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Review

MicroRNAs in pluripotency, reprogramming and cell fate induction

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ABSTRACT

Pluripotent stem cells display a unique expression pattern of microRNAs (miRNAs). These ~22 nucleotide non-coding RNAs have established a crucial role in controlling gene expression of pluripotent stem cells at the post-transcriptional level. Recent studies made important advances in identifying miRNA regulated processes like de novo DNA methylation, progression of the cell cycle and regulation of cell fate decision. miRNAs have also the ability to reprogram somatic cells to pluripotent stem cells and on the other hand, to induce differentiation of pluripotent stem cells into distinct somatic lineages. Previously it was published that miRNAs can direct reprogramming on its own. Here we provide evidence and critically discuss that the effect of miRNA depends on co-expression of the classical reprogramming factors. During transition between these different cell fates distinct miRNAs adjust the levels of specific transcriptional programs and confer robustness to differentiation processes. This results in a complex network between miRNAs and their targets. The fact that miRNAs itself can also be regulated by its targets establishes complex regulatory loops. Based on bioinformatical predictions, each miRNA theoretically has hundreds of target genes making it even more challenging to understand the complete network between miRNAs and their targets.

1. Introduction

Pluripotent cells are capable to differentiate into all cell types within the body including the germ cells. These characteristics are displayed by cells of the inner cell mass (ICM) from early embryos enabling them to form the complete adult organism. The pluripotent state of cells from the ICM can be captured in vitro by placing blastocysts in culture, leading to the generation of pluripotent embryonic stem cells (ESCs) [1].

The derivation of complete animals by transplantation of somatic nuclei into eggs was the first demonstration that cell fates are neither restricted nor irreversible [2]. Subsequent work demonstrated that the determined fate of differentiated cells can be reversed by nuclear transfer, cell fusion or iPS (induced pluripotent stem cell) technology [2–6]. These approaches offer the opportunity to generate pluripotent embryonic-like cells from adult, somatic cells of the same individual, opening the field of personalized regenerative medicine. Furthermore, the developments in this field have been honored by the Nobel Prize for Physiology or Medicine in 2012. The Nobel Prize was awarded to Sir John Gurdon, who cloned a frog by nuclear transfer in 1962 [7] and to Shinya Yamanaka, who invented iPS technology [6].

In contrast to nuclear transfer and cell fusion, induced pluripotent stem cells (iPSCs) are generated by overexpression of the transcription factors Oct4, Sox2, Klf4 and c-Myc and do not rely on the usage of unfertilized eggs making them ethically less controversial. On the other hand, iPSC generation is less efficient, appears to be slower and it still remains questionable if iPSCs are in full terms equivalent to ESCs or pluripotent cells derived by nuclear transfer [8,9]. Several studies indicate that subtle differences in gene expression and chromatin modifications lead to partially reprogrammed iPSCs, which retain an epigenetic memory [10–12]. This manifests in the tendency of tissue-specific iPSCs to more efficiently differentiate to somatic cells of their tissue of origin. Reduced pluripotency has also been associated with loss of imprinting at the *Dlk1–Dio3* locus [13]. iPSC lines that lost imprinting at this specific locus poorly contributed to chimeric mice and were not able to generate "all-iPSC mice" in tetraploid complementation assays [13]. This study also demonstrates the power of tetraploid complementation assays to investigate full developmental potential of iPSCs. Subsequent investigations suggest that imprinting at the *Dlk1–Dio3* locus is not an absolute marker for pluripotency. Expression and stoichiometry of reprogramming factors as well as other parameters rather seem to influence the grade of pluripotency [14]. So far it remains unclear whether such subtle biological differences between ES cells and fully reprogrammed iPSCs have any functional consequences, which might challenge the use of iPSCs in disease research [11,15]. Nevertheless, some promising reports carried out with ESCs demonstrated that pluripotent cells are capable to differentiate into specific cell types in vitro which are capable to integrate and

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participate in tissue repair when transplanted into animal disease models [16,17].

The regulation of pluripotency is controlled by a complex regulatory network including several transcription factors and chromatin modifying enzymes. The core transcriptional network consists of Oct4, Nanog and Sox2. All three transcription factors are highly expressed in the ICM, epiblast and undifferentiated ESCs. Disruption of each of these genes in mice results in early embryonic lethality due to loss of pluripotent cells within the ICM of preimplantation embryos [18,19]. While, c-Myc and Klf4 are used in some reprogramming approaches starting with fibroblasts those seem not to be part of the core transcriptional network in ESCs. In fact even Oct4 alone is able to reprogram neuroectodermal cells from mice and men. The transcriptional regulation of pluripotency and the unique chromatin status of pluripotent cells as well as the different states of pluripotency are extensively reviewed elsewhere [20–24].

Another important role in the regulation of pluripotency and lineage specification has recently emerged for non-coding microRNAs that are associated with the main regulatory circuitry by modulating gene expression at the post-transcriptional level (Fig. 1) [25]. This review focuses on the emerging role of miRNAs in cell fate regulation of pluripotent stem cells. Within the next chapters miRNA mediated regulation of pluripotency and reprogramming is introduced and miRNA mediated cell fate specification will be discussed.

2. Biogenesis of miRNAs in mice and men

MicroRNAs (miRNAs) were initially discovered in *Caenorhabditis elegans* during a genetic screen to uncover molecules involved in the regulation of nematode development [26]. Since then, a fundamental role has been established for these small ~22 nucleotide non-coding RNAs in controlling gene expression at the post-transcriptional level. Hundreds of miRNAs have been discovered in most eukaryotic species [27], located in intergenic regions as well as in exons or introns of other genes.

Most miRNAs are transcribed by Polymerase II as long primary, capped and polyadenylated transcripts (pri-miRNAs) [28], which are processed into their mature form in a complex multistep process.

