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Molecular Basis of Reprogramming: Modulation by microRNAs

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Induced pluripotent stem cells (iPSCs) have opened up a new avenue for customized regenerative medicine. iPSCs can be generated by forced expression of transcription factors, Oct4, Sox2, c-Myc and Klf4. Although reprogramming techniques are well documented, one of the major concerns has been the poor efficiency of reprogramming. The reprogramming efficiency can be enhanced using various chemical compounds and vector systems. However, low reprogramming efficiencies and use of viral based vector systems limit clinical application of iPSCs. microRNAs (miRNAs) are extensively studied due to their critical role in numerous biological activities like cell cycle regulation, growth control and apoptosis. Discovery of embryonic stem cell (ESC) specific unique miRNAs, encouraged researchers to study contribution of miRNAs towards embryonic stem cell development, differentiation and somatic cell reprogramming (SCR). Depletion of mouse embryonic fibroblast (MEF) enriched miRNAs like miR-29a, miR-21 and let-7, are necessary to enhance reprogramming. Furthermore, up regulation of miR-200, miR-106a/b miR-120, miR-93 miR-301, miR-17, miR-721, miR-29b is required for mesenchymal-to-epithelial transition (MET), a critical initial event during the generation of iPSCs from fibroblasts. The expression of embryonic stem cell specific miRNAs like miR-290/miR-302 cluster, miR-367/miR372 is crucial to maintain pluripotent status of iPSCs. In this review, we discuss contribution of miRNAs to generation of iPSCs, their defined role in maintenance of pluripotent state, transcriptional regulatory networks and epigenetic factors to modulate reprogramming.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) are a type of adult stem cells genetically reprogrammed to an embryonic stem cell (ESC) like state. Human ESCs established in 1998 (Thomson *et al*., 1998) are considered promising sources for cell transplantation. However, use of human ESCs has several ethical constraints that hinder its application in regenerative medicine. Moreover, the use of human ESCs for clinical application must overcome barriers such as immune rejection, tissue regeneration and teratoma formation. An alternative to overcome these hurdles is to reprogram a patient's own somatic cells to iPSCs (Takahashi and Yamanaka, 2006), a Nobel prize winning contribution pioneered by Yamanaka and co-workers. The authors demonstrated direct reprogramming of mouse (Takahashi and Yamanaka, 2006) and human

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fibroblasts (Takahashi *et al*., 2007) to a pluripotent state, generating induced pluripotent stem cells (iPSCs). The generation of iPSCs revolutionized regenerative medicine research by introducing a method to supply an adequate number of patient-specific pluripotent cells for therapeutic transplantation, thus obviating the need to use human embryos.

The generation of iPSCs by Yamanaka and colleagues was achieved by overexpressing important pluripotent transcription factors, initially in mouse (Takahashi and Yamanaka, 2006), followed by human fibroblasts using retroviral system. The factor comprised Sex-determining region Y HMG box 2 (Sox2), Krüppellike factor 4 (Klf4), Octamer binding transcription factor 4 (Oct4), and myc myelocytomatosis viral oncogene homolog (c-Myc), are referred as the 'Yamanaka factors' (OSKM) (Takahashi *et al*., 2007). Briefly, Oct-4 and Sox-2 are transcription factors, for maintaining the pluripotency of stem cells (Chen and Daley, 2008). c-Myc plays a major role in early reprogramming stages and enhances generation of partially reprogrammed cells (Koche *et al*., 2011; Schmidt and Plath, 2012). Direct interaction of Klf4 with pluripotent genes, Oct4 and Sox2, is critical for somatic reprogramming (Wei *et al*., 2009). Thomson and colleagues used another combination of reprogramming factors viz., Oct-4, Sox-2, Nanog (homeobox protein Nanog) and Lin-28 (mRNA binding protein expressed in embryonic stem cells) (Yu *et al*., 2007). Such genome integrating viral vectors produce mutagenic lesions that are potentially tumorigenic or influence differentiation potential. Therefore, several approaches have been developed to generate novel, non-integrating methods for iPSC generation. Recent studies have indicated that iPSCs can be obtained with virus-free, expression plasmid or PiggyBac transposons (Jia *et al*., 2010; Kaji *et al*., 2009; Malik and Rao, 2013; Narsinh *et al*., 2011; Woltjen *et al*., 2009). Gonzalez and colleagues (2010), generated iPSCs from mouse embryonic fibroblasts using polycistronic construct co-expressing Oct-4, Sox-2, Kfl4 and c-Myc. However, several rounds of transfection were necessary to maintain expression of transgene at the level required to generate iPSCs (Gonzalez *et al*., 2009). The reprogramming efficiency was significantly lower than using the viral vector systems. Subsequently, modified expression plasmid based technique used a polycistronic non-viral minicircle plasmid vector to genetically reprogram human adult adipose derived stem cells (Jia *et al.,* 2010). The integration free human iPSCs generated by this technique indicated reprogramming efficiency of approximately 0.005%, much lower than integrating viral based method. Further, introduced sequences employed in these approaches, could integrate into the genome as DNA constructs. The safety issue of iPSCs led to the use of protein based

