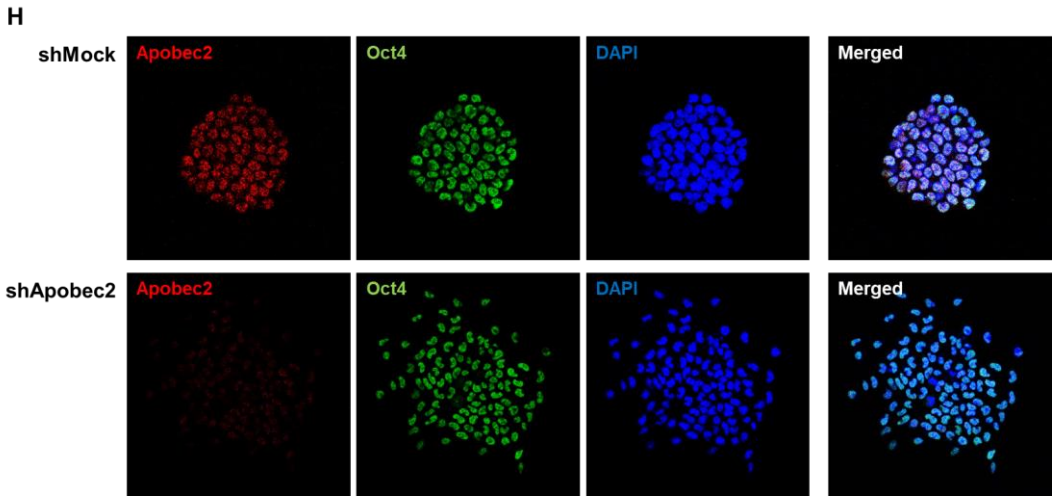


Figure 2. Apobec2 knock down in mouse ESCs.

The E14 cells treated with control shRNA (shMock) or Apobec 1, 2, 3 shRNAs. (A) The expression of Apobec family mRNAs in the E14 cells treated with each shRNA. The relative expression level of Apobec family mRNAs in E14 cells treated with eah shRNA was examined by quantitative real-time PCR and normalized against each control. (B) Knock down of Apobec2 induced dramatic morphological changes in mouse ESCs. The E14 cells were treated with shApobec1, 2, 3 to knock down Apobec family or shMock as a control. The morphological changes were observed by phase contrast microscopy. (C) Cell counting and WST-1 assay of mouse ESCs treated with shRNAs. The E14 cells were treated with shApobec2 or control shRNA and cultured in 2i medium containing puromycin (1ug/ml) in the presence of LIF. Cells were dissociated into single cells and counted before EZ-Cytox was added.

Results shown are the mean viable cell number or normalized O.D. values (percentage against control) ± s.e.m. from at least three independent experiments. (D) Sphere forming assay of Apobec2 knocked down mouse ESCs. The E14 cells treated with shApobec2 or control shRNA were cultured in sphere forming medium containing puromycin (1ug/ml) and spheres were counted on day5 of culture. Results shown are the mean sphere number ± s.e.m. from at least three independent experiments. (E) Alkaline Phosphatase (ALP) staining for the E14 cells treated with shRNAs. (F) The relative expression level of pluiripotency markers in the E14 cells treated with shApobec2 or control shRNA. Results shown are normalized values (percentage against control). (G, H) Expression of Oct4 protein in the E14 cells treated with shApobec2 or control shRNA detected by western blotting and immunostaining, respectively.

Figure 3.

