miRNA screening reveals a new miRNA family stimulating iPS cell generation via regulation of Meox2

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Induced pluripotent stem cells (iPSCs) can be generated by overexpression of Oct4, Sox2 and Klf4 in murine fibroblasts. By conducting a microRNA (miRNA) library screen, we identified a set of miRNAs critically regulating iPSC formation. We revealed a new miRNA family (miR-130/301/721) as an important regulator of iPSC induction by targeting the homeobox transcription factor Meox2 (also known as Gax). Meox2-specific silencing mimicked the effects of this miRNA family on reprogramming. Mechanistically, miRNA-resistant Meox2 overexpression abrogated effects of miR-130/301/721 on reprogramming. In conclusion, the miRNA family miR-130/301/721 enhances iPSC generation via repression of Meox2.

Keywords: induced pluripotent stem cells; Meox2; miRNAs; reprogramming

EMBO reports (2011) 12, 1153–1159. doi:10.1038/embor.2011.176

INTRODUCTION

Induced pluripotent stem cells (iPSCs) can be generated by ectopic overexpression of the transcription factors Oct4, Sox2, Klf4 and c-Myc (Takahashi & Yamanaka, 2006). Although c-Myc is dispensable for iPSC generation from murine embryonic fibroblasts (MEFs; Wernig *et al.*, 2008; Nakagawa *et al.*, 2010), reprogramming with even fewer factors is possible using other cells of origin (Kim *et al.*, 2009). Certain microRNAs (miRNAs),

such as the miR-290 (Judson *et al.*, 2009) and miR-302 (Lin *et al.*, 2010) clusters, are inducers of iPSC generation. Additionally, the miR-302/367 cluster reprograms mouse and human somatic cells to an iPSC state without a requirement for further exogenous factors (Anokye-Danso *et al.*, 2011). Thus, deciphering targets of key miRNAs interfering with the efficacy of iPSC generation might unravel new molecular mechanisms underlying reprogramming of somatic cells. As iPSC generation involves a mesenchymal-to-epithelial transition (MET), factors inducing MET or blocking epithelial-to-mesenchymal transition by suppressing transforming growth factor- β (TGF- β) signalling are important (Li *et al.*, 2010; Wang *et al.*, 2010). Indeed, miR-17, -93, -106a and -106b target the TGF- β receptor II during reprogramming (Li *et al.*, 2011) and miR-200 and miR-205 function in a bone morphogenetic protein–miRNA–MET axis during reprogramming (Samavarchi-Tehrani *et al.*, 2010). MiR-302 and miR-372 accelerate MET during reprogramming and block TGF- β -induced epithelial-to-mesenchymal transition of human epithelial cells (Subramanyam *et al.*, 2011).

Here, by transfecting a miRNA library covering 379 murine miRNAs, we investigated whether additional miRNAs improve reprogramming during the early phase of iPSC generation from MEFs and elucidated further molecular pathways involved in the induction of pluripotency. We identified a newly characterized miRNA family consisting of miR-130, miR-301 and miR-721 specifically targeting the transcription factor Meox2, knockdown of which facilitates and overexpression of which blocks reprogramming.

RESULTS AND DISCUSSION

Novel miRNAs involved in reprogramming

To identify miRNAs hitherto not described to be involved in the induction of pluripotency, we conducted a library screen including 379 murine precursor miRNAs. MEFs derived from OG2 mice (Oct4 promoter-driven green fluorescent protein (GFP) expression; Szabo *et al.*, 2002) were transduced with a polycistronic lentiviral construct encoding the human

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Fig 1 | Impact of miRNA modulation on iPS cell generation. Transfection of a miRNA precursor library to MEFs transduced with a lentiviral OKS reprogramming cassette altered iPS cell generation (A, 7 days p.t.; B, 8 days p.t.). Oct4-GFP⁺ iPS colonies were counted. (C,D) Validation of miRNA screening by transfection of identified miRNAs supporting iPS cell generation (fourfold induction versus control). Oct4-GFP⁺ colonies were counted (C, 7 days p.t.; D, 8 days p.t.); $n=3$ experiments. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. (E) Whole-well imaging demonstrating the effect of the iPS-stimulating miRNA 721 compared with the Scr. Scale bar, 2 mm. GFP, green fluorescent protein; iPS, induced pluripotent stem cell; MEF, murine embryonic fibroblast; miRNA, microRNA; OKS, Oct4/Klf4/Sox2; p.t., post transduction; Scr, scrambled control.

