In Search for the Fountain of Youth: Deciphering the Mechanism behind iPSC Generation

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SUMMARY

Why do we all get old? It is simply because our bodies possess only a limited number of stem cells, which are responsible for regenerating new cells to replace the aged and damaged ones. As the body grows, these stem cells gradually lose the ability to produce sufficient number of new cells to replace aging cells. Hence, old and damaged cells accumulate as the body getting older and older. Numerous environmental and pathogenic factors, such as pollutants, toxic materials, free radicals, stress, microbial/viral infections and various illnesses, can reduce the stem cell population and accelerate the speed of aging. Overcoming stem cell shortages is crucial to rejuvenate our bodies and prevent senescence. Therefore, the key to open up the fountain of youth resides in a natural mechanism underlying stem cell generation and regeneration rather than a method to prevent body cells from aging. Previous studies have found that a small non-protein-coding RNA, miR-302, can induce somatic cell reprogramming (SCR) to form pluripotent stem cells, suggesting its pivotal role in stem cell generation. Recent research further revealed that miR302-induced SCR involves an epigenetic mechanism similar to the natural zygotic reprogramming process in early embryonic development. Based on these advanced discoveries, this review will describe the currently established SCR mechanism—as compared to the natural process of embryonic stem cell (ESC) formation—and will demonstrate how stem cell researchers utilize this mechanism to reprogram somatic tissue cells back to ESC-like "induced pluripotent stem cells" (iPS cells or iPSCs).

In the past, it was widely assumed that a stem cell, once differentiated, could not revert back to an earlier developmental stage. The recent discovery of iPSCs, however, revokes this concept and provides the first evidence that there is an internal mechanism capable of resetting the "stemness" of a differentiated tissue cell back to an ESC-like pluripotent state, indicating a fountain of youth intrinsic to every cell in the body. A stem cell has two principal abilities of stemness: to multiply through unlimited division (referred as self-renewal) and to differentiate into a variety of tissue cells originated from all three embryonic germ layers, ectoderm, mesoderm and endoderm (referred as pluripotency). The breakthrough discovery of such a stemness resetting phenomenon provides us a powerful means to regenerate unlimited pluripotent stem cells directly from the resourceful pool of body tissue cells. Yet, the involved mechanism, called somatic cell reprogramming (SCR), remains elusive.

SCR was first observed by transferring somatic cell nuclei into the cytoplasm of oocytes, which forms ESC-like hybrid cells that can develop into animal clones possessing the same genetic traits as the hosts of the somatic cell nuclei (Wilmut et al., 1997; Wakayama et al., 1998). Although this kind of somatic cell nuclear transfer (SCNT) technology has been intensively practiced for over fourteen years to produce various species of animal clones, the necessity of oocytes is ethically controversial and the mechanism is unclear. In 2006, Takahashi and Yamanaka established a completely new reprogramming method that does not use any oocyte or embryonic components. By introducing four defined transcription factors, Oct4, Sox2, Klf4 and c-Myc, somatic cells were reprogrammed to iPSCs that showed ESC-like properties in almost all aspects (Takahashi and Yamanaka, 2006). Next year, Yu et al. also successfully generated iPSCs using another four defined factors, Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007). Nevertheless, with all these efforts, the SCR mechanism is still unsolved.

Two years after the discovery of iPSCs, another method of iPSC generation was invented which revealed the mechanism of SCR. Lin et al. and their peers showed that a small non-coding RNA, called miR-302, can replace all previously defined factors to reprogram human skin/hair cells to ESC-like iPSCs (Lin et al. 2008 and 2011; Anokye-Danso et al. 2011). MiR-302 is a 23-ribonucleotide microRNA (miRNA) expressed abundantly in human ESCs but is absent in all differentiated tissue cells (Suh et al., 2004). Despite its presence in ESCs, why does a small RNA, not even capable of encoding any protein or peptide, play a pivotal role in regulating SCR? It turns out that miR-302 functions as a gene silencer and simultaneously down-regulates multiple key epigenetic regulators, including lysine-specific histone 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) (Lin et al., 2008 and 2011). Silencing of these epigenetic regulators induces global DNA demethylation, the first sign of SCR. Like a lock DNA methylation sets up the somatic gene expression patterns in cell and defines the cell's properties; the key to SCR, global demethylation, unlocks and resets the cellular gene expression patterns to an ESC-like profile. This "unlocking" of a genome allows transcription machinery access to the ESC-specific genes and is required for iPSC formation.

