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Recent Patents on MicroRNA-Induced Pluripotent Stem Cell Generation

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Abstract: Regenerative medicine using pluripotent stem cells represents the future for curing developmental abnormalities, degenerative disorders and aging-related illnesses. However, today's regenerative medicine faces two major challenges with respect to stem cells: their shortage in supply and uncertain safety in clinical therapy. The recent discovery of induced pluripotent stem cells (iPS cells or iPSCs) derived from patients' somatic cells presents a possible solution for the supply shortage problem. Yet, iPSC generation with the enforced overexpression of previously defined four factors Oct4–Sox2-Klf4-c-Myc (OSKM) also causes oncogenic stimulation, which leads to potential tumorigenicity. The more recent development of miR302-mediated iPSC generation may further solve this problem since miR-302, a tiny 23-nucleotide non-coding microRNA (miRNA), is able to replace all four OSKM in mediating iPSC formation while preventing the onset of stem cell tumorigenicity. MiR-302-reprogrammed iPSCs (mirPSCs) not only possess a highly demethylated genome but also share >92% gene expression similarity with human embryonic stem cells (hESCs). Transplantation of mirPSCs into immunocompromised mice leads to the formation of relatively organized tissue cysts containing various cell types derived from all three embryonic germ layers (ectoderm, mesoderm and endoderm), providing a potential tool for regenerative medicine. Hence, this novel iPSC technology offers a simple, effective and safe method for not only reprogramming somatic cells to hESC-like pluripotent stem cells but also maintaining stem cell pluripotency in tumor-free conditions. Conceivably, mirPSCs present a more suitable choice of iPSCs based on current Food and Drug Administration (FDA) regulations. Due to the novelty of this recent technology, a majority of patent applications are still pending and are mainly led by two major research groups, Lin *et al.* (WJWU & LYNN Institute - 7 filings) and Yamanaka *et al.* (Kyoto University - 3 filings). This review will summarize all relevant patent applications and describe the mechanisms underlying this new miRNA-mediated iPSC generation technology.

Keywords: DNA demethylation, embryonic stem cell (ESC), induced pluripotent stem cell (iPSC), microRNA (miRNA), miR-302, microRNA-reprogrammed iPSC (mirPSC), pluripotency, somatic cell reprogramming.

1. INTRODUCTION

Recent studies in human embryonic stem cells (hESCs) have provided significant insights into methods for developing regenerative medicine. Nevertheless, the sources for collecting hESCs are limited and it is very difficult to control their purity and quality. As shown in Table **1**, Thomson *et al.* isolated the first hESC line from late blastocysts of human embryos [1] and received several US Patents such as US5843780 [2], US6200806 [2], US7029913 [2], and US7220584 [3]. H1 and H9 cells were two typical cell lines resulted from these isolated hESCs. Later, Gearhart *et al.* also developed a method for isolating hESC-like primordial germ cells (PGCs) from post-blastocyst human embryos, which resulted in the filing of US Patents US6090622 [4], US6245566 [5], and US6331406 [5]. Since these hESC isolation methods destroy the donor embryos, a number of ethical concerns have been raised to questioning the use of these hESCs for clinical therapy.

In recent years, the therapeutic safety of hESCs was also concerned. For instance, because hESC growth requires certain undefined factors released by surrounding "feeder" fibroblasts, most of scientists preferably cultivate hESCs on a layer of mouse or human fibroblasts, as described in Reubinoff's US6875607 [6]. However, these fibroblast feeders present different surface antigens, which often contaminate the purity of hESCs and may cause immune rejection after transplantation. Although, some feeder-free culture conditions have been developed, the undifferentiation state of hESC is still very difficult to be maintained. A few (about 5%-10% or more) hESCs always tend to lose their stem cell properties during consecutive culturing. One of the most frequently observed results of hESC differentiation *in vivo* is teratoma. Teratoma is a tumor usually derived from human germ cells, containing multiple primary cell types similar to embryonic endoderm, mesoderm and ectoderm tissues. The presence of teratomas indicates not pluripotency but tumorigenicity of these hESCs. Therefore, preventing feeder cell contamination, increasing stem cell purity and reducing the risk of tumor formation represent the three major roadblocks for using hESCs in therapy.

Induced pluripotent stem cells (iPS cells or iPSCs) were first reported by Takahashi and Yamanaka in 2006 [7]. Using viral delivery of four defined transcription factors (Oct4, Sox2, Klf4 and c-Myc; OSKM) into mouse fetal fibroblasts, they successfully reprogrammed these fibroblasts to iPSCs. The genetic and behavioral properties of these iPSCs were found to highly resemble those of mouse ESCs [8, 9].

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Table 1. Issued Patents Related to *In Vivo* Isolation of Human Pluripotent Stem Cells.

Patent/Application Number & Inventor	Title	Summary/Claim	Ref #
US5843780, US6200806 and **US7029913** Thomson, James A., 1996	Primate embryonic stem cells	A purified preparation of primate embryonic stem cells	[2]
US6090622 Gearhart *et al.*, 1997	Human embryonic pluripotent germ cells	Human pluripotential embryonic germ cells exhibiting certain condition-dependent characteristics in culture	[3]
US6245566 and **US6331406** Gearhart *et al.*, 1998	Human embryonic germ cell line and methods of use	A method for producing human pluripotent embryonic germ (hEG) cells, comprising culturing human primordial germ cells (PGCs) in a defined culture medium	[5]
US6875607 Reubinoff *et al.*, 1999	Embryonic stem cells	A method of modulating the differentiation of undifferentiated, pluripotent human embryonic stem (hES) cell in culture	[6]
US7220584 Thomson *et al.*, 2003	Method of making embryoid bodies from primate embryonic stem cells	A method for producing differentiated human cells from human embryonic stem cells	[4]

Meanwhile, Yu *et al.* also developed new iPSC lines derived from human fibroblasts, using a different set of four defined factors including Oct4, Sox2, Nanog and LIN28 [10], albeit Yu's approach was less efficient than Yamanaka's method. The generation of these iPSCs not only overcame the ethical concerns of hESC usage but also provided patient-friendly stem cell lines for developing somatic cell nuclear transfer (SCNT)-based therapy. Such SCNT-based iPSC therapy has been successfully tested for treating sickle cell anemia in a transgenic mouse model [11]. However, despite holding great therapeutic promises, iPSC technology is still not ready for clinical use because of the following problems: 1) the use of retroviral/lentiviral delivery to enhance reprogramming efficiency, 2) the use of oncogenes (e.g. *c-Myc* and *Klf4*) to increase iPSC survival, and 3) the unknown mechanism underlying iPSC generation. Retroviral/lentiviral infection is the most effective method for simultaneously delivering four large transcription factor genes into a target cell; however, the random insertion of viruses into the cell genome may also affect other non-target cellular genes. This is problematic because uncertain viral insertion often causes genomic mutations, especially when one or more of the delivered transgenes are oncogenes. Although, several one- or two-factor induction methods have been devised to reduce viral insertion [12, 13], the applicability of these methods was limited within certain cell types that already expressed Sox2, Klf4 and/or c-Myc. Due to its unknown mechanism, the development of improvements was also hampered. Hence, understanding the mechanism underlying iPSC generation may help to solve these problems.