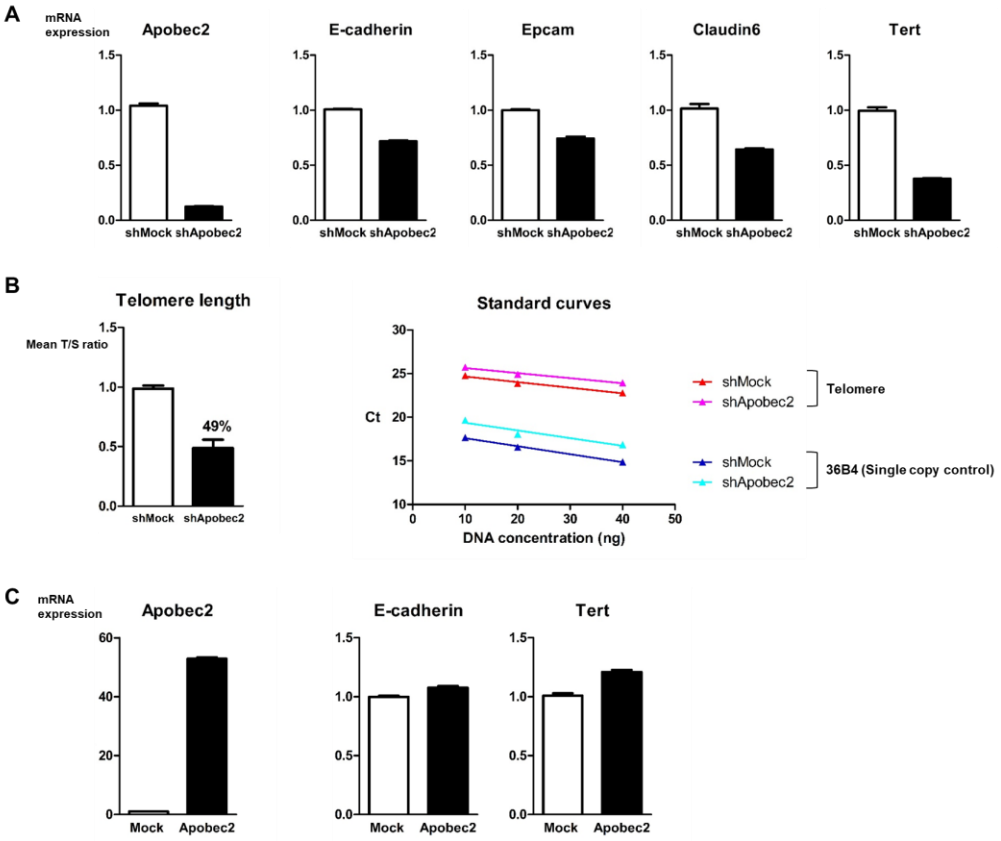

A
mRNA expression
Apobec2
E-cadherin
Epcam
Claudin6
Tert
shMock shApobec2
B
Telomere length
Mean T/S ratio
49%
Standard curves
Ct
DNA concentration (ng)
shMock
shApobec2
Telomere
36B4 (Single copy control)
C
mRNA expression
Apobec2
E-cadherin
Tert
Mock
Apobec2

Figure 3. Apobec2 knock down reduces cell adhesion molecules and Tert mRNA expression and modulates telomere length in mouse ESCs.

(A) Reduced expression of E-cadherin, Epcam, Cloudin6 and Tert mRNAs in Apobec2 knocked down E14 cells. (B) Relative telomere length shown as T/S ratio measured by quantitative real-time PCR. (C) Relative mRNA expression of Tert and E-cadherin in Apobec2 overexpressed mouse dermal fibroblasts was examined by quantitative real-time PCR. Results shown are the mean values normalized against control ± s.e.m. from at least three independent experiments.

Figure 4.