complementary DNAs of the reprogramming factors Oct4, Klf4 and Sox2 (OKS), separated by 2A-peptidase motifs and an internal ribosomal entry site-coupled *dTomato* reporter gene. This lentiviral vector efficiently induces iPS cell generation in MEFs within less than 9–10 days (Warlich *et al*, 2011) even in the absence of c-Myc, which was omitted because of its potential tumorigenicity (Nakagawa *et al*, 2010).

Transduced fibroblasts were distributed to 24-well plates and after 12 h wells were individually transfected with precursor miRNAs, achieving a high transfection rate (supplementary Fig S1A,B online). Counting of GFP⁺ colonies 7 and 8 days after transduction revealed a profound increase of reprogramming efficacy in some wells, whereas other wells showed no differences or even lower colony numbers in comparison with scrambled-RNA transduced wells. MiRNAs described for enhancing iPS cell generation, such as certain miR-290 and miR-302 cluster members (Lin *et al*, 2010; Judson *et al*, 2009; Li *et al*, 2010; Subramanyam *et al*, 2011) were re-identified in our screen (Fig 1A,B; supplementary Table S1 online). Using a fourfold

threshold of increased reprogramming events, however, we identified an additional 14 miRNAs, which were unknown to enhance iPS cell generation at the time the screen was conducted. Meanwhile, other reports have demonstrated enhanced iPS cell generation using some of these miRNAs (Li *et al*, 2011; Liao *et al*, 2011), but miR-130a/b, -148a, -152, -190, -301, -301b, -669b and -721 have not been described in this context. To validate the reprogramming stimulating miRNAs, we repeated experiments for all 14 selected miRNAs and robustly confirmed all miRNA candidates. The miRNA-based induction of iPS cell generation was highly significant for all investigated miRNAs 7 and 8 days after transduction compared with the scrambled controls (Fig 1C,D). Furthermore, as depicted for miR-721 in comparison with scrambled miRNA treatment, most emerging iPS colonies also exhibited an increased size and fluorescence intensity (Fig 1E). In summary, our miRNA screen generated robust and reproducible data and identified several miRNAs, which have not been associated with the induction of pluripotency.

Validation of miRNA candidates

The activation of GFP expression in OG2 mice has been proven to reliably indicate early reprogramming events not only in transcription factor-based reprogramming (Kim *et al*, 2009) but also in somatic cell nuclear transfer (Boiani *et al*, 2002). However, we sought to further characterize the early GFP⁺ colonies as indicators of real reprogramming events. For a representative analysis of reprogramming events in each individual well, we performed immunocytochemistry for the stage-specific embryonic antigen 1 (SSEA1), which is activated during the early steps of iPSC generation. We verified co-expression of GFP and SSEA1 in OG2-MEFs undergoing miRNA-facilitated reprogramming, and similar numbers of GFP⁺ and SSEA1⁺ colonies were detected (Fig 2A), supporting the use of Oct4-GFP expression as a valid indicator for iPSC emergence. As SSEA1 alone is not a sufficient iPSC marker, we performed further analyses of three representative colonies from each well of all tested 15 experimental conditions (including scrambled control) on day 8 after transduction. Using quantitative reverse transcriptase PCR, we analysed reactivation of endogenous Nanog, which is a robust molecular marker for *bona fide* iPSC generation (Gonzalez *et al*, 2011). Expression of Nanog was comparable in all miRNA-treated iPSC colonies and in scrambled controls (Fig 2B). In addition, we expanded full iPSC lines from all experimental conditions and analysed expression of Nanog, Oct4 and Sox2 by quantitative reverse transcriptase PCR. As shown in Fig 2C, the vast majority expressed the pluripotency factors at levels comparable with those in OG2 embryonic stem cells. Next, we analysed silencing of the lentiviral reprogramming cassette. Exogenous human Oct4 expression was significantly reduced in all emerging iPSC colonies (except mir-19b) compared with the initially transduced cells at day 4 (Fig 2D). As the reprogramming factors are expressed from a polycistronic construct, reduction in exogenous Oct4 expression indicates silencing of the entire lentiviral provirus. Profound silencing of the reprogramming cassette was confirmed in all established iPSC lines derived from miRNA-treated MEFs (Fig 2E) and visualized by fluorescence images depicting loss of dTomato expression in colonies that activated the Oct4-GFP reporter construct (Fig 2F). Taken together, these results strongly support the fully pluripotent phenotype of the generated iPSC lines, and we considered *in vivo* tests such as teratoma formation not to be feasible for all individual iPSC lines.