Another disadvantage of the four-factor iPSCs is their heterogeneity. To generate iPSCs, we need to simultaneously deliver three or more defined factors into a single cell genome via viral infection [7-10]. Since viral insertion is highly random, multiple viral infections often result in a variety of transgene combinations in the host cell genomes. Only cells with proper ratios and numbers of the defined four factors can form iPSCs. This problem actually accounted for the low (<0.002%) success rate of iPSC formation due to the presence of incorrect OSKM combinations [7, 8]. To purify iPSCs with a proper OSKM combination, a series of tedious cell selection procedures are required, as described in Jaenisch's WO9955841 [14], US7682828 [14], WO2002097090 [15], and US8071369 [15] and Yamanaka's WO2007069666 [16], US8048999 [16], US8058065 [17], and US8129187 [18]. Alternatively, Irion's US8048675 [19] devised a non-viral electroporation/liposomal transfection method to deliver plasmid vectors that express the defined four factors. Furthermore, other improvements using direct transfection of either OSKM mRNAs or proteins have also been reported [20, 21]. Yet, all these improved methods did not solve the problem of iPSC heterogeneity and their reprogramming efficiency was significantly lower than viral delivery. In view of these drawbacks, the viral delivery of OSKM is still the mainstream method for current iPSC generation. The details of these four-factor iPSC generation methods were summarized in Table **2**.

2. NOVELTY OF THE MICRORNA-MEDIATED IPSC GENERATION

MicroRNAs (miRNAs) are a class of small non-protein-coding RNAs that function to suppress the translation of their respective target genes through binding and formation of RNA-induced silencing complexes (RISCs) in the 3′-untranslated regions (3′-UTRs) of the targeted gene transcripts (i.e. mRNAs). Since this kind of miRNA-target interaction does not require a perfect match in complementarity, miRNAs can concurrently silence multiple target genes via partial binding in order to coordinate the functions and downstream interactions of these target genes. MiR-302 is a 23-ribonucleotide miRNA expressed abundantly in hESCs and iPSCs but is absent in all differentiated normal tissue cells [22, 23]. According to this feature, miR-302 serves as a major gene silencer in hESCs and early human zygotes. Based on the analytic results of online miRNA-target prediction programs TARGETSCAN (http://www.targetscan.org/)

Table 2. Issued Patents Related to Four-Factor iPSC Generation.

Patent/Application Number & Inventor	Title	Summary/Claim	Ref #
US7682828 and **WO199955841** Jaenisch *et al.*, 2004	Method for reprogramming somatic cells	A method for reprogramming somatic cell to multipotent or pluripotent cells, using Oct4, Sox2, and/or Nanog	[14]
US8048999 and **WO2007069666** Yamanaka *et al.*, 2006	Nuclear reprogramming factor	A nuclear reprogramming factor for generating induced pluripotent stem cells (iPSCs), comprising Oct, Klf and Myc familial genes	[16]
US8058065 Yamanaka *et al.*, 2009	Oct3/4, Klf4, c-Myc and Sox2 produce induced pluripotent stem cells	A method for preparing an iPSC using four defined factors Oct3/4, Klf4, c-Myc and Sox2	[17]
US8071369 and **WO2002097090** Jaenisch *et al.*, 2010	Compositions for reprogramming somatic cells	A composition comprising an isolated somatic cell that expresses exogenously introduced Oct4	[15]
US8129187 Yamanaka *et al.*, 2010	Somatic cell reprogramming by retroviral vectors encoding Oct3/4, Klf4, c-Myc and Sox2	A method for preparing at least a mammalian somatic cell derived from an iPSC comprising retroviral Oct3/4, Klf4, c-Myc and Sox2 gene delivery	[18]
US8048675 Irion, Stefan, 2010	Integration-free human induced pluripotent stem cells from blood	A method for generating integration-free human iPSCs from nucleated blood cells, using Oct4, Sox2, Klf4, c-Myc, Nanog and/or Lin28	[19]

and PICTAR-VERT (http://pictar.mdc-berlin.de/), the majority of miR-302-targeted genes are transcripts of differentiation-associated genes and developmental signals, such as members of the RAS-MAPK and TGFß-SMAD/LEFTY pathways, indicating its important role in preventing stem cell differentiation. The genomic sequence encoding miR-302 is located in the 4q25 locus of human chromosome 4, a conserved region frequently associated with longevity [24]. More precisely, miR-302 is encoded in the intron region of the *La ribonucleoprotein domain family member 7* (*LARP7*, *PIP7S*) gene and expressed via an intronic miRNA biogenesis mechanism [25, 26]. Native miR-302 consists of four familial sense homologues (miR-302b, c, a and d) and three distinct antisense members (miR-302b*, c* and a*), all of which are transcribed together as a polycistronic RNA cluster along with another miRNA, miR-367 [22, 25]. Although the overall function of these miR-302 members is largely unknown, their unique expression pattern and abundance in hESCs and iPSCs suggests a pivotal role in regulating stem cell pluripotency.

To improve the efficiency of iPSC generation, an alternative methodology other than four-factor induction is highly desired. In 2008, microRNA (miRNA)-mediated iPSC generation was first invented to replace the previous four-factor induction methods [27, 28]. Up to date, miRNA-mediated iPSC generation is also the only alternative reprogramming method without the need of any previously defined factor. As shown in Table **3**, the majority of this kind of new inventions were accomplished by two leading research groups, Lin *et al*. at the WJWU & LYNN Institute, USA (WO2009058413, WO2009079606, WO2009091659, WO2011025566, US20080293143, US20090203141, and US20100240126 [29-32]) and Yamanaka *et al*. at the Kyoto University, Japan (US20090246875, US20100075421, and EP2202309 [33]), both of which adopted a novel strategy of using miR-302-like miRNAs to induce iPSC formation. The concept of this new iPSC generation method originated from Lin's 2008 studies [27, 28], which showed the first practical evidence of using a small non-coding miRNA, miR-302, to reprogram both human normal and cancer skin cells into a hESC-like pluripotent state. Other miR-302 homologs, such as miR-93, miR-200c, miR-367, miR-371~373 and miR-520, were also found to possess a similar function in reprogramming [29-31]. Yamanaka's inventions confirmed this concept and further devised a method for using these miR-302-like miRNAs to improve the reprogramming efficiency of previous four-factor induction methods [33]. Nevertheless, since miR-302 alone is sufficient to efficiently induce iPSC formation, the combinatory use of miR-302-like miRNAs with the previously defined factors seems practically redundant and more laborious. Also, there is no evidence showing that any of the previously defined factors can actually enhance miR-302-mediated reprogramming, which makes the novelty of Yamanaka's combination method questionable. Other later inventions, including Itescu's US20120121548 [34], Blellock's US20120134966 [35], Rana's WO2011060100 [36] and US20110189137 [36], and Morrisey's WO2011133288 [37], all provided similar methodologies like Lin's and Yamanaka's methods. Taken together, all these research groups have repeatedly confirmed the novelty and feasibility of miRNA-mediated iPSC generation.