Briefly, within the nucleus, the pri-miRNA is converted by a microprocessor-complex to the precursor miRNA (pre-miRNA). The microprocessor-complex consists of the RNase type III endonuclease Drosha, which is associated to the Di George syndrome critical region gene 8 (DGCR8) and additional co-factors. This complex recognizes the hairpin secondary structures embedded in the pri-miRNA [29–32]. The resulting ~70 nucleotide hairpin pre-miRNA is excised from the pri-miRNA and recognized by the Exportin-5/Ran-GTP complex, actively exporting the pre-miRNA out of the nucleus [33–35]. A small subclass of miRNAs, located in small introns, the so called mirtrons, can bypass the Drosha-mediated processing, which is crucial for the vast majority of the other miRNAs [36]. After entering the cytoplasm, the pre-miRNA is further processed by another RNase-type III enzyme, the so called Dicer, to its mature ~22 nucleotide miRNA–miRNA* duplex. Dicer contains a PAZ-domain (Pie/Argonaut/Zwille) recognizing the 3′-overhangs of the pre-miRNAs generated by the microprocessor [37,38]. The dicer-enzyme cuts the pre-miRNAs within the stem loop and creates thereby the ~22–24 nucleotide miRNA-duplex, which remains bound to the dicer.

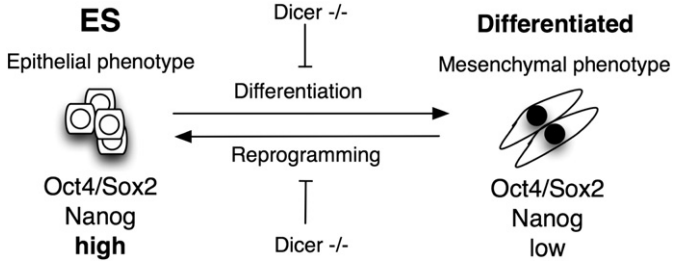

Fig. 1. Differentiation of embryonic stem cells (ESCs). Embryonic stem cells are characterized by a transcriptional network involving the transcription factors Oct4, Sox2 and Nanog, which are downregulated in many cell types during differentiation. The inverse process (reprogramming) can be initiated by forced expression of some of these factors in combination with others such as c-Myc and Klf4. Surprisingly, both processes seem to rely on miRNAs, since knockout of Dicer inhibits both processes.

In most cases only one strand of this duplex, the designated "major" strand, gets incorporated into the multi-protein miRNA ribonucleoprotein complex (miRNP or miRISC complex). The "minor" strand, referred to as miRNA*, gets degraded after exclusion from the miRISC complex. Some large scale studies provided strong evidence, that the miRNA* strand also has a biological function, since a larger number of miRNA* sequences could be detected among the total miRNA population than expected.

The miRNA loaded miRISC complex is used to guide it to its target sites. Previous studies focused on the target sites within the 3′UTR (untranslated region) whereby miRNA targeting within the 5′UTR and cds (coding sequence) has been overlooked. Thus, recent sequencing data using anti-Ago immunoprecipitation and cross-linking have reported target sites e.g. in cultivated HEK 293 cells from 5′UTR to cds and 3′UTR, targeting 4647 transcripts (21% of 22,466 unique HEK293 cell transcripts) [39]. Ago1–4 seemed to bind to the same target sequences: 84% of the miRNA target sites were located in exons, whereas 4% bound to 5′UTR, 50% to cds and 46% to 3′UTR. According to data by Hafner and coworkers miRNA sites in cds seem to result in small significant mRNA destabilization in HEK293 cells.

Target interaction of miRNA and mRNA seems to involve a seed-pairing interaction with a match of approximately 7 nt near the 5′end of the 22nt miRNA (e.g. positions 2–7) with sites in the target region of mRNA [40]. Targeting-sites that cannot be explained by the canonical seed-match model seem to involve non-Watson–Crick base pairing with G-bulges at positions 5–6 and suggest an alternative mode of microRNA target recognition, making target gene identification even more challenging [41].

Initial studies suggested that animal miRNAs exclusively mediate target gene silencing by translational repression. Recently, there is growing evidence that animal miRNAs can induce mRNA degradation as well as translational repression [42]. Translational repression has been proposed to occur at four different stages: initiation of translation, elongation of translation, protein degradation during translation and premature termination of translation. Several studies addressing this question on a genome-wide scale by mass spectrometry and transcriptome analysis suggest that miRNA mediated mRNA degradation alone accounts for most silencing of miRNA targets [43–46]. Nevertheless, the exact molecular mechanisms, which decide at what stage target gene silencing occurs still remain elusive [42].

3. The overall function of miRNAs in embryonic stem cells

For ESCs distinct miRNA signatures could be identified, representing a subset of miRNAs specifically expressed in mESCs and hESCs, respectively [47–50]. Among miRNA-clusters that are highly enriched in mESCs are miR-290, miR-17/19, miR-106b/25, miR-106a/363, miR-302b/367, miR-15b/16 and miR-32. Some of these miRNA families already have been analyzed and specific functions have been revealed [51,52].

Several studies addressing the overall function of miRNAs in mESCs used cell lines deficient for crucial miRNA processing factors like Dicer or DGCR8 [53–56].

In contrast to DGCR8, which only is involved in processing of miRNAs [57], Dicer is involved in both miRNA and siRNA pathways. The phenotype of Dicer deficient cells can therefore be caused by either the absence of siRNA and/or miRNA. Interestingly, parallel deep-

sequencing of Dicer-positive and Dicer-knockout mESCs showed that only miRNA profiles changed in the absence of Dicer. Profiles of other small RNAs e.g. siRNAs seemed not to be affected [58].

Disruption of both *Dicer* alleles in mice leads to embryonic lethality, indicating the importance of Dicer and small RNA processing in mouse development. The possibility to generate homozygous *dicer* knockout cell lines suggests that the overall function of miRNAs is not required for the maintenance of pluripotency, though *Dicer*$^{-/-}$ mESCs show severe defects in growth and differentiation. Dicer-null mESCs fail to differentiate to embryoid bodies (EBs) accompanied with the lack of markers for the endodermal (*Hnf4*) and mesodermal (*T-brachyury*, *Bmp4* and *GataI*) lineage [56]. Loss of Dicer also affects the progression of the cell cycle, resulting in a prolonged G1 and GO phase which compromises the proliferation of *Dicer*$^{-/-}$ mESCs [54]. The importance of miRNAs in regulating cell cycle progression is also stressed by the fact that almost 50% of all miRNA molecules identified by massive parallel RNA sequencing are produced at four loci (miR-21, miR-17/92 cluster, miR-15b/16 cluster and miR-290 cluster), that are involved in regulation of cell cycle and oncogenesis [58].