methods for generation of pluripotent stem cells. Zhou *et al*. (2009), for the first time, reported generation of protein induced pluripotent stem cells (pi-PSCs) from mouse embryonic fibroblasts using recombinant cell penetrating reprogramming proteins. A protein transduction domain, poly-arginine fused to the c-terminus of Yamanaka factors (OSKM) in order to obtain recombinant proteins that can penetrate across the plasma membrane of somatic cells. The approach significantly improved reprogramming efficiency (Zhou *et al*., 2009). However, the procedure involved is technically challenging (Kim *et al*., 2009a; Wang *et al*., 2013). Embryonic stem cells possess a unique set of microRNAs (miRNAs) (Houbaviy *et al*., 2003; Suh *et al*., 2004), with a crucial role in embryonic development and absence of the miRNAs impede cell proliferation and differentiation (Kanellopoulou *et al*., 2005; Murchison *et al*., 2005). Thus, miRNA 302/367 cluster is highly expressed in ESCs and downregulated in cell differentiation, encouraging study of the role of miRNA 302/367 cluster in reprogramming (Lin *et al*., 2011; Miyoshi *et al*., 2011; Subramanyam *et al*., 2011; Zhang *et al*., 2013). Numerous miRNA-mediated iPSCs lines have been derived from mouse fibroblast, human skin and dermal fibroblasts using only miR302 cluster or combination of numerous ESCs specific miRNAs. A key feature in ensuring effective reprogramming is epigenetic remodeling. The crucial role of miRNAs in regulating SCR and various approaches using miRNAs for reprogramming are discussed.

Biogenesis of miRNAs

miRNAs belong to a class of endogenous, single stranded, small non-coding RNAs of 19–22 nucleotides, derived from a 70-nucleotide precursor (Bartel, 2004; Lakshmipathy and Hart, 2008). miRNAs regulate expression of target genes by at least two mechanisms – translational inhibition or by promoting degradation of mRNAs (Krol *et al*., 2010). miRNAs were initially discovered in *Caenorhabditis elegans* (Lee and Ambros, 2001) and subsequently studied in green algae, viruses, plants and mammalian cells (Griffiths-Jones *et al*., 2008; Odling-Smee *et al*., 2007; Pentimalli *et al*., 2007). miRNAs act as key regulators of processes including developmental timing, patterning, growth control, apoptosis and tumorigenesis (Choi *et al*., 2013; Farazi *et al*., 2011; Gangaraju and Lin, 2009; Ivey and Srivastava, 2010; Lima *et al*., 2011; Subramanyam and Blelloch, 2011; Zhao and Srivastava, 2007). miRNAs play a crucial role in maintenance of stem cell pluripotency (Jia *et al*., 2013; Heinrich and Dimmeler, 2012) and critically regulate stem cell fate decisions, including self-renewal and differentiation into specific lineages (Guo *et al*., 2011).

In mammals, the biogenesis of miRNAs and their mechanism of action have been well

characterized (Carthew and Sontheimer, 2009; Huntzinger and Izaurralde, 2011). The miRNA canonical processing pathway, utilizes a 70-nucleotide primary miRNA (pri-miRNA) transcript which gets processed into stem loop precursor miRNA (pre-miRNA) by Drosha-DGCR8 enzyme complex in the nucleus (Carthew and Sontheimer, 2009). In the noncanonical pathway, pre-miRNA is generated from small introns also called mirtrons by alternative splicing and a debranching enzyme that generates a short hairpin, for processing by Dicer. This pathway circumvents requirement of the Drosha-DGCR8 complex that is required in the canonical pathway. In both cases, the pre-miRNA hairpins are translocated in the cytoplasm by exportin 5 where they are processed to mature miRNAs. In the cytoplasm, the ribonuclease type III, Dicer cleaves selectively the terminal loop of precursor to generate approximately 19–22 nucleotide mature miRNA/miRNA* duplex (Kim, 2005). Subsequent to Dicer processing, one of the two strands of the duplex, derived from both canonical and noncanonical pathways, is incorporated into the miRNA-inducing silencing complexes (miRISCs), through its interaction with one of the member of the argonaute (Ago) family. Ago class 2 protein is the only mammalian protein capable of directly cleaving the complementary target of mRNAs. Hence, miRISC silences the expression of target genes predominantly through a posttranscriptional repression, and the silencing of specific targets is dependent on a base-pairing interaction between the incorporated miRNA and the target (Krol *et al.,* 2010). It is postulated that approximately 1–5% of genes in animals encode miRNA and miRNAs target approximately 10–30% protein coding genes (Krol *et al.,* 2010).