Lin's recent studies further demonstrated that elevated miR-302 expression to a level higher than that in hESCs induces somatic cell reprogramming to form iPSCs; yet, direct overexpression of miR-302 can cause cytotoxicity [29, 38, 39]. Direct miRNA or shRNA overexpression has been shown to saturate the cellular miRNA pathways and cause cytotoxicity [39]. Since miR-302 is a natural intronic miRNA, an intronic expression system is required to bypass this cytotoxicity effect. To reiterate the natural mechanism of

miR-302 biogenesis, Lin *et al*. developed an intronic miRNA/shRNA expression vector design mimicking the natural process of intronic miRNA biogenesis [26-29, 38]. As shown in Fig. (**1**), primary miRNA precursors (pri-miRNAs) are first transcribed by type-II RNA polymerases (Pol-II) and excised by spliceosomal RNA splicing to form shRNA-like miRNA precursors (pre-miRNAs), which are then exported out of the nucleus by Ran-GTP and Exportin-5 and further processed by Dicer-like RNaseIII endoribonucleases in cytoplasm to form mature single-strand miRNAs [26, 40-44]. Mature miRNAs are assembled into RISCs that bind and trigger targeted gene silencing effects [40, 44]. Unlike direct miRNA expression, the role of Drosha may not be necessary because some other endoribonucleases can replace Drosha for processing intronic pre-miRNAs [40, 41]. Also, because mammalian introns often contain nonsense (i.e. translational stop) codons recognized by the nonsense-mediated decay (NMD) system, a cellular RNA surveillance mechanism [45, 46], the non-hairpin structures of an intron can be quickly degraded by NMD to prevent the toxicity of excessive RNA accumulation. Due to such tight regulation by the cellular Pol-II transcription, RNA splicing and NMD systems, the strategy of utilizing intronic miR-302 expression has been successfully applied to overcome the cytotoxicity problem of direct miRNA/siRNA expression and has hence led to a better result in iPSC generation [27-29].

Using miRNA microarray analyses, Lin's studies showed that miR-302 does not act alone in reprogramming target cells to form iPSCs. A significant increase in expression of other homologous miRNAs, such as miR-92, miR-93, miR-367, miR-371~373, miR-374, and the whole miR-520 familial members, was also detected following the ectopic expression of miR-302 in target cells [27-31]. Further analyses using the online miRNA-target prediction programs "TARGETSCAN" and "PICTAR-VERT" linked to the Sanger miRBase::Sequences website (http://microrna.sanger.ac.uk/), revealed that miR-302 shares over 400 target genes with these microarray-identified miRNAs, suggesting that they may also play important roles in maintaining stem cell pluripotency and renewal. As shown in Table **4**, the majority of these targeted genes are highly involved in embryonic development and tumor formation. Thus, in view of these overlapping genes and their functions, it suggests that miR-302 can further stimulate these miR-302-like miRNAs, including miR-92, miR-93, miR-367, miR-371~373, miR-374 and miR-520, to enhance its reprogramming efficiency during iPSC formation.

3. MECHANISM UNDERLYING IPSC GENERATION

Global DNA demethylation is required for reprogramming and resetting the epigenetic status of a cell in order to attain stem cell-like pluripotency [47]. Nevertheless, none of previously defined four factors can account for this global DNA demethylation event during somatic cell reprogramming. In principle, DNA methylation acts like a lock that sets up various tissue-specific gene expression patterns in all kinds of somatic cells and defines their different cell properties. Global demethylation, on the other hand, serves as a key that unlocks and resets these different gene expression patterns into an ESC-like profile and hence allows different cells to be reprogrammed to iPSCs.

As shown in Fig. (**2**), miR-302 functions as a gene silencer capable of simultaneously down-regulating multiple key epigenetic regulators, including lysine-specific histone