Target evaluation of the miRNA family miR-130/301/721

Among miRNAs, which enhance iPSC generation, we identified miR-130a, -130b, -301, -301b and -721 as a miRNA family, sharing identical nucleotides 2 to 7 (seed sequence) important for target recognition within the 3'-untranslated region (UTR; Fig 3A). Inline, bioinformatic target predictions suggested similar sets of potential target genes (supplementary Table S2 online). We focused on miR-130b, miR-301b and miR-721 for further analyses, as we assumed similar effects on downstream effectors within this miRNA family. To exclude the possibility that miRNA-mediated effects on reprogramming were caused by altered cell proliferation, we measured the influence of miRNA transfection on the proliferative capacity of MEFs and during the first days of iPSC generation. No significant difference between the treatment groups was observed, suggesting enhance-

ment of iPSC generation by miR-130b, miR-301b and miR-721 to be independent from proliferation (supplementary Fig S1C online). Next, we evaluated endogenous levels of the miR-130b/301b/721 family during OKS-mediated reprogramming (Fig 3B). MiR-130b and -301b were upregulated during the first days of iPSC generation; however, endogenous expression of miR-721 did not increase. The positive impact of the miRNAs 130b, 301b and 721 on iPSC generation was independent of the presence of the histone deacetylase inhibitor valproic acid, although the total number of colonies was lower in all treatment groups without valproic acid (supplementary Fig S1D online).

To identify downstream targets of miR-130b, -301b and 721, we focused on genes encoding transcription factors and chromatin remodellers. Thus, we analysed protein levels of the putative targets Foxo1a, Creb1, Mier1 and Meox2 upon miRNA overexpression in MEFs. Foxo1a, Creb1 and Mier1 were not significantly repressed by ectopic miRNA levels (Fig 3C,D). In contrast, miR-130b, -301b and -721 significantly downregulated protein expression of the transcription factor Meox2, also known as Gax (Perlman *et al*, 1998). Of note, Meox2 repression was not observed at mRNA level (supplementary Fig S1E online), indicating that translational inhibition rather than mRNA degradation is the main mechanism of Meox2 repression by miR-130b, -301b and -721. Meox2 is a homeobox transcription factor and has been associated with limb and somite development (Reijntjes *et al*, 2007). Furthermore, Meox2 has been demonstrated to negatively regulate angiogenesis, which has been linked to downregulation of nuclear factor- κ B signalling in endothelial cells (Patel *et al*, 2005). In addition, Meox2 exerts regulatory effects on cell cycle progression by directly activating the tumour suppressor p21 (Chen *et al*, 2007). However, a potential role of Meox2 in cellular reprogramming was previously unknown. To further validate direct miRNA binding in the 3'-UTR of Meox2, we cloned the respective region encompassing repetitive binding sites for miR-130b, miR-301b and miR-721 into a luciferase reporter vector system as described before (Thum *et al*, 2008) (Fig 3E), which was co-transfected with miRNAs into HEK293 reporter cells. With our current approach, we were not able to perform *luciferase* gene reporter assays in MEF cells because of low transfection efficacy. In comparison with scrambled miRNA treatment, luciferase enzyme activity was significantly lowered when miR-130b, -301b and -721 were transfected (Fig 3F). The combined administration of all three miRNAs did not result in a cumulative effect, which might be explained by the shared seed sequence of all three miRNAs. When performing the luciferase reporter experiments using constructs with mutated miRNA-binding sites, the effect of the miRNAs on luciferase activity was completely abolished. Thus, all three miRNAs (miR-130b, miR-301b and miR-721) specifically bind to the 3'-UTR of Meox2, thereby inhibiting Meox2 expression. We also conclude that miRNA binding to the 3'-UTR must be rigid due to the presence of two neighbouring miRNA-binding sites. In endothelial cells, Meox2 has previously been described as a miR-130- (Chen & Gorski, 2008) and miR-301-responsive target (Cao *et al*, 2010). However, in the context of iPSC generation, the role of Meox2 has not yet been described, and we thus performed further experiments to validate the impact of Meox2 on reprogramming.