The recent high throughput next generation massively sequencing (NGMS)

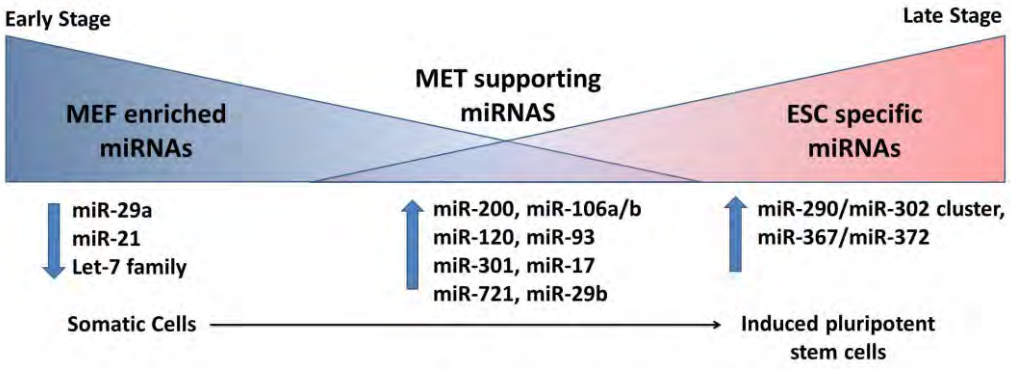

Figure 1: Expression of miRNAs during iPSCs generation. MEF enriched miRNAs downregulate at early stage. Simultaneously miRNAs that positively regulates MET and pluripotent state upregulate. Somatic cells in early stage and late stage of reprogramming show expression patterns of miRNAs that closely resemble somatic cells (fibroblasts) and embryonic stem cells, respectively. Abbreviations – MEFs: Mouse embryonic fibroblasts; MET: Mesenchymal-epithelial transition; ESCs: Embryonic stem cells; miRNA: microRNA.

technology has been used to identify numerous miRNAs. These technologies have modernized genomic research, allowing many mammalian miRNAs to be identified and deposited in miRBase (www.mirbase.org). Till date, 24,521 entries of hairpin precursor miRNAs, with 30,424 matured miRNA products in 206 species have been recognized and deposited in the public miRNA database miRBase (Release 20.0, June 2013). Amongst these, 2578 miRNAs are of human origin. A proper prediction and validation of miRNA targets is essential to understand function of miRNAs. Computational prediction identify that all genes are regulated by miRNAs and single miRNA can target several genes. The analysis of miRNA predicted targets is performed using different algorithms like, TargetScan, PicTar, miRanda. Furthermore, validation of predicted targets can be done by reporter assays for testing predicted functional miRNAs target sites.

miRNAs during initial stage of reprogramming

Early phase of reprogramming includes expression of miRNAs that inhibit apoptosis and enhance cell proliferation (Fig. 1). An elevated level of p53 in the initial stage of reprogramming reduces the overall iPSCs formation efficiency (Sarig *et al*., 2010; Tapia and Schoer, 2010). Moreover, one of the p53 target, cyclin-dependent kinase inhibitor p21, causes cell cycle arrest or favors apoptosis (Bodzak *et al*., 2008; Kawamura *et al*., 2009). An unrevealed crucial role of miR-138 in regulation of p53 pathway and promotion of iPSC generation was first reported by Dan and colleagues (Ye *et al*., 2012) (Fig. 2). Briefly, p53 is down regulated by miR-138 which in turn reduces expression of p21 and miR-34 during somatic cell reprogramming (Choi *et al*., 2011; Ye *et al*., 2012). miR-34 cluster (miR-34a,-34b,-34c) is a barrier to reprogramming as it reduces expression of pluripotent factors like Oct-4, Nanog (Choi *et al*., 2011; Ng *et al*. 2014). Ye *et al.* (2012) reported that, endogenous expression of Oct4 and Sox2 genes is relatively low and retroviral expression remains active in iPSCs generated by OSKM factors from p53-null cells. Moreover, ESC-like morphology cannot be maintained after passage five. Alternatively, in this study, the morphology of miRNA-mediated reprogrammed iPSCs (138-iPSCs), was similar to that of mouse ESCs and were maintained for more than 20 passages *in vitro*. Additionally it was reported that p53 binds to the miR-145 promoter and activates its expression (Sachdeva *et al*., 2009; Suh *et al*., 2011). miR-145 known to induce differentiation of ESCs by suppressing the expression of reprogramming factors, Oct4, Sox2 and Klf4. Hence, miR145-p53 axis is a roadblock to reprogramming (Liu *et al*., 2012; Xu *et al*., 2009) (Table 1 and Fig. 2).

