Stem Cell Research 19 (2017) 21–30

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

MicroRNA-29 impairs the early phase of reprogramming process by targeting active DNA demethylation enzymes and Wnt signaling

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ARTICLE INFO

Article history:
Received 19 May 2016
Received in revised form 29 November 2016
Accepted 15 December 2016
Available online 19 December 2016

Keywords:
Cellular reprogramming
miR-29
WNT signaling
Ten-eleven translocated 1 (TET1) gene

ABSTRACT

Somatic cell reprogramming by transcription factors and other modifiers such as microRNAs has opened broad avenues for the study of developmental processes, cell fate determination, and interplay of molecular mechanisms in signaling pathways. However, many of the mechanisms that drive nuclear reprogramming itself remain yet to be elucidated. Here, we analyzed the role of miR-29 during reprogramming in more detail. Therefore, we evaluated miR-29 expression during reprogramming of fibroblasts transduced with lentiviral OKS and OKSM vectors and we show that addition of c-MYC to the reprogramming factor cocktail decreases miR-29 expression levels. Moreover, we found that transfection of pre-miR-29a strongly decreased OKS-induced formation of GFP^+-colonies in MEF-cells from Oct4-eGFP reporter mouse, whereas anti-miR-29a showed the opposite effect. Furthermore, we studied components of two pathways which are important for reprogramming and which involve miR-29 targets: active DNA-demethylation and Wnt-signaling. We show that inhibition of Tet1, Tet2 and Tet3 as well as activation of Wnt-signaling leads to decreased reprogramming efficiency. Moreover, transfection of pre-miR-29 resulted in elevated expression of β-Catenin transcriptional target sFRP2 and increased TCF/LEF-promoter activity. Finally, we report that Gsk3-β is a direct target of miR-29 in MEF-cells. Together, our findings contribute to the understanding of the molecular mechanisms by which miR-29 influences reprogramming.

1. Introduction

MicroRNAs are known to have the capability to modulate multiple cell signaling pathways simultaneously (Hashimoto et al., 2013; Shalgi et al., 2009; Subramanyam et al., 2011). Many of these small molecule regulators of gene expression have been described to modulate somatic cell reprogramming to pluripotency (Anokye-Danso et al., 2011; Li and He, 2012; Subramanyam et al., 2011). For example, the miR-302/367 cluster is highly upregulated during cellular reprogramming to induced pluripotent stem cells (iPSCs) and highly expressed in embryonic stem

Abbreviations: microRNA, miR; OKS.dT, hOCT4-hKLF4-hSOX2-dTom; OKSM.dT, hOCT4-hKLF4-hSOX2-hC-MYC-dTom; MEF, mouse embryonic fibroblasts; iPSC, induced pluripotent stem cells; ESC, embryonic stem cells; shRNA, short-hairpin RNA; siRNA, small interfering RNA; OG2, Oct4 promoter-driven GFP expression.

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http://dx.doi.org/10.1016/j.scr.2016.12.020

cells (ESC) (Card et al., 2008; Lee et al., 2013; Lipchina et al., 2012). It is known that OCT4, SOX2 and NANOG upregulate transcription of the miR-302/367 cluster (Card et al., 2008; Sandmaier and Telugu, 2015), which in turn targets components of TGF-β (Lipchina et al., 2011; Subramanyam et al., 2011), and RHOC pathways (Subramanyam et al., 2011). Alongside, miRs of the miR-290/295 cluster (Parchem et al., 2014) have been associated with targeting components of the cell cycle's G1-S barrier (Wang et al., 2008), as well as the WNT signaling inhibitor Dkk1 (Zovoilis et al., 2009) and the pro-apoptotic genes Caspase 2 and EiI24 (Zheng et al., 2011) and were found to increase reprogramming efficiency (Parchem et al., 2014). On the other hand, the miR-143/145 cluster was described as a major inhibitor of somatic cell reprogramming (Barta et al., 2015). This cluster has increased expression levels during ESC differentiation exerting its function by targeting OCT4, SOX2 and KLF4 (Xu et al., 2009), but also genes related to cell proliferation pathway (Ding et al., 2015) and c-Myc expression (Sachdeva et al., 2009).

The miR-29 family of microRNAs is highly conserved amongst mammalian species and its members have as well been investigated for their roles in modulation of somatic cell fate reprogramming from fibroblasts to iPSCs (Guo et al., 2013; Pfaff et al., 2011; Yang et al., 2011). The family comprises of three variants where miR-29b-1 and miR-29a are located in the same cluster on chromosome 7q32.3, while miR-29b-2 and miR-29c are located on chromosome 1q32.2 in human cells (Mott et al., 2010). In mice the respective miR-29 clusters are located at chromosomes 6qA3.3 and 1qH6. Importantly, the mature sequence of all miR-29 variants is conserved amongst human and mouse species (Slusarz and Pulakat, 2015). During reprogramming of mouse embryonic fibroblasts (MEFs) into iPSCs using separate retroviral vectors encoding for the four transcription factors Oct4, Sox2, Klf4 or c-Myc, it was found that c-Myc is an inhibitor of miR-29 clusters transcription, whereas Sox2 and Klf4 were reported to induce miR-29 expression levels (Yang et al., 2011). Moreover, blocking of miR-29a by anti-miRs resulted in enhanced reprogramming efficiencies and the effect of miR-29a inhibition was partially attributed to indirect down-regulation of p53, orchestrated by high levels of p85α and CDC42 proteins, both of which are targets of miR-29 (Yang et al., 2011). In our own previous work we analyzed the effects of a set of 379 microRNAs in OG2-MEFs from Oct4-GFP reporter mice undergoing nuclear reprogramming after transduction with OKS lentiviral vector. In this work, we confirmed that transfection of miR-29 family members at the early stage of somatic cell reprogramming rather decreases the total number of Oct4-GFP$^+$ colonies compared to scramble controls (Pfaff et al., 2011).