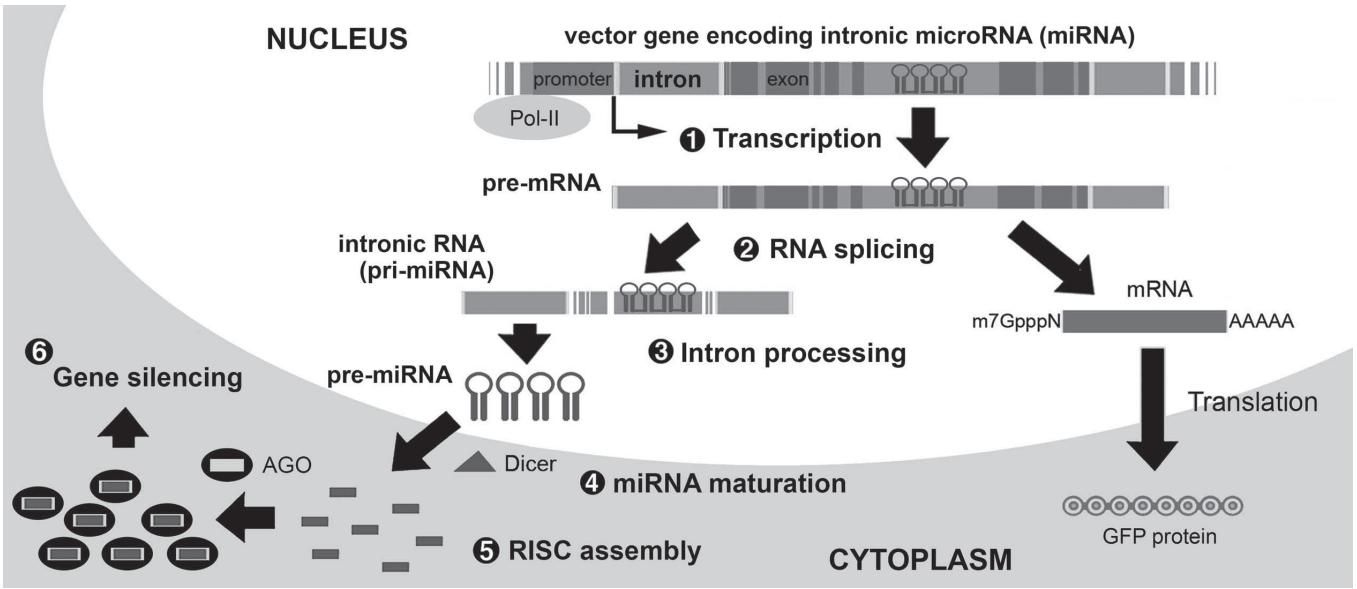

Fig. (1). Mechanism of natural miR-302 biogenesis. The intronic miR-302 familial cluster is transcribed together within a host gene by mammalian RNA polymerase II (Pol-II) and spliced out of the host gene transcripts by spliceosomal components and/or Drosha-like RNases to form precursor miR-302 (pre-miR-302), which is then exported out of the nucleus by Ran-GTP and Exportin-5 and further processed by Dicer-like RNaseIII endoribonucleases in the cytoplasm to form mature miR-302. For gene silencing, mature miR-302 is assembled into a RNA-induced silencing complex (RISC) with argonaute proteins and Dicer endoribonucleases and then functions to suppress the translation or cause direct degradation of targeted messenger RNAs (mRNAs).

Table 3. Published Patent Applications Related to miRNA-Mediated iPSC Generation.

Patent/Application Number & Inventor	Title	Summary/Claim	Ref #
US20080293143 and **WO2009058413** Lin, Shi-Lung *et al*., 2008	Generation human embryonic stem-like cells using intronic RNA	A composition and method for reprogramming mammalian somatic cells to induced pluripotent stem cells (iPSCs) using miR-302 and/or miR-302-like intronic microRNAs/shRNAs/siRNAs, such as miR-93, miR-367, miR-371~373, and miR-520	[29]
WO2009079606 Lin, Shi-Lung *et al*., 2008	MicroRNA-induced ES-like cells and uses thereof	A method of generating embryonic stem (ES)-like cells by contacting non-ES cells with an isolated nucleic acid homologous to miR-302	[30]
US20090203141 and **WO2009091659** LIN, Shi-Lung *et al*., 2009	Generation of tumor-free embryonic stem-like pluripotent cells using inducible recombinant RNA agents	An inducible non-transgenic composition and method for generating tumor-free iPSCs using miR-302 and/or miR-302-like microRNAs/siRNAs	[31]
US20090246875, **US20100075421** and **EP2202309** Yamanaka, Shinya *et al*., 2009	Effective method for nuclear reprogramming	A method for generating iPSCs or increasing the reprogramming efficiency of iPSCs using miR-290~295, miR-302 and/or miR-302-like microRNAs in conjunction with at least one of the previously defined four factors comprising Oct4, Sox2, Klf4, c-Myc, and/or Nanog	[33]
US20100240126 and **WO2011025566** Lin, Shi-Lung *et al.*, 2010	Development of universal cancer drugs and vaccines	A method for inducing the miR-302-mediated tumor suppression effect in tumor/cancer cells	[32]
US20120121548 Itescu, Silviu, 2010	Production of reprogrammed pluripotent cells	A method of producing reprogrammed cells using "potency-determining" factors, such as Oct4, Sox2, Klf4, Nanog, Lin28, c-Myc, and/or microRNAs/siRNAs	[34]
US20120134966 Blellock, Robert, *et al*., 2010	Embryonic stem cell-specific microRNAs promote induced pluripotency	A method of inducing cell pluripotency using microRNAs, such as miR-290, miR-302, miR-17~92, miR-106a, and miR-370 family in the presence or absence of Oct4, Sox2, and Klf4	[35]
US20110189137 and **WO2011060100** Rana, Tariq M., *et al*., 2010	Method for generation and regulation of iPS cells and compositions thereof	A method for generating iPSCs or increasing the related induction efficiency using using microRNAs, such as miR-302, miR-17~92, miR-21, miR-25, miR-29a, miR-106a, and let-7, in the presence of Oct4, Sox2, Klf4, and c-Myc	[36]
WO2011133288 Morrisey, Edward E., 2011	MicroRNA induction of pluripotent stem cells and uses thereof	A composition and method for the formation of an iPSC from a somatic cell, using a miR-302~367 cluster or miR-302~367 mimics	[37]

demethylases 1 and 2 (namely AOF2/1, LSD1/2, or KDM1/1B), DNA (cytosine-5-)-methyltransferase 1 (DNMT1), and methyl-CpG binding proteins 1 and 2 (MECP1/2) [29-31, 38, 48]. As a result, silencing of these important epigenetic regulators induces global DNA demethylation, the first sign of nuclear reprogramming in the process of iPSC formation [29-31, 38, 47]. Previous transgenic animal studies have shown that, in the absence of AOF1, germ cells failed to undergo *de novo* DNA methylation during oogenesis [49], while AOF2 deficiency could cause embryonic lethality due to a progressive loss of genomic DNA methylation and lack of cell differentiation [50]. Therefore, inhibition of AOF1 and/or 2 is sufficient to induce global demethylation. Meanwhile, the additional suppression of MECP1/2 may help to change the chromatin structure and hence further enhances the DNA demethylation effect of AOF1/2 inhibition [27, 29-31, 38].

Since AOF2 is required for stabilizing DNMT1 [50], miR-302-mediated silencing of AOF2 expression inhibits the DNMT1 activity in maintaining replication-dependent DNA methylation [38, 48]. DNMT1 is known to maintain inherited CpG methylation patterns by methylating the newly replicated DNA during the S-phase of cell cycle. Loss of DNMT1 activity has been shown to cause passive DNA demethylation in early zygotic cells during embryonic development [51-54]. This finding is further supported by the analytic data of online miRNA-target prediction program provided by the European Bioinformatics Institute EMBL-EBI (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/detail_view.pl?transcript_id=ENST00000359526), pre-