miRNAs expressed in mouse embryonic fibroblasts (MEFs) interfere with

reprogramming efficiency (Melton *et al*., 2010). Depletion of MEFs enriched miR-29a and miR-21 result in an enhanced reprogramming efficiency mediated by regulation of ERK1/2 and p53 pathways (Yang *et al*., 2011; Yang and Rana, 2013). The depletion of miR-29a using inhibitors, decreased p53 protein levels by elevating p85α and CDC42 expression (Yang *et al.,* 2011), and depletion of miR-21, decreased ERK1/2 phosphorylation (Yang *et al.,* 2011). Further, c-Myc has shown to repress miR-29a and miR-21 to promote reprogramming (Yang *et al.,* 2011) (Table 1).

Let-7 family of miRNAs (Let-7a1, -a2, -a3, -b, -c, -d, -e, -f,1,-f2, -g, -i) are abundantly expressed in MEFs (Pasquinelli *et al*., 2000; Reinhart *et al*., 2000) (Fig. 1), leading to investigation of the role of let-7 family of miRNAs in reprogramming. The miRNAs are pluripotent silencing miRNAs as they inhibit expression of a number of pluripotent regulators, including Sall4, Lin-28b, Hmga2 and c-Myc, n-Myc (Kim *et al*., 2009b; Melton *et al*., 2010; Park *et al*., 2007; Rybak *et al*., 2008; Sampson *et al*., 2007). c-Myc inhibits expression of let-7 through Lin-28b transactivation and depletion of let-7 elevates reprogramming efficiency four fold with OSK reprogramming factors (Melton *et al.,* 2010). The let-7 family of miRNAs act as a barrier to reprogramming via expression of prodifferentiation genes including early growth response protein 1 (EGR1) (Worringer *et al*., 2013). The inhibition of let-7 with the OSK cocktail increases the reprogramming efficiency of human dermal fibroblasts (HDF) comparable to that with OSKM. Further let-7 inhibition augments OSK mediated reprogramming, at least in part through promoting LIN-41 expression. EGR1 mRNA is bound and negatively regulated by LIN-41 and blocks reprogramming. Together these findings delineate the role of a let-7-based pathway that counteracts the activity of reprogramming factors through promoting the expression of prodifferentiation genes (Chang *et al*., 2009; Worringer *et al.,* 2013) (Table 1).

c-Myc, one of the four reprogramming factors, induces expression of a number of miRNAs that favor initiation of the early transitional stage (Yang *et al.,* 2011; 2013). c-Myc induces repression of MEF enriched miRNA, miR-21 and miR-29a enhancing the early phase of reprogramming events (Yang *et al.,*, 2013). Furthermore, c-Myc alone can augment expression of miR-17`92 cluster, miR-106b`25 cluster and miR106a`363 cluster expressions (Li *et al*., 2011; Mendell, 2008). Recently, He and colleagues (2014), reported that miR 19a/b of cluster miR17`92 were significantly induced by c-Myc during initial stage, suggesting a crucial role of the miRNAs during somatic cell reprogramming. The enhancement of reprogramming by miR-19a/19b was mediated by repressing expression of tumor suppressor protein, phosphatase and tensin homolog (PTEN),

causing cell cycle arrest (Weng *et al*., 2001). These results suggest that cMyc-miR-19a/b-PTEN axis plays a crucial role in reprogramming human somatic cells. The approach circumvents the use of c-Myc, hence miR17`92 cluster can be used to reprogram somatic cells into iPSCs for clinical purpose (He *et al*., 2014) (Table 1).