None of the previously defined transcription factors can provide an explanation for the process of global demethylation. To clarify this unsolved mechanism, Lin's studies reveal that miR-302 binds to the gene transcripts (mRNAs) of AOF2/1 and MECP1/2 and forms a RNA-induced silencing complex (RISC) with argonaute proteins and Dicer endoribonucleases to repress the translation of these epigenetic regulators (Lin et al., 2010 and 2011). In the absence of AOF1 protein synthesis, germ cells do not undergo de novo DNA methylation during oogenesis (Ciccone et al., 2009), while AOF2 deficiency causes embryonic lethality due to a progressive loss of genomic DNA methylation and therefore no cell differentiation (Wang et al., 2009). Hence, silencing of AOF1 and/or 2 is sufficient to induce global demethylation. Silencing of MECP1/2 further enhances this demethylation effect of AOF1/2 deficiency (Lin et al., 2008 and 2011).

In addition to global demethylation, Lin's studies also found that miR302-targeted AOF2 silencing can destabilize DNMT1 activity and prevent replication-dependent DNA methylation during iPSC division, subsequently resulting in a passive demethylation mechanism (Lin et al., 2011). Based on the analytic results 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), miR-302 also directly targets DNMT1 for gene silencing. Given that DNMT1's function is to maintain inherited CpG methylation patterns by methylating the newly replicated DNA during S-phase of the cell cycle, loss of DNMT1 activity has been shown to cause passive DNA demethylation in early zygotic cells during embryonic development (Monk et al., 1987; Mayer et al., 2000; Santos et al., 2002; Hirasawa et al., 2008). Taken together, all the above studies indicate that SCR begins 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 observed or reported. Passive demethylation is unable to remove the methylated sites originally left in the somatic genome before SCR; therefore, only the newly divided daughter cell genomes are demethylated and reprogrammed. Whether these hemi-methylated cells are quickly degraded by programmed cell death (apoptosis) during SCR or further demethylated by another active mechanism remains unknown.

Global demethylation is not a new biological event in view of natural embryonic development. Global demethylation occurs twice during development: first during migration of primordial germ cells (PGCs) into the embryonic gonads (approximately embryonic day E10.5 to E13.5), and next in the 2-to-8-cell-stage zygotic cells after fertilization (Hajkova et al., 2002; Szabo and Mann, 1995; Mayer et al., 2000; Santos et al., 2002). Parental methylation imprints are erased and re-established in germline PGCs but largely preserved in postfertilized zygotic cells (Stoger et al., 1993; Tremblay et al., 1995; Chamberlain et al., 2010), demonstrating that the germline and zygotic demethylation mechanisms are not identical. The recent discovery of global demethylation in iPSCs introduces a new kind of man-made SCR demethylation mechanism comparable to the natural ones (Lin et al., 2011). Similar to zygotic demethylation, SCR demethylation triggers massive erasure of genomic methylation sites but preserves parental imprints (Figure 1). Hence, SCR demethylation is like a forced zygotic demethylation mechanism in somatic cells. However, unlike zygotes, somatic cells do not receive parental elements from oocytes or sperms, such as paternal protamines, maternal proteins and RNAs. As a result, some zygotic components required for the completion of epigenetic reprogramming during early embryogenesis are missing in SCR. This observation is further supported by recent evidence that the reprogramming induced by SCNT results in more similar epigenomic modification and transcriptomic expression patterns to those of ESCs than does the direct reprogramming by four previously defined factors (Kim et al., 2010). In other words, the clones derived from iPSCs, if available, are likely to be defective due to their lack of some natural components. This puts the necessity of animal clones for regenerative medicine highly questionable.

Figure 1

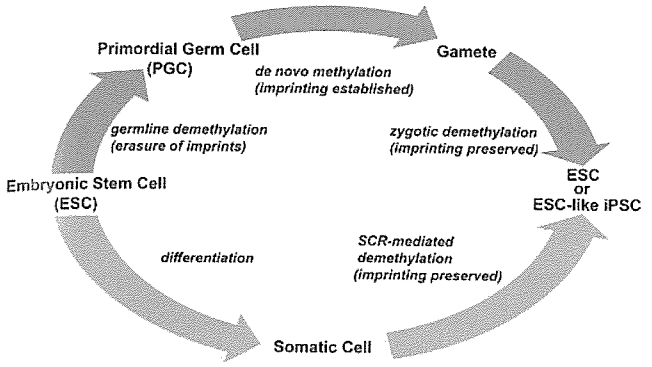

Figure 1. Comparison of natural germline-zygote and manually forced SCR demethylation pathways. Global demethylation occurs naturally in two developmental stages, one during formation and migration of PGCs into the embryonic gonads (germline demethylation) and a second time in 2-to-8-cell-stage zygotes before the morula stage (zygotic demethylation). Parental imprints are erased and re-established during germline demethylation but not in zygotic demethylation, resulting in different genomic imprinting patterns from the parents. On the other hand, SCR demethylation can be forced in somatic cells by manually introducing the expression of either miR-302 or four defined reprogramming factors (Oct4-Sox2-Klf4-c-Myc or Oct4-Sox2-Nanog-Lin28). Notably, Oct4 and Sox2 can also induce miR-302 expression. Due to the effects of miR-302-targeted epigenetic gene silencing, the somatic cell genome is forcedly demethylated without the erasure of parental imprints. As a result, expression of parental germline elements is still inactivated by the imprints. Given that parental elements are essential for normal zygotic development, their deficiency may cause developmental defects in SCR-induced iPSCs.