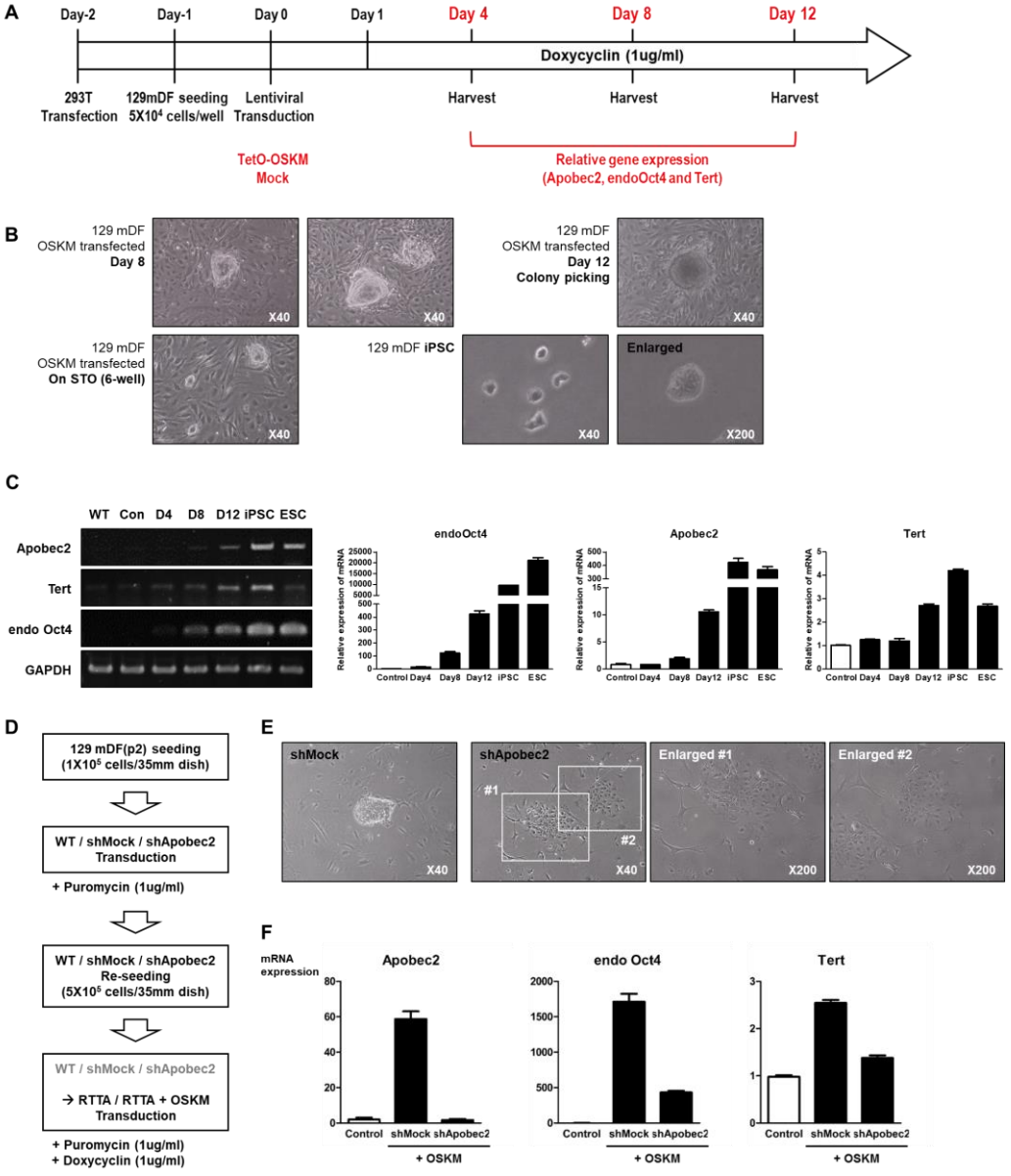

A
Day-2
Day-1
Day 0
Day 1
Day 4
Day 8
Day 12
Doxycyclin (1ug/ml)
293T Transfection
129mDF seeding 5X10⁴ cells/well
Lentiviral Transduction
Harvest
Harvest
Harvest
TetO-OSKM Mock
Relative gene expression (Apobec2, endoOct4 and Tert)
B
129 mDF OSKM transfected Day 8
129 mDF OSKM transfected Day 12 Colony picking
129 mDF OSKM transfected On STO (6-well)
129 mDF iPSC
Enlarged
X40
X200
C
WT Con D4 D8 D12 iPSC ESC
Apobec2
Tert
endo Oct4
GAPDH
endoOct4
Apobec2
Tert
Relative expression of mRNA
Control Day4 Day8 Day12 iPSC ESC
D
129 mDF(p2) seeding (1X10⁵ cells/35mm dish)
WT / shMock / shApobec2 Transduction
+ Puromycin (1ug/ml)
WT / shMock / shApobec2 Re-seeding (5X10⁵ cells/35mm dish)
WT / shMock / shApobec2
→ RTTA / RTTA + OSKM Transduction
+ Puromycin (1ug/ml)
+ Doxycyclin (1ug/ml)
E
shMock
shApobec2
#1
#2
Enlarged #1
Enlarged #2
X40
X200
F
mRNA expression
Apobec2
endo Oct4
Tert
Control shMock shApobec2
+ OSKM

Figure 4. Apobec2 is essential for reprogramming of mouse iPSCs.