Controversial results regarding the effect of loss of Dicer on the epigenetic modification of centromeric DNA were described. On the one hand, a reduction in DNA methylation and histone modification of centromeric repeat sequences was described [56], whereas another study reported that loss of Dicer affects the abundance of transcripts from centromeres without affecting histone modifications or DNA methylation of centromeric DNA [54].

Disruption of the *Dgcr8* gene also results in a global defect of miRNA maturation, indicated by the complete absence of neither fully mature nor intermediate pre-miRNA products [53]. Similar to loss of Dicer, *Dgcr8* knockout embryos die during early development and like Dicer$^{-/-}$ mESCs, *Dgcr8*$^{-/-}$ mESCs display defects in differentiation and cell cycle progression. *Dgcr8*$^{-/-}$ deficient mESCs accumulate in the G1 phase of the cell cycle and showed a delay in the upregulation of differentiation markers after withdrawal of LIF in EB cultures. Furthermore, *Dgcr8*$^{-/-}$ was not able to generate teratomas after subcutaneous injection, further stressing the observed differentiation defect. Both studies demonstrate that complete loss of miRNAs in ESCs compromises the exit of self-renewal during initiation of differentiation as well as the fast progression of the cell cycle.

Exit from self-renewal of *Dgcr8*$^{-/-}$ mESCs can be promoted by introduction of the let-7 miRNAs into DGCR8 deficient mESCs. The let-7 miRNA family is highly expressed in somatic cells and represses genes involved in self-renewal. The inhibitory effect of let-7c on self-renewal can be rescued by co-transfection of let-7 and miR-294, a member of the ESC specific miR-290 cluster. Introduction of miR-294 indirectly enhances the expression of Lin28 and c-Myc. Lin 28 is known to directly block the maturation of let-7 [59,60], whereas c-Myc together with n-Myc for the transcriptionally activates the miR-290 cluster [25,61]. The let-7 miRNA family thereby gets silenced and self-renewal is maintained.

During differentiation self-renewal normally becomes inhibited by downregulation of Oct4, Sox2 and Nanog resulting in a decrease of Lin28 and miR-290 cluster expression. Without Lin28, let-7 maturation is not blocked any longer, resulting in the further suppression of self-renewal promoting genes. These results demonstrate that self-renewal is regulated by opposing miRNA families. The let-7 miRNA family is required for inhibition of self-renewal, which is normally initiated during differentiation, whereas miR-290 cluster indirectly represses let-7 function in the state of self-renewal. Depletion of all miRNAs in ESCs therefore results in an undifferentiated state with no exit from self-renewal (Fig. 1).

Deletion of crucial miRNA processing factors like Dicer or DGCR8 [53–56] also enables the investigation of distinct miRNA families. This strategy leads to an identification of important cellular functions of the ESC specific miR-290 cluster, which is discussed in more detail within the following section.

The murine miR-290 cluster was initially identified as a 2.2-kb region on chromosome 7 [47,62]. The spliced, capped and polyadenylated primary transcript produced at this locus gives rise to 14 mature miRNAs. Among these, miR-290-3p, miR-291a-3p, miR-291b-3p, miR-292-3p, miR-294 and miR-295 share the hexamer seed ′AAGUGC′ (Table 1). The other miRNAs of the miR-290 cluster (miR-290-5p, miR-291a-5p, miR-291b-5p, miR-292-5p, miR-293, miR-293*, miR-294* and miR-295*) differ in their seed but are still highly expressed in ESCs with the exception of the hardly detectable minor forms of miR-293, miR-294 and miR-295 (miR-293*, miR-294* and miR-295*) [48].

Expression of this miRNA cluster is downregulated during ESC differentiation and not detectable in adult mouse organs [47]. The miR-290 cluster contributes to about 60% of the complete miRNAs in mESCs making it the most abundant miRNA family in mESCs [25]. Though its structure stays highly variable, the miR-290 cluster is conserved among human, chimpanzee, rat, mouse, dog and cow but stays restricted to placental mammals [62,63]. Sequence comparisons indicated that the mouse homologue of miR-290–295 is present at two human loci, the highly similar miR-371 cluster and the moderately similar miR-512 cluster.

Recent studies reported that miR-290 cluster regulates various important cellular processes by post-transcriptional inhibition of a broad range of different target genes. Two independent studies revealed the regulation of *de novo* DNA methylation by miR-290 cluster using Dicer deficient mESCs [64,65]. Microarray data indicated a downregulation of methyltransferases (Dnmt3a, Dnmt3b and Dnmt3l), which could be rescued by overexpression of miR-290 cluster, indicating an indirect control of the expression of methyltransferases by miR-290 cluster [64]. Retinoblastoma-like 2 (*Rbl2*), a known transcriptional repressor [66], was identified as a potential miR-290 cluster target with two conserved binding sites within the 3′UTR. Transfection of siRNAs directed against *Rbl2* resulted in an increase of Dnmt, supporting the hypothesis, that Rbl2 is a miR-290 cluster target that acts as a repressor of Dnmt expression, resulting in a decreased methylation in *Dicer*$^{-/-}$ mESCs. During in vitro differentiation of these cells, expression of Oct4 is only partially decreased, caused by a missing de novo methylation of its promoter. This defect could be rescued by transfection of all three methyltransferases as well as by transfection of the miR-290 cluster. This favors strongly the notion, that the defect in the methylation of the Oct4 promoter is a result of a repressed expression of methyltransferases [64]. Therefore, in *Dicer*$^{-/-}$ cells, a reduced miR-290 cluster expression causes an improper silencing of *Rbl2*, which is responsible for the transcriptional repression. Another study also described such defects in *Dicer*$^{-/-}$ cells and identified *Rbl2* as a target of miR-290 cluster, indirectly affecting telomerase-length homeostasis as a result of a global decreased DNA methylation [65]. Another important function of miR-290 cluster is the regulation of G1/S transition in ESCs, ensuring a rapid proliferation [67]. *Dgcr8* knockout mESCs, lacking microprocessor activity and hence all canonical miRNAs, were used to study the proliferation defect. p21, an inhibitor of the

Table 1
Sequences of mature miRNAs generate by miR-290 cluster.