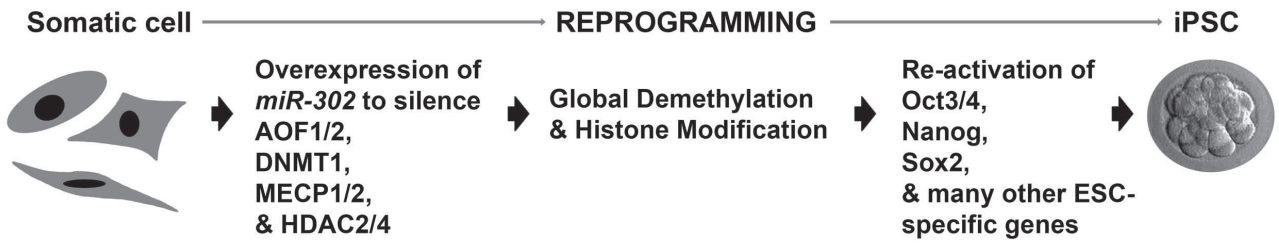

Fig. (2). Currently established reprogramming mechanism for iPSC generation. Briefly, miR-302 silences AOF1/2 and DNMT1 and, further in conjunction with the co-suppression of MECP1/2 and histone acetylase 2/4 (HDAC2/4), results in global DNA demethylation and histone modification. Subsequently, these epigenetic reprogramming events induce ESC-specific gene expression, particularly Oct4, Sox2 and Nanog, which in turn function to further complete the process of reprogramming.

Table 4. List of the target genes shared by miR-92, miR-93, miR-302, miR-367, miR-371~373, miR-374, and miR-520.

Function	Gene
Oncogene	RAB/RAS-related oncogenes, ECT-related oncogenes, pleiomorphic adenoma genes
Transcription factor or regulator	E2F transcription factors, cyclin D binding Myb-like transcription factors, HMG-box transcription factors, Sp3 transcription factors, transcription factor CP2-like proteins, NFkB activating protein genes, PCAF
Kinase	Cyclin-dependent kinases (CDKs), MAPK-related kinases, SNF-related kinases, myosin light chain kinases
Developmental signal	IGF receptors, endothelin receptors, left-right determination factors, TNF-alpha-induce protein genes, DAZ-associated protein genes, LIM-associated homeobox genes, DEAD/H box protein genes, forkhead box protein genes, SMAD2, BMP regulators, inhibin, Rho/Rac guanine nucleotide exchange factors, Max-binding protein genes, c-MIR cellular modulator of immune recognition
Adhesion/Migration	Protocadherins, integrin ß4/ß8, ankyrins
Cell cycle regulator	Cyclins, p53 inducible nuclear protein genes, RB-like 1, RB binding protein genes, Bcl2-like apoptosis facilitator
Epigenetic regulator	MECP2, histone acetyltransferase MYST3, nuclear RNP H3
Others	SENP1, NUFIP2, FGF9/19, CXCR4, EIF2C

dicting that miR-302a-d can directly target DNMT1 for gene silencing. Taken together, the above observations indicate that iPSC formation starts with a passive global demethylation mechanism. However, this passive demethylation model will generate two hemi-methylated cells in every single iPSC colony, an event that has not yet been reported. Passive demethylation is unable to remove the methylated sites originally left in the somatic genome before reprogramming; therefore, only the newly divided daughter cell genomes are demethylated and reprogrammed. Whether these hemi-methylated cells are degraded via programmed cell death (apoptosis) during reprogramming or further demethylated by another active mechanism remains elusive.

Global demethylation has been found to promote Oct4–Nanog overexpression in early mouse embryos and mouse-human fused heterokaryons [55, 56]. Recent studies further showed that an increase beyond the miR-302 level found in normal hESCs triggers both global demethylation and co-expression of Oct4, Sox2 and Nanog in human iPSCs [29-31, 38]. The expression of Lin28 and many other hESC marker genes was observed 1-3 days after the induction of Oct4–Sox2–Nanog co-expression. A similar miR-302 transfection approach was also shown to increase Oct4–Nanog expression by two folds in hESCs [57]. Further studies revealed that miR-302 directly silences nuclear receptor subfamily 2, group F, number 2 (NR2F2), a transcriptional repressor against Oct4 expression, to activate Oct4 expression [58]. These findings suggest that miR-302 induces global demethylation to remove transcriptional blocks and hence activates hESC-specific gene expression, particularly Oct4 and Nanog. Furthermore, microarray analyses have shown that the genome-wide gene expression patterns between iPSCs and hESCs share over 92% similarity [27, 29-31, 38], indicating that miR-302 is able to induce global demethylation and Oct4–Nanog activation essential for the initiation of reprogramming to form iPSCs.

Natural global DNA demethylation occurs in two developmental stages; first, during migration of primordial germ cells (PGCs) into the embryonic gonads (at around embryonic day E10.5), and second, in 2-to-8-cell-stage zygotic cells after fertilization [51, 52, 59, 60]. Parental methylation imprints are erased and re-established in PGCs but are largely preserved in postfertilized zygotic cells [59-62], demonstrating the key difference between germline and zygotic demethylation. The recent discovery of global demethylation in iPSCs introduces a new level of DNA demethylation comparable to the endogenous ones [27-29, 38]. Reprogramming-associated global demethylation triggers massive removal of genomic methylation sites but preserves many parental imprints [29-31, 48, 63, 64], resembling a zygotic

demethylation mechanism in somatic cells. However, unlike zygotes, somatic cells do not possess any germline element, such as sperm protamines, oocyte proteins and mRNAs. As a result, some zygotic components required for the completion of reprogramming during early embryogenesis are missing in iPSCs. This observation is further supported by recent evidence showing that SCNT-mediated reprogramming results in more similar epigenomic and transcriptomic patterns to those of ESCs than does the induced reprogramming by the four previously defined factors [63]. Hence, it is conceivable that iPSCs may not contain sufficient materials to support the full development of an embryo. In other words, the animal clones derived from iPSCs, if available, are likely to be defective due to their lack of certain essential components from germ cells.

4. ADVANTAGES OF miRNA-REPROGRAMMED IPSCS (mirPSCs)

Tendency in tumor formation is one of the most challenging problems inherent in stem cell therapy using ESCs or iPSCs. Development of applications for preventing ESC/iPSC tumorigenicity is extremely critical for the success of stem cell therapy. Tumor-free pluripotency is one of the major advantages of mirPSCs compared to the four-factor iPSCs. Oncogenic factors such as c-Myc and Klf4 are frequently used to boost the survival and proliferative rates of four-factor iPSCs, creating an inevitable problem of tumorigenicity that hinders the therapeutic usefulness of these pluripotent cells. On the other hand, mirPSCs are generated using a tumor suppressor miRNA, miR-302, which essentially induces somatic cell reprogramming while preventing stem cell tumorigenicity [65]. As the mechanism of stem cell tumorigenicity is still poorly understood, breaking through this knowledge barrier is necessary for us to develop a safer and more effective stem cell therapy.