(A) Schematic figure of mouse iPSC reprogramming. (B) Establishment of mouse iPSCs. The morphological changes during reprogramming process were observed by phase contrast microscopy. (C) The expression level of Apobec2 mRNA during reprogramming of mouse iPSCs. The relative expression of Apobec2 and endogenous Oct4 mRNAs was examined by quantitative real-time PCR. Results shown are the mean values normalized against control ± s.e.m. from at least three independent experiments. (D) Schematic figure of Apobec2 knock down reprogramming process. (E) Cell morphologies on day9 of knock down reprogramming. The morphological changes were observed by phase contrast microscopy. (F) The mRNA expression of Apobec2, endogenous Oct4 and Tert on reprogramming day9. The relative expression of mRNAs was examined by quantitative real-time PCR and results shown are the mean values normalized against control ± s.e.m. from at least three independent experiments.

Figure 5.

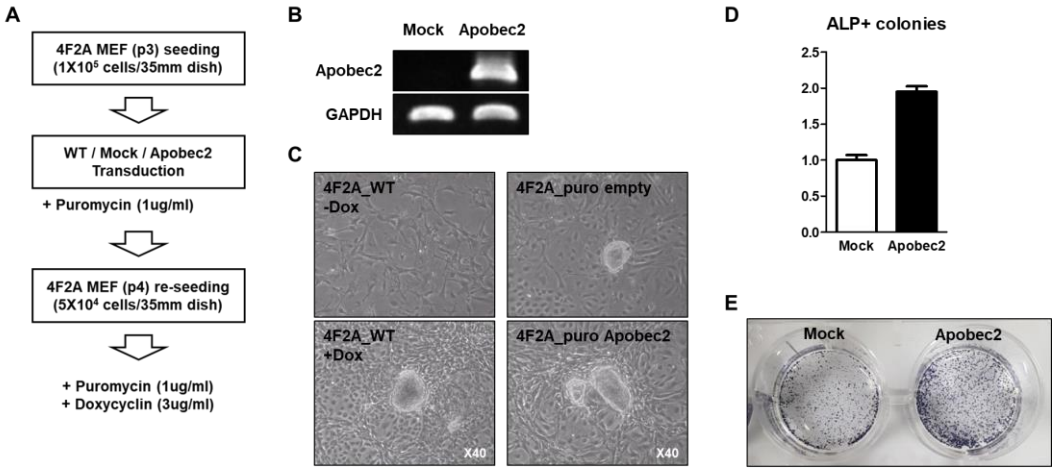


Figure 5. Apobec2 overexpression reprogramming of 4F2A MEFs.

4F2A MEFs were overexpressed by exogenic Apobec2 before transduction of reprogramming 4 factors – Oct4, Sox2, c-Myc and Klf4 – and examined reprogramming efficiency compared to control. (A) Schematic figure of Apobec2 overexpression reprogramming process. (B) Apobec2 mRNA was overexpressed in 4F2A MEFs (C) iPSC colonies on day18 of overexpression reprogramming. The cell morphologies were observed by phase contrast microscopy. (D) The relative numbers of ALP positive iPSC colonies in Apobec2 overexpressed cells compared to control. Results shown are the mean colony numbers normalized against control ± s.e.m. from at least three independent experiments. (E) Alkaline Phosphatase (ALP) staining of 4F2A iPSCs established on day21 of reprogramming.

Figure 6.