Name of miRNA	Sequence of miRNA
miR-290-3p	aaagugccgccuaguuuuaagccc
miR-291a-3p	aaagugcuuccacuuugugugc
miR-291b-3p	aaagugcauccauuuuguuugu
miR-292-3p	aaagugccgccagguuuugagugu
miR-294	aaagugcuucccuuuugugugu
miR-295	aaagugcuacuacuuuugagucu
miR-290-5p	acucaaacuaugggggcacuuu
miR-291a-5p	caucaaaguggaggcccucucu
miR-291b-5p	gaucaaaguggaggcccucucc
miR-292-5p	acucaaacuggggcucuuuug
miR-293	acucaaacugugugacauuuug

cyclinE-Cdk2 complex was identified as a direct target of miR-290 cluster. Knockdown of p21 as well as overexpression of members of the miR-290 cluster enabled a rescue of the defect in proliferation, indicating the importance of miR-290 cluster for the rapid proliferation of mESCs [67].

Besides regulating de novo DNA methylation and the G1/S transition, miR-290 cluster has also been linked to cell survival by protecting mESCs against genotoxic stress and thereby preventing apoptosis. This protective effect is mediated by direct repression of Caspase 2 and Ei24, which are activated upon damage of DNA [68].

miR-290 cluster deficiency in developing mice leads to a partially penetrant embryonic lethality and defective germ cells, indicating an important role for miR-290 cluster in early mouse development [69]. Notably, mESCs deficient for the miR-290 cluster could be generated and showed no obvious defects.

Members of the miR-290 cluster were also demonstrated to enhance the efficiency of reprogramming somatic cells to induced pluripotent stem cells, a recently described process, which is introduced in the next chapter.

4. Diverse functions for miRNAs in reprogramming

Besides deriving ESCs from the ICM of early embryos, pluripotent cells can also be generated by overexpression of the transcription factors Oct4, Sox2, Klf4 and c-Myc [6,70]. Somatic cells can be reprogrammed by these factors to induced pluripotent stem cells (iPSCs), which have all distinct features with "naïve" mESCs in common. iPSCs are able to contribute to chimeric mice, show the expression of key pluripotency markers and both X-chromosomes are activated in female cells [71]. Furthermore, full developmental potential of iPSCs was demonstrated by tetraploid complementation creating mice entirely derived from iPSCs [72–74].

Since its first description in 2006 by Takahashi and Yamanaka [6], different combinations of genes including Nanog, Esrrb, Nr5a2, Lin28 and E-cadherin have been used to reprogram fibroblasts and other types of somatic cells [75–78]. To induce pluripotency in neural stem cells (NSCs) even Oct4 alone seems to be sufficient [79]. Among all factors used for induction of pluripotency, Oct4 is the only one, which cannot be replaced by other factors. This indicates a unique role for Oct4 during reprogramming [80]. The mechanism by which the indicated factors accomplish the dedifferentiation and establishment of the pluripotent state is poorly understood but involves reactivation of the endogenous pluripotency genes, massive chromatin remodeling and morphological changes towards an epithelial phenotype.

Various small molecules and specific miRNAs were employed in the improvement of the efficiency of reprogramming [81–85]. During reprogramming of mouse embryonic fibroblasts (MEFs), a subset of miRNAs belonging to the miR-290 cluster (miR-291-3p, miR-294 and miR-295) was able to significantly enhance the colony number of induced pluripotent stem cells (iPSCs) [82]. Transient transfection of miR-291-3p, miR-294 or miR-295 at day 0 and 6 post-infection with the reprogramming cocktail consisting of Oct4, Klf4 and Sox2 (OKS) enhanced the induction of pluripotency, whereas two miRNAs of the same cluster with different seed sequences had no effect. The strongest effect was observed after OKS transduction with addition of miR-294 and iPSCs generated by this approach were injected into blastocysts confirming pluripotency of these cells by contribution to all three germ layers as well as the germ line. Though miR-294 is able to replace c-Myc during the process of reprogramming, the mechanisms by which canonical members of the miR-290 cluster can substitute c-Myc remain elusive [82].

The binding of c-Myc to the promoter of these miRNAs suggests that miR-291-3p, miR-294 and miR-295 act downstream of c-Myc, though the effects of c-Myc and the indicated miRNAs on the cell population are not identical. Unlike c-Myc, miR-294 did not induce proliferation of MEFs during the early phase of dedifferentiation. Furthermore, in contrast to exogenous c-Myc, miR-294 yielded a homogenous population of iPSC colonies. These data indicate overlapping as well as independent functions of c-Myc and miR-294 during the process of reprogramming [82].

Three studies so far even suggested that induction of pluripotency is possible by miRNAs alone [86–88]. Retroviral mediated overexpression of miR-302/367 cluster was demonstrated to be sufficient to induce pluripotency of mouse and human fibroblasts without exogenous expression of other transcription factors [86]. Pluripotency of miRNA-mediated reprogrammed iPSCs was confirmed by teratoma formation for human iPSCs and contribution to chimeric mice as well as germ-line contribution for mouse iPSCs. Notably, the efficiency in reprogramming was claimed to be two magnitudes higher in comparison to the usual approach using Oct4, Klf4, Sox2 and c-Myc [86]. Transient transfection of a combination of mature miR-200c, miR-302 and miR-367 was also used to induce pluripotency of mouse and human somatic cells without integration of any viral-based vectors [88]. Nevertheless, miRNA mediated reprogramming was not used in other studies so far, making it a very controversial issue in this field. We and our collaborators were not able to reproduce the published results in our laboratories. Though additional expression of miR-302 cluster and miR-290 cluster leads to a slight increased reprogramming efficiency when using Oct4-GFP mouse fibroblasts with the classical reprogramming factors Oct4, Sox2, Klf4 and c-Myc (OSKM) (Fig. 2). Nevertheless, both miRNA clusters fail to induce pluripotency without additional factors (unpublished data, Fig. 2). Furthermore, reprogramming of human fibroblasts with OSKM and miR-302/367 leads to an enhanced expression of TRA-1–60 from 5.7% to 13.3% (unpublished data, Dr. Holm Zaehres, Prof. Dr. Hans Schöler, Department Cell and Developmental Biology, Max–Planck-Institute for Molecular Medicine, Münster, Germany). Again miR-302/367 alone did not induce reprogramming (personal communication, Dr. Holm Zaehres). Further work has to be carried out, to solve the question why miRNAs alone are not able to reprogram fibroblast to iPS cells without the classic reprogramming factors.