To solve the problem of stem cell tumorigenicity, we must first understand the natural mechanism by which embryonic cells in early zygotes control their cell division. It is known that early zygotes before the morula stage (16-32 cell stage) often exhibit a very slow cell cycle rate, whereas such stringent cell cycle regulation is not found in later blastocyst-derived hESCs. Naturally occurring embryonic cells in early zygotes possess two unique stemness properties: potential of pluripotent differentiation into almost all cell types and unlimited self-renewal in the absence of tumor formation. These two features are also important for the clinical application of hESCs or iPSCs. However, despite overwhelming reports regarding hESC/iPSC pluripotency, there is little understanding about the mechanism underlying tumor prevention in zygotes. To clarify this point, Fig. (**3**) demonstrates that miR-302 inhibits iPSC tumorigenicity through co-suppression of both cyclin E-CDK2 and cyclin D-CDK4/6 cell cycle pathways, subsequently blocking >70% of the G1-S phase transition [31, 65]. Additionally, miR-302 also silences BMI1, a cancer stem cell marker, to promote the expression of two tumor suppressor genes, p16Ink4a and p14/p19Arf [31, 65]. p16Ink4a inhibits cyclin D-dependent CDK4/6 activities via phosphorylation of retinoblastoma protein Rb and subsequently prevents Rb from releasing E2F-dependent transcription required for S phase entry [66, 67], while p14/p19Arf prevents HDM2, an Mdm2 p53 binding protein homolog, from binding to p53 and permits the p53-dependent transcription responsible for G1 arrest or apoptosis [68]. Collectively, the combined effect of reducing G1-S cell cycle transition and increasing p16/p14(p19) expression results in an attenuated cell cycle rate similar to that of 2-to-8-cell-stage embryonic cells in early zygotes (20-24 hour/cycle). Taken together, these findings have clearly identified the dual function of miR-302 in both iPSC induction and tumor suppression.

Similar to its function in iPSC generation, the effect of miR-302-mediated tumor suppression is also dose-dependent, occurring only when its expression level exceeds the level found in hESCs [31, 65]. When miR-302 level is equal to or lower than hESCs', however, only large tumor suppressor homolog 2 (LATS2), but not CDK2, was silenced. On the other hand, when miR-302 level is higher than that in hESCs, both CDK2 and LATS2 are silenced and hence cell cycle is attenuated at the G1-phase checkpoint [31, 65]. Given that LATS2 inhibits the cyclin E–CDK2 pathway to block G1-S cell cycle transition [69], the direct silencing of CDK2 by miR-302 can enforce this LATS2 function even in the absence of LATS2. Moreover, the silencing of CDK2 can also counteract the suppressive effect of miR-367 on CDKN1C (p57, Kip2), a cell cycle inhibitor against both CDK2 and CDK4, subsequently leading to a reduced cell cycle rate (Lin *et al*. unpublished). In light of

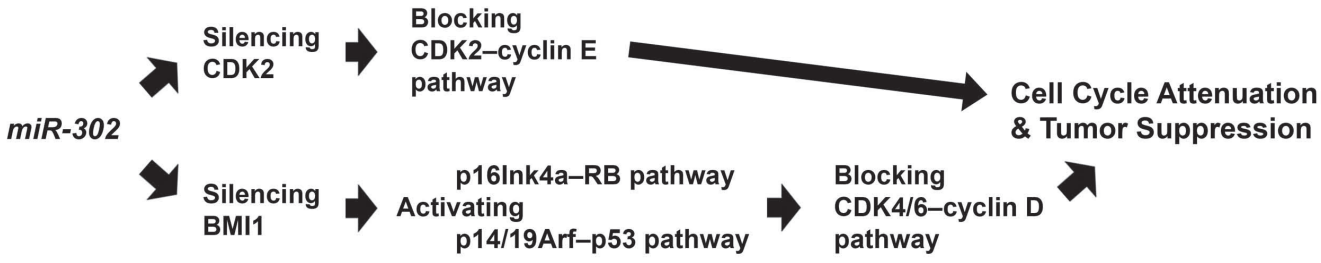

Fig. (3). Currently established mechanism underlying miR-302-mediated tumor suppression in iPSCs. MiR-302-mediated cell cycle attenuation is effected via co-suppression of multiple G1-checkpoint regulators such as cyclin-dependent kinase 2 (CDK2), cyclins D1/D2 and polycomb ring finger oncogene BMI1 as well as activation of tumor suppressors p16Ink4a and p14/p19Arf to suppress most of the cell cycle activities during reprogramming. Quiescence at the G0/G1 cell cycle phase also prevents any tumor cell growth or transformation in the reprogrammed iPSCs. Collectively, the synergistic effect of these two tumor suppression events results in a more accurate and safer reprogramming process, by which premature cell differentiation and tumorigenicity are both inhibited.

this dose-dependent tumor suppression effect of miR-302, we may use an inducible method to control the miR-302 expression level for generating tumor-free iPSCs or improving the tumorigenicity problem of hESCs. As shown in Table **3**, this kind of approach has been adopted by Lin's US20090203141 [31], US20100240126 [32], WO2009091659 [31], and WO2011025566 [32].

Based on the above advance in understanding of miR-302 function in both iPSC generation and tumor suppression, many tumor-free iPSC lines have been generated and tested as early as 2008 [27-32, 38, 65]. These tumor-free iPSCs include mirPSCs derived from normal human keratinocytes and melanocytes as well as cancerous melanoma Colo829, prostate cancer PC3, breast cancer MCF7, hepatocellular carcinoma HepG2, and embryonal teratocarcinoma Tera-2 cell lines. Notably, normal and cancerous cells respond very differently to miR-302-mediated tumor suppression effect. In humans, miR-302 triggers massive reprogrammed cell death (apoptosis) in fast proliferative tumor/cancer cells whereas slow growing normal cells can tolerate this inhibitory effect on cell proliferation [32, 65]. It is understandable that tumor/cancer cells may not survive in such a slow cell cycle state due to their high metabolism and rapid proliferation rates. This result provides a very beneficial advantage in preventing iPSC tumorigenicity. Further, *in vivo* teratoma formation assays indicate that these mirPSCs are highly pluripotent but not tumorigenetic, as they preferentially form teratoma-like tissue cysts in the uteruses and sometimes in peritoneal cavities but rarely in other tissues of pseudopregnant female immunocompromised SCID-beige mice [27-31, 38, 65]. Unlike tumor-prone embryonal teratomas, these tissue cysts contain various but relatively organized tissue regions that are derived from all three embryonic germ layers (ectoderm, mesoderm and definitive endoderm) [27-31, 38, 65]. When xenografted into normal male mice, these mirPSCs can be easily differentiated and assimilated by the surrounding tissues, indicating a potential application for regenerative medicine [32, 65]. Taken together, these findings suggest that miR-302 reprograms both normal and cancerous somatic cells to hESC-like iPSCs with a high degree of pluripotency but without tumorigenicity.