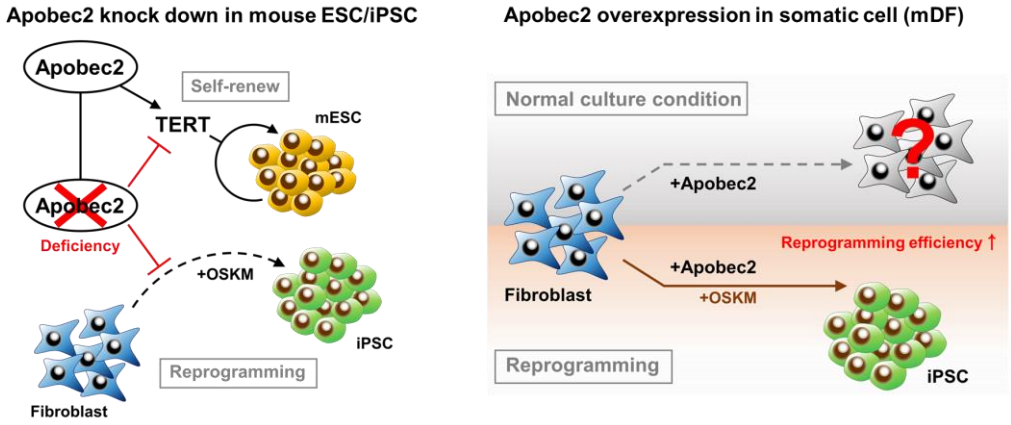


Figure 6. Schematic figure of the results.

Apobec2 is essential for self-renewal of mouse ESCs and reprogramming of mouse iPSCs. However, overexpression of Apobec2 in somatic cells is not enough to initiate reprogramming process of mouse iPSCs without reprogramming 4 factors – Oct4, Sox2, c-Myc and Klf4.

국 문 초 록

생쥐 배아줄기세포와 유도만능줄기세포의 self-renewal 유지 및 유도만능줄기세포 유도과정에서 Apobec2의 역할 규명

서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과
분자의학 및 바이오제약전공
유 지 혜

Apolipoprotein B mRNA-editing enzyme catalytic subunit 2 (Apobec2)는 심장, 근육 세포에서 높게 발현하고, 근육 세포 발달 조절에 중요한 역할을 하는 것으로 잘 알려져 있다. 그러나, 근육 세포 발달을 제외하고는 다른 세포에서의 역할은 밝혀진 바가 없으며, 특히 배아줄기세포(Embryonic Stem Cells, ESCs)나 유도만능줄기세포(induced Pluripotent Stem Cells, iPSCs)에서 Apobec2의 발현과 역할에 대해 보고된 바가 없다.

배아줄기세포는 배반포(Blastocyst)의 미분화배아세포(Inner Cell Mass, ICM)에서 분리, 배양된 미분화 세포로, 여러 종류의 신체 조직으로 분화할 수 있는 다분화능(Pluripotency)과 무한한 자가복제능력(Self-renewal)을

가진다. 이러한 줄기세포의 다분화능 및 자가복제능력 유지를 위해서는 적절한 말단소체(Telomere)의 유지가 필수적인데 말단소체복원효소(Telomerase)가 말단소체의 연장을 관할하는 효소로 잘 알려져 있다. 말단소체복원효소는 생쥐의 배아줄기세포와 유도만능줄기세포의 자가복제능력 유지와 유도만능줄기세포의 유도과정에서 중요한 역할을 하는 것으로 보고되었으나, 정확한 기전은 아직 밝혀지지 않았다.

이 논문에서는, Apobec2가 생쥐의 배아줄기세포와 유도만능줄기세포 특이적으로 발현하며, Tert의 발현을 조절함으로써 생쥐 배아줄기세포와 유도만능줄기세포의 자가복제능력 유지 및 유도만능줄기세포의 유도과정에서 중요한 역할을 한다는 것을 밝혔다. 생쥐의 배아줄기세포에서 Apobec2의 발현을 억제하였을 때, 생쥐 배아줄기세포의 증식(Proliferation)과 콜로니 형성(Colonization)이 억제되었으며, 세포구 형성 실험(sphere forming assay)을 통해 자가복제능력이 저하되는 것을 발견하였다.

또한, Apobec2의 발현이 유도만능줄기세포의 유도과정이 진행됨에 따라 점차 증가하는 것을 발견하였으며, Apobec2의 과발현과 발현 억제에 따른 유도만능줄기세포 리프로그래밍(Reprogramming) 실험을 통해 Apobec2가 리프로그래밍 과정에서 필수적인 역할을 하는 것을 밝혀내었다.

이 연구를 통해, 생쥐 배아줄기세포 및 유도만능줄기세포의 자가복제능력 유지와 리프로그래밍에서 Apobec2의 역할을 밝히고, 나아가 줄기세포 기전 연구의 기반을 다지는 계기가 되기를 기대한다.

주요어: Apobec2, 생쥐 배아줄기세포, 유도만능줄기세포, 역분화, 자기재생, 말단소체(telomere), Tert

학번: 2016-27474