Another study indicating the importance of miRNAs in reprogramming demonstrated that fibroblasts lacking all mature miRNAs are not able to generate iPSCs [89]. Therefore, miRNAs are not only necessary for proper differentiation of pluripotent cells, but also play a crucial role during de-differentiation of fibroblasts [55,89] (Fig. 1).

Defining the target genes of miRNAs during the induction of pluripotency provides further insight into the mechanism of reprogramming. Indeed, various studies revealed a role for distinct miRNAs in the establishment of an epithelial character during reprogramming of MEFs [83–85]. These reports underline the emerging notion that pluripotency is closely related to an epithelial phenotype and that reprogramming requires a mesenchymal to epithelial transition (MET).

During development epithelial to mesenchymal transitions (EMTs) and mesenchymal to epithelial transitions (MET) are naturally occurring processes, which involve dramatic cytoskeletal and morphological changes enabling adoption to specific cell fates for proper formation of organs and tissues. The discovery of MET as an early event during reprogramming of fibroblasts, which represent a typical mesenchymal phenotype, leads to the emerging notion that pluripotency is closely related to an epithelial phenotype.

Loss of the epithelial character is characterized by EMT. This process involves an upregulation of Snail, Slug and Zeb transcription factors which repress the transcription of E-cadherin, followed by the loss of cell adhesion and massive cytoskeletal rearrangements. As a result, cells acquire a motile phenotype. EMTs occur at different stages during development and cancer progression and different signaling pathways like TGF-β, NF-κB or Wnt are known as potential EMT inducers [90].

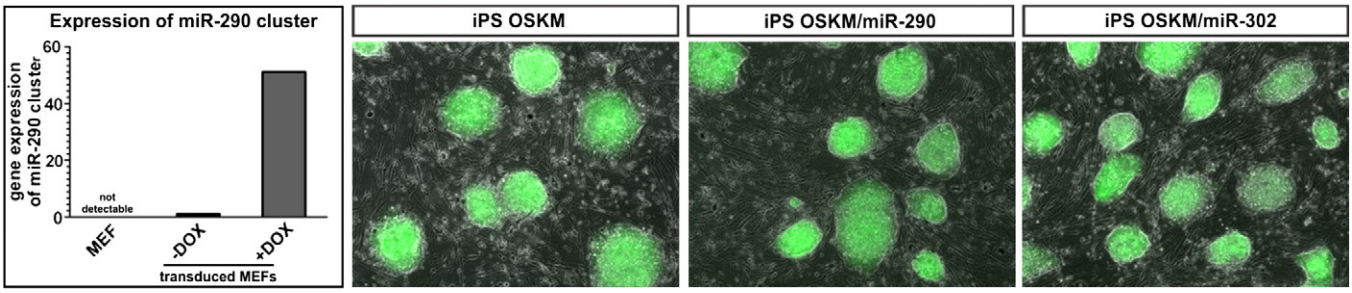

Fig. 2. Reprogramming with miR-290 cluster and/or miR-320 cluster failed to induce pluripotency. Doxycycline-inducible lentiviral vectors were used to express miR-290 and/or mir-302 cluster. Left image shows the expression of miR-290 cluster after induction with doxycycline (DOX). Reprogramming efficiency of Oct4-GFP mouse fibroblasts with Oct4, Sox2, Klf4 and c-Myc (OSKM) could be only slightly enhanced by the additional expression of miR-290 and/or miR-302 cluster (right images). Reprogramming strategies using miR-290 cluster and/or miR-302 cluster alone failed to reprogram fibroblast to iPSCs (Hauser et al., unpublished data).

ESCs are characterized by a small, densely packed morphology and a high expression of E-cadherin, resembling rather characteristics shared with an epithelial phenotype than a mesenchymal one. These characteristics easily enable morphological and cytoskeletal adjustment triggered by specific cues during differentiation and furthermore this morphology is likely an adaption to the fast progressing cell cycle of ESCs since each mitotic division requires massive cytoskeletal rearrangements.

So far it remains unclear whether the epithelial character of pluripotent cells is directly involved in cell signaling and maintenance of pluripotency or if this is just a result of the undifferentiated, pluripotent state.

During the initial phase of reprogramming several miRNA families could be identified as mediators of MET. Among them, miR-205 and the miR-200 clusters were investigated in more detail. Both were induced by BMP-signaling, which is also known in participating in the maintenance of pluripotency in addition with LIF. A MET occurs as an early event during reprogramming and seems to have an initiating character on the induction of pluripotency [84]. Suppression of reprogramming by BMP antagonists could be rescued by transfection of mimics for miR-200b and miR-200c. As previously reported, the miR-200 family directly targets the E-cadherin repressors *Zeb1* and *Zeb2*, as well as *Snail* and *Slug*, key regulators of EMT [91–94] and thereby triggers the conversion to an epithelial phenotype.

In addition to miR-200 cluster, several other miRNA clusters were identified as potential enhancers of reprogramming. Expression analysis of miRNA clusters that were highly expressed in mESCs during the initial phase of reprogramming revealed an induction of miR-17/92, miR-106b/25, miR-106a/363, and miR-302b/367 within the first four days of the reprogramming process [83]. Further investigation of miR-106b/25 cluster suggested an enhancement of iPSC induction by promoting MET. Transfection of mimics for miR-93 and miR-106b promoted an increase in the colony number of iPSCs, whereas inhibition of the indicated miRNAs resulted in a decrease of the colony number. *Tgfbr2* and *p21* were identified as direct targets of miR-93 and miR-106b and subsequent siRNA-mediated knockdown of both genes resulted in an increase of iPSC colony number. In contrast, overexpression of TGF-β receptor II diminished the improving effect of miR-106b. In agreement with the notion that MET is an initiating event during induction of pluripotency, these data indicate that miR-106b/25 cluster promotes the establishment of an epithelial phenotype by inhibition of TGF-β signaling, a known inducer of EMT [90].