miRNAs promote mesenchymal-to-epithelial transition

An early event during iPSC generation is mesenchymal-to-epithelial transition (MET). Factors that promote MET or inhibit epithelial-to-mesenchymal transition (EMT) help in reprogramming. A prominent observation in early days of reprogramming is the transformation into cluster of cells resembling epitheloid morphology. Inhibition of EMT occurs by suppression of transforming growth factor β (TGF-β) pathway (Li *et al*., 2010; Miyazono, 2009). miR-106a, miR-106b, miR-93 and miR-17 accelerate reprogramming by targeting TGF βII (Li *et al.,* 2011) (Fig. 1). Another family of miRNAs, miR-130, miR-301 and miR-721 enhanced mouse fibroblast reprogramming by reducing expression of homeobox transcription factor, Meox2 (or Gax) (Pfaff *et al*., 2011). Meox2 is associated TGFβ pathway (Valcourt *et al*., 2007) (Fig. 2). miR-200 downregulates expression of MET barriers including ZEB1 and ZEB2 (Burk *et al*., 2008; Gregory *et al*., 2008; Korpal *et al*., 2008) (Fig. 2). ZEB1 and ZEB2 (mesenchymal markers) are transcriptional repressors of E-Cadherin and master regulators of epithelial polarity (Bracken *et al*., 2008). Although it is now known that fibroblasts can be reprogrammed to an ES like state, the underlying mechanism is not clear. He *et al.* (2014) reported Oct-4 and Sox-2 positively regulate expression of miR-200, which in turn down-regulates mesenchymal marker ZEB2 through directly targeting the 3'UTR. ZEB2 is member of the ZFHX1 family of two-handed zinc finger/homeodomain proteins, initially shown as a binding partner of SMAD1 and SMAD2/3. Thus, miR-200s regulate expression of Sox-2/Oct-4 during iPSCs generation, and miR-200s/ZEB2 axis play crucial roles in Sox-2/Oct-4-initiated MET process during reprogramming (He *et al*., 2014). During iPSCs generation, a change in DNA methylation pattern is essential for epigenetic remodeling and the re-establishment of the ESCs-specific gene expression profile (Mikkelsen *et al*., 2008). In addition, DNA methylation leads to reactivation of epithelial specific markers in the MET process. The reactivation of imprinted regions, like *Dlk1-Dio3* locus, is essential for development of fully pluripotent iPSCs (Liu *et al*., 2010). DNA hyper-methylation leading to silencing of *Dlk1-Dio3* locus prevents cells from becoming fully pluripotent iPSCs (Liu *et al.,* 2010; Li *et al*., 2010). Hence, DNA methyl transferases

Table1: miRNAs: Regulators of induced pluripotency

microRNAs	Targets	Function in reprogramming	Reference
miR-34 cluster-miR-34a, miR34b, miR-34c	Oct-4, Nanog	Barriers	Choi *et al* 2011; He *et al*., 2007, Ye *et al.,* 2012
miR-145	P53/ Oct-4, Sox-2,Klf-4	Barrier	Liu *et al*. 2012; Xu *et al*., 2009
miR-29a	P53 pathway	Barrier	Yang *et al*., 2011
miR-21	ERK1/2 phosphorylation	Barrier	Yang *et al*., 2011
Let-7 family	Sall4, Lin-28b, Hmga2, c-Myc, N-Myc	Barrier	Kim *et al*., 2009b; Melton *et al.*, 2010; Park *et al.*, 2007 Rybak *et al*., 2008; Sampson *et al*., 2007
miR19a/miR19b	PTEN	Promoters	He *et al*., 2014
miR106a, miR106b, miR-93, miR-17	TGFβII	Promoters	Li *et al.*, 2011
miR-130,miR301, miR-721	Meox1	Promoters	Pfaff *et al.*, 2011
miR-200	ZEB1 and ZEB2	Promoter	He *et al*., 2014; Burk *et al*., 2008; Gregory *et al*., 2008; Korpal *et al.*, 2008
miR-29b	Dnmt3a, Dnmt3b	Promoter	Guo*et al*., 2013
miR 302, miR372, miR-367	TGFβ, MECP2, MBD2,SMARCC2, NR2F2	Promoters	Hu *et al*., 2013; Subramanyam*et al*., 2011
Mouse: miR-291a-3p, 291b3p, 294, 295, 302a-d	cdkn1a,Rb1, Rb2	Promoters	Judson *et al*., 2009
miR-138	P53 Pathway	Promoters	Ye *et al*., 2012

(DNMTs) act as barriers to early stage reprogramming. However, its expression is up-regulated during later stages of iPSCs generation (Pawlak and Jaenisch, 2011). Guo *et al*. (2012) demonstrated that Sox2 directly regulates miR-29b expression during reprogramming and expression of miR-29b is essential for OSKM- and OSK-mediated reprogramming. miR-29b targets Dnmt3a and Dnmt3b, thus enhancing expression of MET promoting factors, Cldn3, E-Cadherin and EPCAM, while suppressing expression of mesenchymal specific genes like Cdh2, Snail and Zeb1 during reprogramming events (Guo *et al*., 2012). Expression of miR-29b in OSKM-mediated iPSCs generation with low transcriptional activity of the Dlk1-Dio3 locus reactivates expression of miRNAs and genes in the imprinted region (Stadtfeld *et al*., 2012) (Fig. 2).