5. APPLICATIONS OF mirPSCS IN CANCER THERAPY AND REGENERATIVE MEDICINE

Since miR-302 has a dual function in iPSC generation (reprogramming) and tumor suppression, it is highly conceivable that it can be used for developing regenerative medicine and cancer therapy. Several studies have demonstrated the utilization of miR-302 in cancer therapy, showing that inducible miR-302 expression higher than 1.1 folds of the hESC level inhibited an average > 78% of tumor/cancer cell growth, eliminated > 98% of the tumor/cancer cell populations but not normal cells, and completely prevented any malignant cell invasion and metastasis [32, 65]. Multiple cancer cell types were successfully treated in these studies, including human skin cancer, prostate cancer, breast cancer, liver cancer, and embryonal teratocarcinoma (hEC) cells. Further *in vivo* tumorigenicity assays testing the drug effect of miR-302 on hEC Tera2-derived teratomas in eight-week-old male athymic mice (BALB/c nu/nu strain) also confirmed the feasibility of this therapeutic strategy *in vivo* [32, 65]. Due to their similar pluripotency, hEC cells were often served as a malignant model of hESC transformation into cancer stem cells. The results of these *in vivo* miR-302 treatments revealed a marked decrease in average tumor size by > 90% compared to that of non-treated ones. Interestingly, miR-302 treatment only inhibited the tumorigenicity but not pluripotency of hEC cells, strongly suggesting a novel therapeutic approach whereby miR-302 eliminates >98% tumor/cancer cells while reprogramming the remaining <1%~2% cells to iPSCs for repairing the tumor/cancer damages. These consistent *in-vitro* and *in-vivo* data indicate that miR-302′s tumor suppression mechanism can be applied to develop an effective therapy or drug for not only preventing the malignant transformation of iPSCs/hESCs into tumor/cancer cells but also treating a variety of tumors and cancers.

Another currently available application for the miR-302-mediated iPSC (mirPSC) technology lies in regenerative medicine. Although engineering iPSC-derived tissues/organs for autotransplantation is still far from reach at this point, some applications using miR-302-induced tissue regeneration for wound healing have been proposed [65]. Recent studies have found that miR-302 functions to stimulate stem cell generation and hence may promote tissue regeneration *in vivo* [32, 65]; based on this practical idea, the results of Fig. (**4**) demonstrate the possible applicability of this novel regenerative medicine approach in wound healing (Lin *et al.* unpublished). In animal trials using mouse, 10μg/mL of miR-302 was mixed with a pre-prepared ointment base containing cocoa butter, cottonseed oil, olive oil, sodium pyruvate, and white petrolatum. Skin open wounds were generated by scalpel dissection and sized about 25mm^2. Ointments with blank base, miR-434 (negative control), or miR-302 were directly applied on the wounds, respectively, and covered the whole wounded area. The treated area was subsequently sealed by liquid bandage. As shown in Fig. (**4**), within 4 days, the miR-302-treated wound was clearly healed better than all other controls. At day 11, miR-302-treated skin area was almost completely recovered with new hair regrowth, while other controls still presented large scar tissues with no hair. These results clearly validated the therapeutic effects of miR-302-stimulated tissue regeneration on wound healing, providing a principle-of-proof drug design and application for today's regenerative medicine.

6. CURRENT & FUTURE DEVELOPMENTS

Competition in the technology of miRNA-mediated iPSC generation is extremely intense in the recent years. Many research groups have attempted to claim the first in this invention. To clarify this issue, Table **3** summarizes the currently published patent applications related to miRNA-mediated iPSC technology in a chronological order. The list of Table **3** showed that Lin's WO2009058413, WO2009079606 and US20080293143 [29] first disclosed the composition and method for reprogramming human somatic cells to iPSCs using miR-302 and/or miR-302-like miRNAs, such as miR-93, miR-367, miR-371~373, and miR-520. In early 2009, their WO2009091659 and US20090203141 [31] further revealed the intrinsic mechanisms underlying miR-302-mediated tumor suppression in iPSCs, leading to a novel non-viral approach for preventing

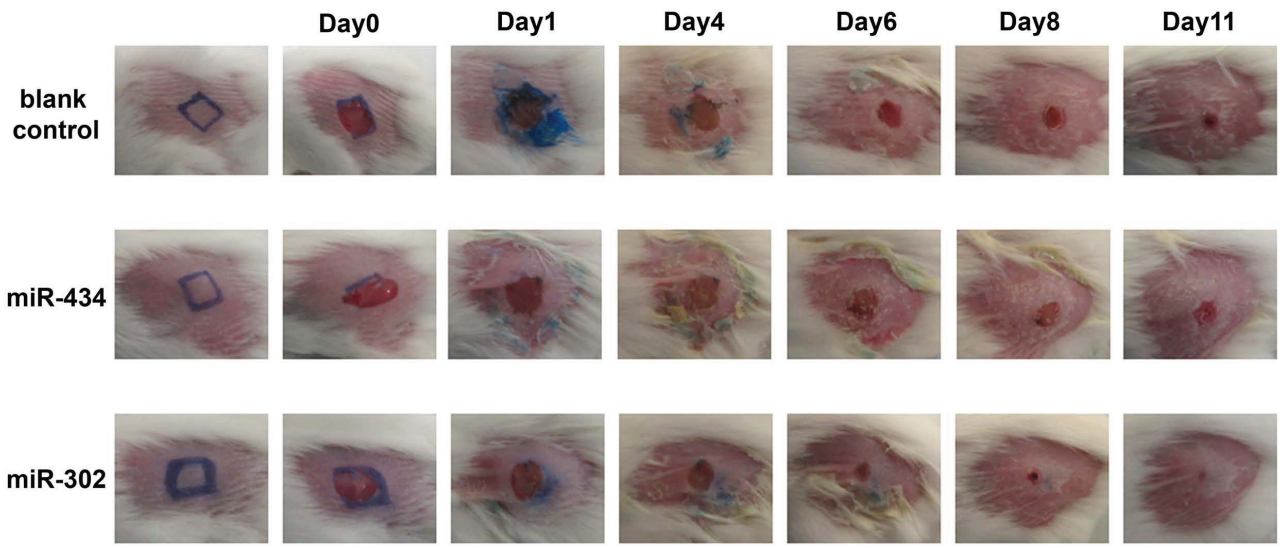

Fig. (4). Therapeutic results of an *in-vivo* pre-investigational new drug (pre-IND) study using microRNA miR-302-containing ointment to treat skin open wounds in mice. All animal tests were performed following the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) regulations. Three different treatments were tested: top, blank ointment only; middle, 10 μg/mL of miR-434 in ointment (negative control); and bottom, 10 μg/mL of miR-302 in ointment.