Tgfbr2 was also discovered to be a target of the human miR-302b and miR-372. During reprogramming of human fibroblasts [85], transfection of both miRNAs at days 2 and 7 after infection with the reprogramming cocktail enhanced the number of colonies with a hESC like morphology [85]. Among different potential target genes identified by expression analysis, *Tgfbr2* and *Rhoc* were validated by reporter assays and mutation of miRNA binding sites. Furthermore, miR-302b, miR-372 and miR-294, which share the same seed sequences, were able to block TGF-β induced epithelial to mesenchymal transition in HaCaT cells. Notably, a modified form of miR-294 with a mutated seed sequence was not able to inhibit a TGF-β mediated epithelial to mesenchymal transition [85], indicating the importance of the seed sequence for probable targeting of these miRNAs

Another miRNA family (miR-130/301/721) regulating induction of iPSCs was recently identified in a miRNA library screen [95]. miR-130/301/721 enhances the efficiency of iPSC generation by repressing the homeobox transcription factor Meox2. Overexpression of miRNA-resistant Meox2 abolished the effect of miR-130/301/721 on iPSC formation. It is unclear, how Meox2 interferes with reprogramming, but Meox2 might inhibit TGF-β signaling or help overcome p21 mediated cell cycle arrest [95].

In contrast to enhancing the generation of iPSCs, some miRNA families (miR-34, miR-21 and miR-29a) interfere with reprogramming [96,97]. Inhibition of these miRNAs results in an enhanced reprogramming efficiency. MEFs with a genetic ablation of miR-34a exhibited an increased reprogramming efficiency, suggesting that miR-34a interferes with reprogramming. miR-34 cluster consists of miR-34a, miR-34b and miR-34c. Knockout of miR-34a or miR-34a/b promotes reprogramming of somatic cells while ablation of miR-34a showed a stronger effect on iPSC generation than miR-34b and miR-34c. MEFs deficient for all three miRNAs exhibited the greatest effect on reprogramming. Deficiency of these miRNAs also results in more rapid kinetics during reprogramming. miR-34 cluster was previously identified as bona fide target of p53 [98], a known repressor of reprogramming [99–101]. Therefore, miR-34 cluster seems to act in response to p53 to repress its target genes. Among these targets, Sox2, Nanog and N-Myc were identified to be post-transcriptionally regulated by miR-34 cluster during induction of iPSCs [97].

Further examples for miRNAs interfering with iPSC generation are miR-21 and miR-29a [96]. Both miRNAs are abundantly expressed in MEFs. Depletion of both miRNAs results in an enhanced reprogramming efficiency mediated by regulation of p53 and ERK1/2 pathways. Notably, c-Myc seems to repress MEF enriched miRNAs like miR-21 and miR-29a to promote reprogramming [96]. Both studies suggest that miRNAs are not only involved in maintaining the undifferentiated cellular state, but also trigger maintenance of the differentiated cell fate (Fig. 5).

5. miRNA mediated cell fate determination

Besides being involved in the regulation of pluripotency, there is increasing evidence that miRNAs also play a crucial role in the cell fate specification during differentiation of pluripotent cells and more restricted progenitor cells (Fig. 5). This is either by inhibiting distinct differentiation processes or by promoting the exit of self-renewal and commitment to specific lineages. Conversion of pluripotent stem cell to distinct lineages enables the identification of

specific miRNA signatures for the different differentiation processes. This opens the opportunity to study individual candidate miRNAs for their potential to regulate lineage commitment. A large body of work has been carried out to identity distinct miRNAs and their target genes. Table 2 gives an overview about several studies identifying miRNA mediated cell fate regulation. Within the following chapter we would like to introduce some examples of miRNA mediated cell fate regulation.

The orphan nuclear receptor NR2F2 (COUP-TFII) is able to promote neuronal differentiation of ESCs by inducing the expression of neural genes during early differentiation [102]. Within the undifferentiated state of ESCs, COUP-TFII is inhibited by OCT4 as well as by miR-302. OCT4 represses COUP-TFII at the transcriptional level, whereas miR-302 acts on the post-transcriptional level. Conversely, during differentiation COUP-TFII is able to repress OCT4, creating a positive feedback loop. Thereby, the OCT4 driven expression of miR-302 is reduced, resulting in increasing levels of COUP-TFII [102] (Fig. 3). This regulatory circuitry controls early neuronal lineage commitment in human embryonic stem cells and demonstrates how ES-specific miRNAs participate in the maintenance of pluripotency and also control lineage commitment during exit of self-renewal (Fig. 5).

Another example how cell fate determination is regulated by ESC-specific miRNAs are miR-293 and miR-291b-5p. Both, miR-293 and miR-291b-5p belong to the miR-290 cluster and inhibit the NF-κB subunit p65 in mESCs (Fig. 4) [103]. Thereby protein levels of NF-κB/p65 are kept at a low level within the undifferentiated state of mESCs. To prevent translated NF-κB from activating target gene transcription, the ESC specific transcription factor Nanog interacts with NF-κB by direct protein–protein interaction, which inhibits target gene activation of NF-κB [104]. Both NF-κB inhibiting pathways, Nanog and the expression of miR 290 cluster are directly regulated by the ESC core transcription factors Oct4 and Sox2 [25]. Thus undifferentiated ES cells are functionally null for NF-κB subunits. In this line forced expression of NF-κB transactivating subunits resulted in differentiation of mESCs towards a mesodermal lineage [103]. It is tempting to speculate that miR-290 cluster keeps the p65 protein level at a certain threshold, which ensures that enough Nanog protein is left to act as a transcriptional activator. Nevertheless, it has yet not been investigated, if NF-κB is able to inhibit Nanog.

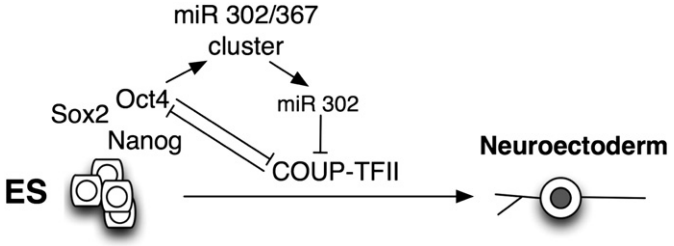

Fig. 3. Regulation of cell fate by COUP-TFII and miRNA 302. Orphan nuclear receptor COUP-TFII (also called NR2F2) can shift cell fate in ES cells to neuroectoderm. Within pluripotent ES cells a network of transcription factors such as Oct4, Sox2 and Nanog direct the expression of miR 302/367 cluster. From this cluster miR 302 is processed which represses translation of COUP-TFII. Furthermore COUP-TFII expression is directly repressed by Oct4. Thereby a feedback loop is created to regulate COUP-TFII mediated differentiation.