miR-302b (orthologous to mouse miR-302s) and miR-372, miR-373 (orthologous to mouse miR-291, miR-294, miR-295) enhances human somatic cell reprogramming by increasing the kinetics of MET, by suppressing TGF-β induced EMT and by targeting epigenetic modifiers (MECP2, MBD2, SMARCC2) (Subramanyam *et al*., 2011). In addition, miR-302 expression in reprogramming leads to DNA hypomethylation and DNMT1 deficiency (Lin *et al*., 2011). In conclusion, these findings

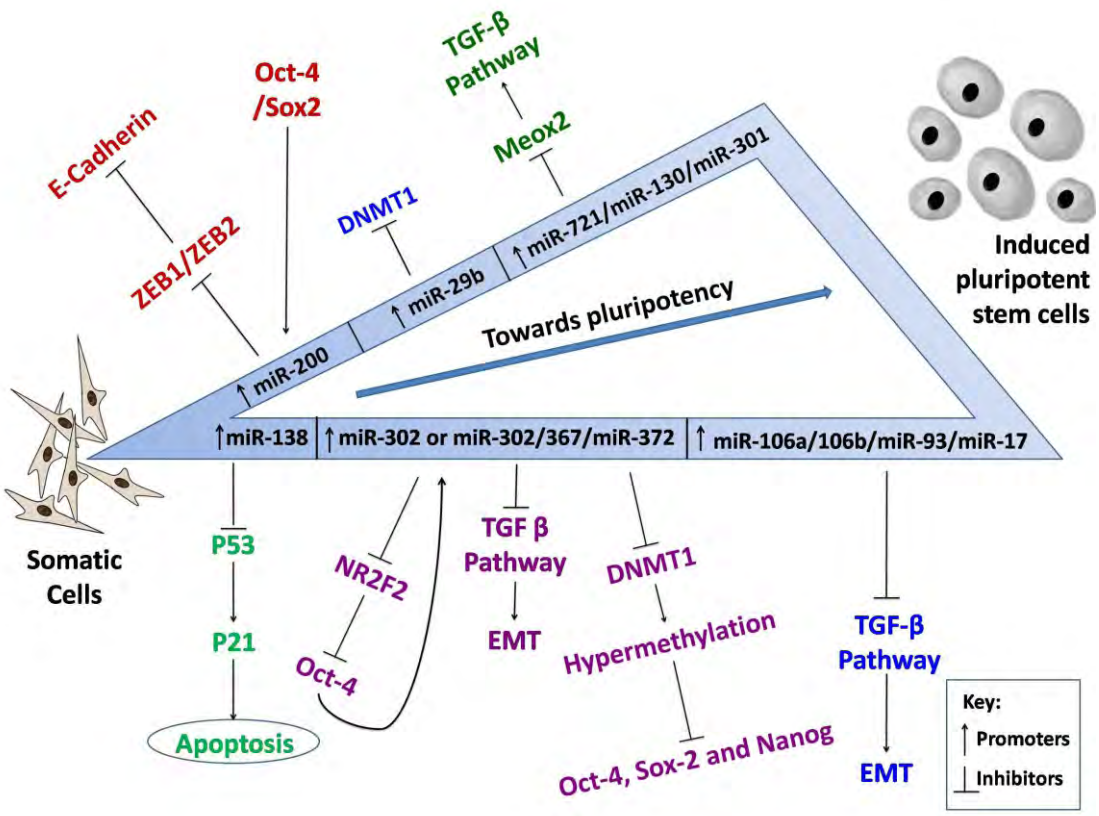

Figure 2: miRNAs that modulate reprogramming along with their downstream effectors. miR-138 promotes reprogramming by suppressing inhibiory effects of P53 pathways. P53 induces apoptosis by promoting expression of cyclin-dependent kinase P21. miR-302 cluster or miR-302/367/372 has multiple targets. miR-302 suppresses expression of Oct-4 inhibitor, NR2F2, cluster inhibits TGF β pathway thus blocking EMT transition and augments expression of pluripotent genes, Oct-4, Sox-2 and Nanog by inhibiting DNMT1 thus suppressing hypermethylation. miR-106a, miR-106b miR93 and miR17 accelerate reprogramming by targeting MET inhibitor TGF βII. Pluripotent genes, Oct-4/Sox-2 positively regulate expression of miR-200 which in turn down regulates transcriptional repressor of E-Cadherin, ZEB1/ZEB2 expression. miR-29b inhibits DNMT1. miR-130, miR-301 and miR-721 cluster of miRNAs enhance mouse fibroblast reprogramming by reducing expression of homeobox transcription factor, Meox-2. Abbreviations – miRNAs: microRNAs; NR2F2: Nuclear receptor subfamily 2, group F, member 2; TGFβ: Transforming growth factor beta; DNMT: DNA methyltransferase; MET: Mesenchymal-to-epithelial transition; EMT: Epithelial-to-mesenchymal transition; ZEB: Zinc finger E-box binding homeobox.

show that miRNAs are necessary for gene expression and epigenetic remodeling during OSKM-mediated somatic cell reprogramming. Thus, it is observed that miRNAs are differentially expressed and possess critical functions during reprogramming of somatic cells.