ging iPSC colonies in OKS-transduced MEFs after downregulating Meox2 (Fig 4C). However, the effect of Meox2 silencing was less pronounced than that of the three individual miRNAs. Thus, other miRNA downstream effectors might have additional beneficial effects on iPSC generation. Again, the higher number of iPSC colonies in these experiments was not a result of increased proliferation rates, as Meox2 knockdown did not accelerate proliferation of MEFs under our experimental conditions (Fig 4D). To confirm complete reprogramming, we analysed expression of Nanog, Oct4 and Sox2 in an iPSC line derived from MEFs transfected with Meox2 siRNA, which showed robust reactivation of endogenous pluripotency factors and efficient silencing of the

Fig 3 | Evaluation of predicted miR-130b/301b/721 targets. (A) Murine miRNA family members 130a/b/301/301b/721 share an identical seed sequence (details obtained from <http://www.targetscan.org>). (B) Time course for miRNA expression during reprogramming of MEFs. Expression of miRNAs 130b/301b/721 was analysed in OKS-transduced MEFs at different time points by qRT-PCR. Untransduced MEFs are designated as Ctr; $n = 3$ experiments per group. * $P < 0.05$ and ** $P < 0.05$. (C) Exemplary western blots for bioinformatically predicted targets of miR-130b/301b/721 in MEFs transfected with 50 nM precursor miRNA. (D) Densitometric analysis for western blots of putative miR-130b/301b/721 target genes; $n = 3$ transfections per group. ** $P < 0.01$, *** $P < 0.001$. (E) Binding of miRNAs to Meox2-3'-UTR region. (F) Luciferase gene reporter assays in HEK293 cells confirmed miRNA binding to murine Meox2-3'-UTR. Reporter gene assay was performed with wild-type and mutated Meox2-3'-UTR; $n = 3$ experiments per group. * $P < 0.05$ and ** $P < 0.01$. D, day; FC, fold-change; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MEF, murine embryonic fibroblast; miRNA, microRNA; Mut., mutated; OKS, Oct4/Klf4/Sox2; qRT-PCR, quantitative reverse transcriptase PCR; Scr, scrambled miRNA; UTR, untranslated region.

lentiviral reprogramming cassette (Fig 4E). Taken together, siRNA-mediated Meox2 knockdown mimicked the effects of miR-130, -301b and -721. To further underline the functional relationship between these miRNAs and Meox2 during reprogramming, we tested whether the miRNA-mediated effects on iPSC generation were abrogated by ectopic overexpression of Meox2. To this end, we constructed a lentiviral vector expressing Meox2 from a SFFV (spleen focus-forming virus) promoter/enhancer. This vector yielded consistent Meox2 overexpression in MEFs as monitored on day 4 after transduction (Fig 4F). It is noteworthy that the construct lacked a 3'-UTR and therefore was unresponsive to miRNA-130b/301b/721 modulation. Upregulation of Meox2 significantly blocked the enhancing effects on reprogramming efficacy mediated by miR-130b, -301b and -721 (Fig 4G), although we cannot rule out the possibility that other

targets of this miRNA family contribute to iPSC formation. Thus, our data suggest that decreased Meox2 expression supports efficient iPSC generation and calls for efforts to elucidate Meox2 downstream targets. In the context of reprogramming, the interference of Meox2 with TGF- β signalling might be of particular importance (Valcourt *et al*, 2007), as suppression of TGF- β signalling is a key mechanism in iPSC generation (Ichida *et al*, 2009). Furthermore, a miRNA-mediated reduction in Meox2 levels might also overcome p21-mediated cell cycle arrest, which was described as a roadblock for iPSC generation (Hong *et al*, 2009).