Notably, in *Drosophila* the NF-κB ortholog dorsal has already an established role for cell fate determination during embryogenesis [105]. Since Bilateria show a common plan for patterning the dorsoventral axis [106], NF-κB might not only regulate cell fate specification in ESCs [103,104,107], but also play a role for cell fate specification during developmental processes in vivo (Fig. 4).

Both examples given above describe miRNAs which inhibit distinct differentiation processes. It is also possible that distinct miRNAs promote commitment to specific lineages. This is demonstrated for miR-125b, which promotes neural lineage commitment by posttranscriptional repression of SMAD4 [108]. miR-125 regulates the levels of SMAD4 in response to different levels of extracellular signals of the TGF-β superfamily. Whereas Activin and Bmp4 inhibit miR-125, Noggin and the small molecule SB431542 promote miR-125 expression, which subsequently inhibits SMAD4 mediated transcriptional activation of its target genes. This study demonstrates how miR-125 secures neural commitment of human embryonic stem cells and gives further insight how dual inhibition of SMAD signaling efficiently converts pluripotent cells into a neural lineage [109].

Table 2
Summary of miRNAs and their corresponding targets involved in cell fate regulation.

MicroRNA	Target	Process	Reference
Let7	TLX	Increases neuronal differentiation	[119]
miR-1/miR-206	Klf4	Regulates myoblast differentiation	[120,121]
miR-7a	Pax6	Controls dopaminergic differentiation of ventral neural stem cells	[122]
miR-9	TLX, Rest, Gsh2, Foxg1	neurogenic fate induction	[123–125]
miR -9*	Co-Rest, BAF53a, BAF45a	See above	[124,126]
miR-10a	Bcl-6, Ncor2	Constrains plasticity of helper T cells	[127]
miR-22	HDAC6	Promotes osteogenic differentiation	[128]
miR-23a cluster		Inhibits B-cell differentiation	[129]
miR-23b	SMAD 3,4,5	differentiation of liver stem cells	[130]
miR-27b	Dll4, Spry2	promotes endothelial tip cell fate and venous differentiation	[131]
miR-124	BAF53a, BAF45a	Promotes neural transcriptome, neurogenic fate induction	[126,132–135]
miR-125	SMAD4	Early neural specification	[108]
miR-137	LSD1	inhibits cell proliferation and accelerates differentiation of neural stem cells	[136]
miR-145	Oct4, Sox2, Klf4	Promotes differentiation of pluripotent cells	[111]
miR-143 and miR-145	Klf4, ELK1	Promote smooth muscle cell differentiation	[113]
miR-290 cluster	Rbl2, Cdkn1a, Lats, caspase 2, Ei24, RelA	De novo methylation, cell cycle progression, regulation of differentiation	[64,65,67,68,103]
miR-302 cluster	COUP-TFII, Lefty1, Lefty2	Promotes mesodermal lineage at the expense of mesoderm	[102,137]
miR-669a, miR-669q	MyoD	Prevent differentiation of postnatal myogenic progenitors	[138]
miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-205, and miR-217	RUNX2, TRPS1	Block of chondrocyte and osteoblast maturation, redirect mesenchymal fate to adipogenic	[38]

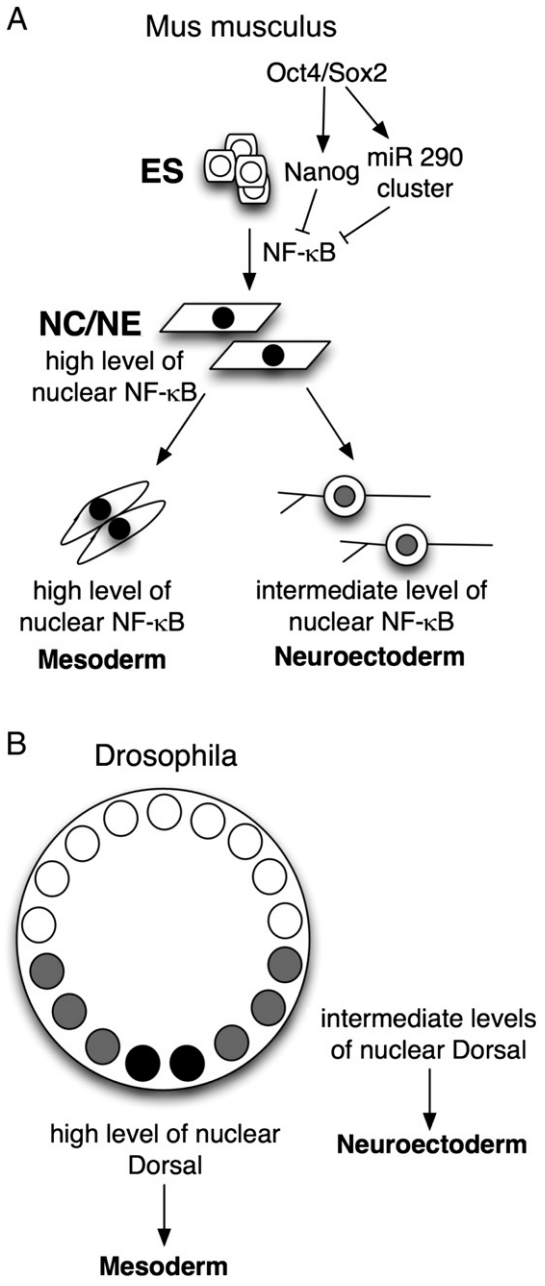

Fig. 4. Regulation of cell fate by a network of miRNAs and transcription factor NF-κB. (A) In embryonic stem cells (ESCs) a transcriptional network directed by the transcription factors Oct4 which can heterodimerize with Sox2 and its target gene Nanog direct the expression of miRNA cluster 290. Members of this cluster e.g. miR290 can repress translation of NF-κB subunits such as RelA. Furthermore NF-κB activity could be repressed by direct interaction with Nanog. This repression seems to be crucial for the maintenance of pluripotency. During differentiation a neural crest like neuroectodermal intermediate (NC/NE) appeared, which relies on NF-κB activity. A high amount of NF-κB resulted in the formation of mesoderm, whereas a low amount of NF-κB shifts cell fate from mesoderm to neuroectoderm. (B) A similar regulation occurred during Drosophila development. During the formation the dorso-ventral axis, the NF-κB ortholog Dorsal is translocated to totipotent nuclei in the dorsal region. Nuclei within the syncythium containing high amounts of Dorsal give rise to mesoderm, whereas nuclei with intermediate levels of Dorsal are directed to a neuroectodermal cell fate (modified from [107]).