ESCC regulating miRNAs for reprogramming

The embryonic stem cell-specific cell cycle-regulating (ESCC) family of miRNAs promote reprogramming of somatic cells to iPSCs (Judson *et al*., 2009; Subramanyam *et al*., 2011). The role of ESC-specific miRNAs in iPSCs generation was first demonstrated by

Judson and Blelloch (2009). MEFs were reprogrammed by viral transfection vector system using transcription factors Oct4, Sox2 and Klf4 (OSK) and miRNA-290 cluster mimics including miR-291 – 3p, miR-292-3p, miR-293, miR-294, and miR-295. The reprogramming efficiency enhanced with miR-291-3p, miR-294, and miR-295, whereas miR-292 and miR-293 were not effective. Optimum results were obtained by overexpressing miR-294, with increasing efficiency from 0.01–0.05% to 0.1–0.3%. These reports show that miR-294 can substitute for c-Myc in order to enhance reprogramming in presence of other transcription factors (OSK) (Judson *et al*., 2009). Importantly, iPSCs generated without c-Myc will be safer for future use in clinical research. miR-290 (mouse), miR-372 and miR-302 cluster, ESCC specific miRNAs directly target inhibitors of cyclin-Cdk2 pathway, thereby ensuring fast G1-S transition. The miRNAs has reported to augment reprogramming of human fibroblasts (Guo *et al*., 2014; Subramanyam *et al*., 2011) (Fig. 2 and Table 1). Bone morphogenic protein (BMP) is necessary for efficient reprogramming along with OSKM, promoting MET by inducing expression of miR-200 and miR-205 (Tehrani *et al.,* 2010). The various targets of miR302/367 were revealed using photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation method (PAR-CLIP). miR-302/367 promotes BMP signaling by targeting BMP inhibitors TOB2, DAZAP2 and SLAINI (Lipchina *et al.,* 2011)

miRNAs modulate late stages of reprogramming

Activation of pluripotent markers occurs in late stages of reprogramming. miR-302/367 regulates expression of pluripotent markers, Oct-4, Sox-2 and Nanog (Hu *et al*., 2013; Marson *et al*., 2008; Rosa and Brivanlou, 2013). Human adipose tissue derived stem cells (hASCs) were reprogrammed into iPSCs using Yamanaka factors (Klf4, c-Myc, Oct4, and Sox2) with miR-302 (combination is referred to as 'KMOS3'). Thus, miR-302 blocks expression of Oct-4 inhibitor, NR2F2 and promotes pluripotency by regulating Oct-4 through indirect mechanism. The positive feedback loop represents a novel mechanism for inducing pluripotency status in somatic cells (Hu *et al*., 2013). Study in hESCs, showed that expression of NR2F2 increases with differentiation and simultaneously down regulation of Oct-4 and miR-302/367 expression was observed (Marson *et al*., 2008). The transcription factors, Oct-4, Nanog and Sox-2 enhances expression of miR-302/367 by binding to its promoter region (Anokye-Danso *et al*., 2012; Marson *et al*., 2008). miR-302/367 cluster indirectly modulates expression of multiple pluripotent factors by targeting several epigenetic modifiers leading to global genomic

hypomethylation. Often, genomic hypomethylation occurs at the promoter region of ESCs specific important transcription factors (Lin *et al.,* 2011). During somatic reprogramming, miR-302 supresses expression of DNA methyltransferases 1 (DNMT1) which inhibits expression of AOF2 (lysine specific histone DNA methylases). This leads to genomic hypomethylation and subsequently reactivation of essential pluripotent factors (Lin *et al*., 2011; Reik *et al*., 2001) (Figs. 1 and 2). Tay *et al.* (2008) demonstrated that miR-134, miR-296 and miR-470 act as barriers to reprogrammimg by inhibiting pluripotent transcription factors.