stem cell tumorigenicity. Shortly after Lin's findings, Yamanaka's US20090246875, US20100075421 and EP2202309 [33] also identified a similar method for generating iPSCs and further showed another method for enhancing the reprogramming efficiency of iPSCs by using a combination of a miR-302-like miRNA and at least one of the previously defined factors (OSKM, Lin28 and/or Nanog). Thus far, all other later inventions, such as Itescu's US20120121548 [34], Blellock's US20120134966 [35], Rana's WO2011060100 and US20110189137 [36], and Morrisey's WO2011133288 [37], shared a highly similar concept and principle with Lin's and Yamanaka's methods. However, none of these later inventions disclosed any prior contribution from Lin's or Yamanaka's works. In view of current patent law requirements for "prior art" disclosure, the patentability of these later inventions is therefore questionable.

The original idea of using miRNAs for iPSC generation is derived from the natural process of ESC formation in early embryogenesis. It has been known that miR-294/miR-302 is expressed as early as in two-cell-stage zygotes, whereas the presence of Oct4, Sox2 and Nanog starts after the onset of zygotic demethylation (after two-cell stage). Before the presence of Oct4, Sox2 and Nanog, many miR294/miR302-like homologous miRNAs, such as miR-92, miR-93, miR-200c, miR-367 and miR-371~373, have also been abundantly expressed in various stages of oocytes and early zygotes. The technology of SCNT has shown that oocyte cytoplasm can reprogram somatic cells to pluripotent stem cells in the absence of Oct4, Sox2 and Nanog, which are all nuclear transcription factors [38, 70, 71]. Since miRNA functions as a gene silencer to suppress mRNA translation in cytoplasm, it is conceivable that miR-302-like miRNAs may play a pivotal role in reprogramming prior to the activation of Oct4, Sox2 and Nanog expression. Most importantly, only miR-302, but not any of the previously defined factors, can explain the mechanisms underlying global DNA demethylation in iPSCs, the first sign of nuclear reprogramming. To clarify this unsolved issue, Lin *et al*. have deciphered the mechanism by which miR-302 triggers global DNA demethylation to activate Oct4–Sox2–Nanog co-expression and hence subsequently induces reprogramming to form iPSCs [38, 48]. Based on this established mechanism comparable to the natural zygotic reprogramming, it suggests that miR-302 is a reprogramming initiator upstream to the previously defined four factors and thus can be used to replace all previously defined factors for more efficient iPSC induction.

The technology of miRNA-mediated iPSC (mirPSC) generation is still in its early stage, yet very promising. MirPSCs represent tumor-free iPSCs essential for overcoming the two major problems of stem cell therapy: supply and safety. Currently, the majority of iPSC studies are focused on the revelation of the mechanisms underlying somatic cell reprogramming and prevention of stem cell tumorigenicity. Deciphering these mechanisms will lead to the identification of methods for improving current iPSC generation methods as well as developing new iPSC applications. Due to the recent advances in understanding of these mechanisms [38, 48, 58, 65], several applications in regenerative medicine and cancer therapy have been successfully developed and tested in animal trials. Compared to the previous four-factor induction methods, the present mirPSC technology has great advantages in reprogramming efficiency and safety. Simply by determining the success rate between delivering a single miRNA and the four large transcription factors, the mirPSC technology clearly provides better efficiency and less damage to the reprogrammed cells; not to mention, miR-302 is a tumor suppressor in humans whereas Klf4 and c-Myc are known oncogenes. In fact, it has been reported that the opti-

mal reprogramming efficiency for miR-302 and four-factor induction methods is >10% and <1%, respectively, showing at least a ten-fold improvement [27, 38, 72].

It took five years from the discovery of iPSCs by Yamanaka *et al*. [7] to the identification of its mechanisms by Lin *et al*. [38, 48, 65]. The progress of modern iPSC technology is fast and becoming important for the development of today's regenerative medicine. Given that most of current research was performed in isolated cells under *in vitro* conditions, applying these findings for developing *in vivo* therapy will be the next challenge. However, the iPSC tumorigenicity is still a problem hindering its usefulness *in vivo*. Lin's US20090203141, US20100240126, WO2009091659 and WO2011025566 have solved this problem by using mirPSCs [31, 32]. Without the risks of stem cell tumorigenicity, the mirPSC technology could deliver better therapeutic outcomes [32, 65]. Data from these studies suggest that miR-302 not only stimulates *in vivo* stem cell generation to facilitate wound healing and tissue regeneration but also inhibits tumor cell growth to prevent any possible cancer transformation, indicating a very beneficial potential for both regenerative medicine and cancer therapy. Therefore, the development of this novel technology has progressed beyond simply iPSC generation; in fact, certain advanced applications have extended to the level of using its related reprogramming mechanism for therapy. For example, direct stimulation of *in vivo* stem cell generation to facilitate tissue recovery is one of the most desirable trends that have been attempted [32, 65]. Following the recent revelation of the reprogramming mechanism, further development in this direction is highly expected.

Today, regenerative medicine is no longer just a concept of future medicine. It holds great potentials in curing aging-related illnesses by repairing and rejuvenating body cells. It was assumed in the past that, once differentiated, a cell cannot revert back to its earlier undifferentiated state. The discovery of iPSCs, however, totally changes this concept and proves that there is an internal mechanism capable of reprogramming differentiated cells back to the earliest ESC-like pluripotent stage. In recent years, the revelation of this reprogramming mechanism may further advance the design and development of iPSC-based regenerative medicine to a whole new era for not only disease therapy but also anti-aging medicine. Since numerous environmental and pathogenic factors, such as pollutants, toxic materials, free radicals, stress, microbial/viral infections and various illnesses, can reduce our stem cell number to accelerate the speed of aging, the invention of miRNA-mediated iPSC generation may provide us sufficient stem cells for studying the rejuvenation mechanism and hence facilitating the development of anti-aging treatments. As now we have plentiful body cells to serve for stem cell generation, a variety of new regenerative medicine methods may be exploited in the near future for improving our quality of life.

CONFLICT OF INTEREST

Jack S.K. Chen has no conflict of interest with the listed patents or pending applications. Shi-Lung Lin is an inventor of the cited patent applications US20080293143, US20090203141, US20100240126, WO2009058413, WO2009079606, WO2009091659, and WO2011025566.

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