Several miRNAs participating in cell fate determination seem not to be restricted to a specific lineage which rather indicates a general involvement of these miRNAs in cellular differentiation. miRNA-145 for example has been proposed to be involved in regulating cell fate decisions across different lineages [110–113].

Within the undifferentiated state of hESCs, miR-145 is only weakly expressed, but dramatically upregulated during differentiation. Ectopic expression of miR-145 causes loss of pluripotency by repressing Oct4, Sox2 and Klf4 [111], whereas inhibition of miR-145 leads to an impaired differentiation.

Another function of miR-145 is the regulation of smooth muscle cell fate decisions [112,113]. miR-145 is transcripted as a bicistronic primary transcript together with miR-143, the most enriched miRNA during differentiation of mESCs into multipotent cardiac progenitors. Expression of both miRNAs is controlled by the essential cardiac transcription factors SRF (serum response factor) and NKx2.5 [113]. Knockout of miR-143 and miR-145 in mice revealed a crucial role for both miRNAs in the differentiation of vascular smooth muscle cells (VSMCs). Ablation of miR-143 and miR-145 resulted in structural defects due to incomplete differentiation of VSMCs [112].

To further investigate the role of miR-145 or miR-143 in cell fate regulation, both miRNAs were tested for their ability to modulate myocardin (myocd)-induced reprogramming of fibroblasts into VSMCs [113]. Myocd is a smooth muscle and cardiac muscle-specific transcriptional coactivator of serum response factor, which is able to induce muscle-specific expression. Whereas miR-145 was able to strongly increase the Myocd mediated conversion of fibroblasts, miR-143 had no effect. Without Myocod, miR-145 was not able to induce reprogramming of fibroblasts into VSMCs. The potential for miR-145 to trigger VSMC differentiation was also demonstrated using multipotent neural crest stem cells. In contrast to miR-143, which had no effect, miR-145 was able to drive a large amount of these cells into VSMCs [113].

Though only miR-145 is capable to directly regulate smooth muscle cell fate, both miRNAs cooperate to regulate a network of transcription factors, resulting in the inhibition of proliferation and entry into differentiation.

6. Conclusion

miRNAs and their targets form tightly regulated networks that control pluripotency as well as differentiation processes into distinct lineages. Triggered by specific extracellular cues signaling cascades are initiated determining cellular fates. Activation of lineage specific transcription factors promotes the cellular programs which are necessary for proper differentiation. Since their discovery a crucial role for miRNAs has emerged during these processes. Maintenance of pluripotency as well as differentiation of pluripotent stem cells depends on the action of specific miRNAs. Within the pluripotent state, specific miRNAs repress transcription factors promoting cellular differentiation. Once exit from self-renewal is committed, lineage-specific miRNAs are upregulated inhibiting transcription factors specific for the pluripotent state. Thereby, miRNAs ensure that the level of specific transcriptional programs does not exceed a certain threshold and provides an additional level of regulation (Fig. 5). In this context it is also noteworthy to

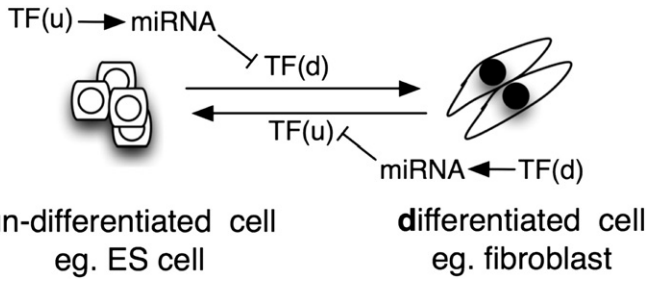

Fig. 5. General model for cell fate specification by a network of miRNAs and transcription factors. A transcription factor in an un-differentiated cell type (TF(u)) might regulate the expression of a miRNA, which could destabilize the mRNA of a transcription factor inducing differentiation (TF(d)). In a differentiated cell a transcription factor expressed in this state might regulate a miRNA, which in turn inhibits the expression of a transcription factor necessary in un-differentiated cells. Thus, the combination of positively acting transcription factors and negatively acting miRNA might stabilize cell fates.

speculate about the mechanisms regulating the availability of functional miRNAs. Recent studies indicate that miRNAs can also be regulated by their targets, which creates the possibilities to form complex regulatory loops enabling further options to adjust transcriptional program. This regulatory mechanism also provides an option to adjust the availability of functional miRNAs.

Though a large number of studies have been carried out to identify miRNA regulated genes, the large number of potential targets regulated by one miRNA makes it difficult to unravel complete networks. Furthermore, some miRNAs share the same seed sequences encoded at distinct genetic loci, creating a functional redundancy that makes it difficult to carry out loss of function studies. In this line, knockout of specific miRNAs in mice often results in partially penetrant phenotypes [69,114–116], which might be explained by the function of miRNAs itself. Given that miRNAs have exclusively regulatory functions to confer robustness to cellular processes [117], knockout cells or animals that can adjust their transcriptional program do not develop a phenotype. This adjustment can either be performed by the redundancy of seed sequences or by the randomness of gene expression during developmental processes even in genetically identical organism [118]. The fact that miRNA targeting goes beyond the canonical seed match, even makes the prediction of a subset of target sites even more difficult.

Taken together, though a lot of studies during the last couple of years shed some light into the role of miRNA mediated cell fate induction, the complete network of miRNAs and their targets still remains elusive. Further studies will benefit to our understanding how miRNAs help to guide cells to their designated fate.

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