After the revelation of miR-302-mediated global demethylation, the next question is how this mechanism relates to the reprogramming by previously identified reprogramming factors (Oct4, Sox2, Naong and Lin28). Klf4 and c-Myc are not preferred in the process of iPSC generation due to their potential oncogenic activities. Global demethylation has been reported to promote Oct4-Nanog overexpression in mouse embryos and mouse-human fused heterokaryons (Bhutani et al., 2010; Popp et al., 2010). Lin's studies further demonstrated that elevated miR-302 expression to about 1.1~1.3 folds of the normal human ESC level (approximately 0.9 to 1.0 million copies per ESC) triggers both global demethylation and co-expression of Oct4, Sox2 and Nanog in human iPSCs (Lin et al., 2010 and 2011). The expression of Lin28 and many other ESC marker genes was observed 1~3 days later than the presence of Oct4-Sox2-Nanog elevation. A similar miR-302 transfection approach was also shown to increase Oct4 and Nanog expression by two folds in human ESCs (Rosa et al., 2009). Further research in these ESCs revealed that miR-302 directly silences nuclear receptor subfamily 2, group F, number 2 (NR2F2), a transcriptional repressor against Oct4 expression, to activate Oct4 transcription (Rosa and Brivanlou, 2011). These findings have clearly confirmed that global demethylation activates ESC-specific gene expression in particular, Oct4, Sox2 and Nanog. Through silencing of AOF1/2, MECP1/2 and DNMT1, we now understand that miR-302 induces global demethylation and subsequently leads to Oct4-Sox2-Nanog activation essential for the initiation of SCR to form iPSCs. Also, Oct4 and Sox2 have been found to be the transcriptional activators for miR-302 expression (Marson et al., 2008; Card et al., 2008). As shown in Figure 2, the mutual stimulation between miR-302 and Oct4-Sox2-Nanog forms a positive feedback regulation loop to maintain the stem cell status of reprogrammed iPSCs. This finding explains why miR-302, Oct4, Sox2 and Nanog are all essential markers for human ESCs.

How can we show the interaction between miR-302 and global demethylation? As global DNA demethylation occurs in the cell nucleus while miR-302 functions in the cytoplasm, SCNT is the best method to test this epigenetic reprogramming mechanism. SCNT is a well established technology to generate ESC-like pluripotent stem cells by transferring a somatic cell nucleus into the oocyte cytoplasm (Wilmut et al., 1997; Wakayama et al., 1998). When following the same strategy to transfer somatic cell nuclei into the cytoplasm of miR302-induced iPSCs, most (93%) of the hybrid cells were successfully reprogrammed to iPSC-like pluripotent cells possessing ESC-like properties (Lin et al., 2011). Global demethylation was quickly detected a few days after SCNT. Conversely, transferring iPSC nuclei into the somatic cell cytoplasm failed to form any viable cell. Hence, the earliest reprogramming effector, the"initiator", lies in the cytoplasm rather than nucleus of an iPSC. This finding is coincident with the previous SCNT results using the oocyte cytoplasm, which contains several miR-302 homologues that may have the same reprogramming function, such as miR-200c and miR-371~373. Given that miR-302 is a cytoplasmic effector whereas Oct4, Sox2 and Nanog are all nuclear transcription factors, it is clear that miR-302 is responsible for initiating SCR through global demethylation in the SCNT-induced iPSCs. On the other hand, the transfer of iPSC nuclei fails to induce pluripotency, suggesting that somatic cytoplasm may lack a key reprogramming effector required for iPSC generation. Alternatively, somatic cytoplasm may contain an inhibitor directed against iPSC formation; however, this possibility has been ruled out by further induction of miR-302 elevation in the hybrid cells containing the miR302-induced iPSC nuclei, showing that the efficiency of iPSC generation is increased proportional to the induced miR-302 elevation (Lin et al., 2011).