Intriguingly, more recently published work showed contrasting findings regarding the effects of miR-29 during OSKM reprogramming (Guo et al., 2013). Guo and colleagues demonstrated that forced expression of miR-29b throughout the reprogramming process by γ-retroviral vectors improved reprogramming efficiency, which they related to knockdown of Dnmt3a and Dnmt3b (Guo et al., 2013). However, Guo et al. did not study in detail whether the observed effects resulted exclusively from ceaseless overexpression of miR-29 during the entire process or if temporary overexpression at the early phase could help to explain the discrepancies between their work and previously published data. Nevertheless, these contrary results call for an in-depth investigation about the actual role of miR-29 family members during reprogramming.

Differential expression of miR-29 family has been associated with many types of cancer; however, several reports have attributed contrasting roles to these miRs. Depending on the context, miR-29 members were reported as onco-miRs or as tumor suppressors (Garzon et al., 2009; Oliveira et al., 2015; Zhang et al., 2013). Those reports highlight the interplay of miR-29 with several alternative pathways and mechanisms, attributing context-specific roles to these miRs. In human, miR-29 family members have been related to the regulation of expression of genes such as de novo methyltransferases (Morita et al., 2013; Oliveira et al., 2015; Robaina et al., 2015), active DNA demethylation enzymes TET1 and TDG (Zhang et al., 2013) as well as components of WNT signaling such as DKK1, sFRP2 and Kremen2 (Kapinas et al., 2009), GSK3-β (Liu et al., 2011) and Icat (Shin et al., 2014). In mouse, miR-29 was shown to modulate p85α and CDC42 from the p53 signaling pathway (Yang et al., 2011).

Of these pathways, active DNA demethylation mediated by Ten-eleven translocation methylcytosine dioxygenase (Tet) family members is highly related to ESC pluripotency maintenance (Mohr et al., 2011). Tet1 transcription is directly activated by transcription factors Oct4 and Sox2, whereas the promoter regions of Oct4 and Nanog are bound by Tet1 during somatic cell reprogramming, which enriches their 5hmC content, leading to their demethylation and reactivation (Koh et al., 2011). ShRNA-mediated knockdown of Tet1 in mESCs (mouse ESCs) decreases total 5hmC levels and leads to increased DNA methylation at the Nanog-proximal promoter, resulting in reduced Nanog expression and inhibition of cell proliferation (Ito et al., 2010). Moreover, double knockdown of Tet1 and Tet2 in ESC cells leads to down-regulation of several other pluripotency-related genes (Ficz et al., 2011). Importantly, it was shown that Oct4 can be efficiently substituted by Tet1 during reprogramming of MEF cells, and that an all-in-one lentiviral vector containing Tet1 (TKSM) is more efficient compared to OKSM for generation of Nanog-GFP$^+$ iPSC colonies (Gao et al., 2013).

Another important pluripotency-related pathway affected by miR-29 is WNT-signaling. During osteoblastic differentiation from human mesenchymal stem cells, miR-29a binds to and decreases the expression levels of DKK1, sFRP2 and KREMEN2; which in turn allows the activation of canonical WNT signaling and increases levels of WNT transcriptional targets, i.e. c-MYC and miR-29a (Kapinas et al., 2009). DKK1, sFRP2 and KREMEN2 are natural inhibitors of WNT/LRP6/Frizzled binding, blocking the downstream canonical WNT signaling activation. Moreover, it was shown that during neurogenesis in mice, miR-29 inhibits Icat (inhibitor of β-Catenin and Tcf receptor), thereby activating WNT signaling pathway (Shin et al., 2014). Of notice, it was also shown that miR-29b targets GSK3-β, the major component of β-Catenin destruction complex, in human cells (Liu et al., 2011).

The role of canonical WNT signaling during reprogramming process has been described controversially. Some studies showed that inhibition of canonical WNT signaling during reprogramming process results in higher efficiencies of ESC-like colony generation (Aulicino et al., 2014; Ho et al., 2013). Ho et al., used the small molecule inhibitor of canonical WNT signaling IWP2 at the early phase (1–3 days post iPSC induction) of reprogramming by OSMK vector in Nanog-GFP MEFs, resulting in higher number of GFP$^+$ colonies compared to DMSO control. Moreover, they also show that the activation of WNT signaling by using recombinant WNT3a during the same period decreases the colony number (Ho et al., 2013). Aulicino and colleagues showed that a WNT "off" state at the early phase of reprogramming is beneficial to this process (Aulicino et al., 2014). However, Zhang et al. engineered MEF cells employing a construct for overexpression of β-Catenin for constitutive activation of WNT signaling throughout the entire reprogramming process, which resulted in increased efficiency of iPSC generation after OKSM transduction (Zhang et al., 2014). Moreover, they assessed the consequences of activation of WNT signaling by recombinant Wnt3a or CHIR99021 during reprogramming, and they found that both molecules improve reprogramming efficiency. However, both Wnt-activators were added to the culture medium during the entire reprogramming process (Zhang et al., 2014). Another study showed that the addition of Wnt3a conditioned media (Wnt3a-CM) in MEFs under doxycycline inducible OSK cassette during the entire reprogramming process allowed for more Oct4-GFP$^+$ iPSC colonies to emerge compared to the control system without Wnt3a-CM (Marson et al., 2008). Together, current literature suggests that ceaseless activation of Wnt signaling is beneficial for somatic cell reprogramming but its temporary activation at the early phase may have the opposite effect. In this context it was suggested that canonical WNT signaling undergoes a