miRNAs alone for reprogramming

The strategies currently employed for iPSC generation involves, ectopic expression of Yamanaka factors (OSKM) (Takahashi and Yamanaka 2006; Takahashi *et al*., 2007). Although numerous alternate approaches have been documented to augment iPSC generation, including use of signaling molecules, additional transcription factors and pharmacological molecules (Jia *et al*., 2010), the different approaches require at least one pluripotent stem cell transcription factor. Lin *et al.* (2011) have reported use of miRNA-302 cluster for successful reprogramming without need of any transcription factor. There are four major advantages in miRNA-based reprogramming, compared to conventional methodology used for reprogramming. Firstly, the transfection of miRNA cluster expressing transgene is safe, easy and efficient for generating iPSCs, thus bypassing the tedious adeno- or retro-viral insertion of huge transcription factors (OSKM) into single somatic cell. Secondly, since the size of miRNA transgene is approximately 1kb the efficiency of transfection will be increased. Thirdly, generation of iPSCs by using miRNA-based approach circumvents use of proto-oncogenes. Several investigators have employed exclusively miR-302a/b/c/d or in combination with miR-302a/b/c/d and miR-369 or miR-302a/b/c/d and miR200c and miR-369 for reprogramming without oncogenes, c-Myc or Klf-4 (Anokye-Danso *et al*., 2011; Lin *et al*., 2011; Miyoshi *et al*., 2011). Finally, the transfection of miRNA cluster transgene is done by electroporation instead of retroviral vector system (Lin *et al*., 2011). Hence, somatic cells can be successfully reprogrammed without use of pluripotent transcription factors in the miRNA-based approach (Anokye-Danso *et al*., 2011; Lin *et al*., 2011). miRNA-based reprogramming approach has circumvented most of the problems encountered in SCR using conventional method. The future challenge will be to apply this technique to generate patient-specific iPSCs in a large scale with better quality and safety for transplantation therapy.

miRNAs in regulation of lineage differentiation

A self renewal process is normally inhibited during differentiation due to down regulation of pluripotent genes, Oct-4, Sox-2 and Nanog, hence resulting in a decrease of miR-290 and lin-28 cluster expression. A down regulation of lin-28 leads to maturation of let-7, resulting in the suppression of self-renewal promoting genes, hence facilitating differentiation. miR-290 family indirectly represses let-7 in order to maintain pluripotent state (Guo *et al*., 2014). Depletion of miR-290 family results in differentiation of pluripotent stem cells. Bernardini *et al*. (2014) reported crucial role of miR-21 during endodermal differentiation of iPSCs. PTEN/Akt pathway is a direct target of miR-21 and augments TGF-β2 expression, thus promoting endodermal differentiation of iPSCs. Okamoto *et al.* (2012) demonstrated that miR-181a, miR-24a, miR-9-3p, miR-19b, miR-10b, miR-10a are important regulatory factors in osteoblastic differentiation of mouse iPSCs. Specifically, miR-124a and miR-181a directly targets the transcription factors Dlx5 and Msx2. Hence, down regulation of these miRNAs are necessary to enhance expression of osteoblastic differentiation markers such as Rux2, Msx2 and osteopontin.

SUMMARY

Somatic cell reprogramming is a ground breaking discovery in the field of stem cells and regenerative medicine. The iPSC technology has opened avenues for personalized medicine, since patient-specific somatic cells can be employed. However, to realize the therapeutic potential of iPSCs, comprehension of the molecular mechanisms involved in pluripotency and cell fate decisions are critical. Despite numerous advancements in iPSC research, the search for a methodology that is safe, effective with high efficiency, for engineering somatic cells into a versatile embryonic-like state is ongoing. miRNA-based reprogramming methods seem promising for generation of iPSCs/progenitor cells using defined approaches, and will be more efficient than the conventional (Oct 4/Sox2/Klf4/c-Myc mediated) methods. miRNAs are differentially expressed in an organized manner during the entire reprogramming process. A key cellular process, MET, is an early stage event that occurs immediately after forced expression of core transcription factors required for reprogramming in fibroblasts. Simultaneously, inhibition of EMT is a prerequisite for efficient reprogramming. A distinct set of miRNAs modulate the EMT/MET transitions, a critical step towards an altered pluripotent state. Finally, another distinct set of ESC specific miRNAs is observed, ensuring that the pluripotency regulatory network is maintained. miRNA-based reprogramming methods are relatively new and a number of challenges have to be addressed including, the ideal cluster of

miRNAs for reprogramming, the different types of clusters that can be used for reprogramming at a single time point, a distinct signature of miRNAs that could serve as a fingerprint for a particular stage of reprogramming. We have progressed immensely in understanding of several molecules for reprogramming with several unknowns to be unraveled.

CONFLICT OF INTEREST

The authors claim no conflict of interest.

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