Regenerative medicine holds the key to future therapies for curing genetic disorders, physical abnormalities, injures, cancers/tumors, and aging. Supply and safety of the stem cells are two of the most important bottlenecks in regenerative medicine. Prior efforts have succeeded in isolating various pluripotent/

multipotent stem cells from embryos, umbilical cords/amniotic fluids and reproductive organs; yet, none of these stem cells can be made in a large quantity for patients. Without the knowledge of stem cell generation mechanism, no one can provide sufficient stem cells for regenerative medicine. Following the recent revelation of miR302-mediated SCR mechanism (Figure 2), an induced reprogramming process mimicking the natural zygotic development event, we have now insight into the regulatory mechanism necessary for stem cell generation and a method of reprogramming somatic cells to ESC-like iPSCs. Most importantly, we learn that this miR302-mediated SCR mechanism is able to not only generate unlimited iPSCs but also prevent tumor formation from these iPSCs (Lin et al., 2010). During SCR, miR-302 functions to silence both cyclin E-CDK2 and cyclin D-CDK4/6 cell cycle pathways during G1-S phase transition, consequently preventing iPSC tumorigenicity. Furthermore, miR-302 also silences polycomb ring finger oncogene BMI1, a cancer stem cell marker, to promote the expression of two tumor suppressor genes, p16Ink4a and p14/p19Arf. The combination of these two tumor suppression effects results in a tightly regulated cell cycle rate similar to that of 2-to-8-cell-stage embryonic cells in early mammalian zygotes (20~24 hours/cycle). Given that human ESCs express abundant miR-302, this newly identified tumor suppressionfunction of miR-302 may explain how early embryonic development prevents tumor formation. Thanks to this advancement, we now have the ability to generate tumor-free iPSCs for developing safer regenerative medicine.

Figure 2

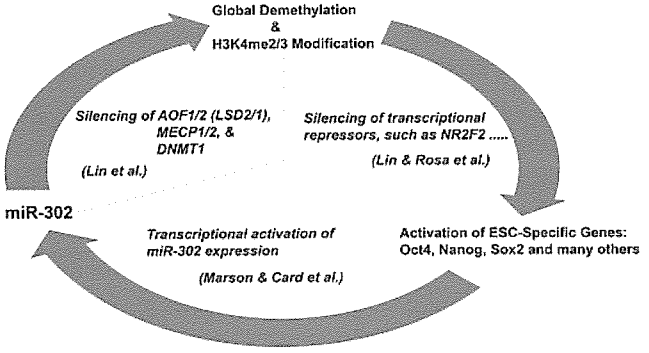

Figure 2. Currently established SCR mechanism for iPSC generation. MiR-302 silences AOF1/2 and DNMT1 activities and, in conjunction with the co-suppression of MECP1/2 and HDAC2, results in global DNA demethylation and chromosomal H3K4me2/3 modification. Subsequently, these epigenetic reprogramming events induce ESC-specific gene expression, in particular Oct4, Sox2 and Nanog, which in turn further stimulate miR-302 expression to form a positive feedback loop cycle crucial for the maintenance of SCR. Based on this mechanistic model, there are two methods for inducing iPSC formation: one is to force the miR-302 expression and the other is to introduce the co-expression of four defined factors (either Oct4-Sox2-Klf4-c-Myc or Oct4-Sox2-Nanog-Lin28). Both methods can trigger the activation of this cycling SCR mechanism; however, miR-302 directly induces global demethylation to initiate SCR while the four factors indirectly function through miR-302 expression.

Researchers have spent over five years—from the first discovery of iPSC generation to the disclosure of its mechanism—to understand the process of ESC-like pluripotent stem cell generation and regeneration. Since previous studies were all performed in isolated cells under an in vitro condition, extending these in vitro findings for in vivo applications is the next challenge. Rejuvenation involves a systemic stem cell regeneration process throughout the whole body. This process requires a large stem cell supply and that must be maintained for a relatively long time. Hence, supply and safety of the used stem cells will significantly affect the final results of rejuvenation. Fortunately, from now on plentiful body cells can serve as an unlimited resource for iPSC generation using miR-302 while introduction of miR-302 elevation in these iPSCs also prevents tumor formation. These solutions rely on an inducible SCR mechanism similar to natural zygotic reprogramming.

For modern regenerative medicine, the dual function of miR-302 in both reprogramming and tumor suppression has provided us a convenient means to control the quantity and quality of iPSCs, opening up a new avenue toward the fountain of youth. Recent identification of the same reprogramming and tumor suppression function preserved in the cell lysates of these miR302-induced iPSCs further leads to a novel SCNT application for iPSC generation without using any transgenic or genomic materials. Based on this groundbreaking advance, the development of a feasible strategy and therapy for not only local regenerative medicine but also whole systemic rejuvenation is highly expected in the near future.

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