In conclusion, by using a miRNA library screen we here identified a new set of miRNAs enhancing reprogramming of murine fibroblasts. Specifically, the miRNA family miR-130b/301b/721 strongly promotes cellular reprogramming, at least in

Fig 4 | Impact of direct Meox2 modulation on iPSC generation. (A) Meox2 expression in iPSCs derived from MEFs or murine BM cells; $*P < 0.05$. (B) Western blot for Meox2 expression in MEFs 72 h after transfection of scrambled or Meox2-siRNAs; $n = 3$. $*P < 0.05$. (C) Meox2 silencing enhanced iPSC generation; $n = 3$, s.d. depicted. $***P < 0.001$. (D) Proliferation of MEFs after scrambled or Meox2-siRNA transfection; $n = 3$. $*P < 0.05$. (E) Analysis of pluripotency factor expression in an iPSC line derived from OG2-MEFs treated with anti-Meox2-siRNA. (F) Lentiviral Meox2 overexpression construct (vector map depicted; left). Western blot of Meox2 and vinculin (housekeeping control) after Meox2 overexpression. (G) Meox2 overexpression blocked stimulating effects of the miRNA-130b/301b/721 family on iPSC generation; $n = 3$, s.d. depicted. $*P < 0.05$ and $**P < 0.01$. BM, bone marrow; cPPT, central polypurine tract; FC, fold-change; GFP, green fluorescent protein; iPSC, induced pluripotent stem cell; MEF, murine embryonic fibroblast; miRNA, microRNA; RRE, rev-responsive element; Scr, scrambled control; SFFV, spleen focus-forming virus promoter; SIN, self-inactivating element; siRNA, short interfering RNA; wPRE, woodchuck-hepatitis virus posttranscriptional regulatory element.

part, by repression of the common target Meox2. Hence, our data provide a valuable source for a better understanding of miRNA-mediated regulation of cellular reprogramming.

METHODS

iPSC generation. MEFs were obtained from OG2 fetuses (13 days post-coitum). To generate iPSCs, 8×10^6 MEFs were

transduced with the lentiviral OKS construct and $8 \mu\text{g}/\mu\text{l}$ protamine sulphate at a multiplicity of infection of 15 for 12 h as described previously (Warlich *et al*, 2011). Next, 20,000 transduced MEFs per well were seeded in 24-well plates 12 h before miRNA transfection. At day 4, 50% MEF medium/50% embryonic stem cell medium was applied. Two mM valproic acid (Sanofi-Aventis) was added during days 2 to 7. Efficiency of iPSC

generation was calculated by dividing the numbers of GFP⁺ colonies by 20,000 input cells before individual emerging iPS colonies (5–10 of each experimental condition) were picked and expanded to obtain full iPS cell lines.

miRNA/siRNA transfection. Lipofectamine 2000 (Invitrogen) transfection of siRNAs or miRNAs (supplementary Table S3 online) was done according to the manufacturer's instructions using 150 nM siRNA and 50 nM miRNA/anti-miRNA, respectively. Expression analysis of cells transfected with specific and control siRNAs/miRNAs/anti-miRs (Ambion or MWG-Biotech) was performed 24–72 h (siRNA) or 72 h (miRNAs) after transfection.

miRNA target prediction. The miRNA databases and target prediction tools Microcosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>), PicTar (<http://pictar.mdc-berlin.de/>) and TargetScan (<http://www.targetscan.org>) were screened to identify potential miRNA targets.

Statistical analysis. GraphPad Prism was applied to perform unpaired Student's *t*-test for two treatment groups or one-way analysis of variance following Bonferroni or Dunnett's post-test analysis for more than two groups. Unless otherwise stated, s.e.m. is plotted. Asterisks mean **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank Doreen Lüttge for technical assistance and Ulrich Martin for discussion and support of the project. Parts of the study were funded by the German Research Foundation (EXC 62/1; DFG-TH903/7-2), the DAAD and the German Federal Ministry of Education and Research (IFB-Tx, BMBF 01EO0802; 01GN0812; 01GM0854; and 01GP1007c).

Author contributions: N.P., J.F. and A.H. performed the experiments and evaluated the data with the help of A.S., T.C. and T.T. N.P., J.F., T.M., T.C. and T.T. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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