Ezh2 mediated H3K27me3 activity facilitates somatic transition during human pluripotent reprogramming

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Factor induced reprogramming of fibroblasts is an orchestrated but inefficient process. At the epigenetic level, it results in drastic chromatin changes to erase the existing somatic “memory” and to establish the pluripotent state. Accordingly, alterations of chromatin regulators including Ezh2 influence iPSC generation. While the role of individual transcription factors in resetting the chromatin landscape during iPSC generation is increasingly evident, their engagement with chromatin modulators remains to be elucidated. In the current study, we demonstrate that histone methyl transferase activity of Ezh2 is required for mesenchymal to epithelial transition (MET) during human iPSC generation. We show that the H3K27me3 activity favors induction of pluripotency by transcriptionally targeting the TGF-β signaling pathway. We also demonstrate that the Ezh2 negatively regulates the expression of pro-EMT miRNA's such as miR-23a locus during MET. Unique association of Ezh2 with c-Myc was required to silence the aforementioned circuitry. Collectively, our findings provide a mechanistic understanding by which Ezh2 restricts the somatic programme during early phase of cellular reprogramming and establish the importance of Ezh2 dependent H3K27me3 activity in transcriptional and miRNA modulation during human iPSC generation.

Forced expression of the transcription factors Oct4, Sox2, Klf4 and c-Myc (OSKM) alter the fate of somatic cell to a pluripotent state1-3. These induced pluripotent cells iPSCs share molecular and functional features of embryonic stem cells ESCs and therefore hold great promise for understanding human development and disease4. Although a variety of somatic cell types can be reprogrammed, our current understanding of the molecular mechanisms and cellular nature of reprogramming is almost exclusively derived from fibroblasts5-8. At the chromatin level, reprogramming of fibroblasts is initiated by inhibition of somatic gene expression along with rapid acquisition of H3K4me2 on several promoters and enhancers of genes that are transcriptionally activated later during the reprogramming process9. This widespread remodeling of histone modifications acts as an immediate response and is consistent with the fact that the perturbation of somatic gene expression is a prerequisite for cellular reprogramming10. To accomplish such massive epigenomic changes, pluripotency transcription factors direct the recruitment of chromatin modulators to repress the fibroblast specific programme. In this regard, studies have documented that the deletion of repressive chromatin modulators such as Polycomb proteins (PcG), Ehm1 and Ehm2 inhibited iPSC generation while knockdown of their respective demethylases UTX, JmJD3 and JARID2c enhances the process11-13.

PcG proteins are comprised of multiprotein complexes PRC1 and PRC2 that are required for conveying cellular memory by transcriptional silencing of a subset of genes14. The PRC2 core is composed of the catalytic subunit Ezh2, non-enzymatic Suz12, and EED components that catalyzes the histone H3 methylation at lysine-27 residue14. Genome-wide binding of PRC2 in human and mouse pluripotent cells demonstrated binding overlap with pluripotency factors on the promoters of genes encoding developmental regulators that are required for lineage specification later during development15. Consistently, these genes are enriched for the domains containing repressive H3K27me3 and activating H3K4me3 that are deposited by polycomb (PcG) and trithorax (Trx) complexes, to hold the promoters of developmental regulators in a poised state16. The importance of PcG is underscored by the deletion of PRC2 components in mouse embryonic stem cells, which results in global de-
repression of target genes\textsuperscript{15,17,18} followed by spontaneous differentiation\textsuperscript{18}. Furthermore, genetic ablation of PRC2 components in mice results in developmental failures and early embryonic lethality\textsuperscript{19,20}. Ezh2 also plays an important role in maintaining the identity of multipotent adult hematopoietic, neural and muscle precursors stem cells\textsuperscript{21,22}. A recent report has demonstrated the increased expression of PRC2 components during mouse fibroblast reprogramming\textsuperscript{23}. Moreover, knockdown of PRC2 components, including Ezh2, Suz12 and Eed, have been shown to drastically reduce iPSC generation from human and mouse fibroblasts\textsuperscript{12,23–25}. Besides, the requirement of PRC2 components in factor-induced reprogramming is supported by Pereira et al.’s (2010) observation\textsuperscript{24} wherein PRC2 deficient ESCs failed to reprogram differentiated cells to pluripotency in heterokaryon assays.

Given the importance of the PcG protein complex in resetting the epigenetic barrier, it is necessary to uncover the mechanism by which these regulators govern iPSC generation. Therefore, in the current study we investigated the requirement of Ezh2 and its methyltransferase activity in human iPSC generation using gain/loss of function approaches and by using specific small molecular inhibitor of H3K27 activity. We discovered Ezh2 and its H3K27me3 activity represses pro-EMT signaling during reprogramming. We also determined that Ezh2 along with c-Myc assist in somatic transition by silencing TGF-\(\beta\) signaling and the miR-23a locus. Our data provides a mechanistic understanding by which Ezh2 overcomes the initial impediments of cellular reprogramming.

**Results**

**PRC2 components are induced during human fibroblasts reprogramming.** We first decided to verify the kinetics of PRC2 expression during human iPSC (hiPS) generation. Towards this hFibs (human fibroblasts) were transduced with OSKM and cultures were monitored for phenotypic changes by phase contrast microscopy along with molecular analysis. Consistent with previous reports\textsuperscript{7}, clusters of cells with epithelial morphology indicative of mesenchymal to epithelial transition (MET) appeared at day 7, which eventually matured to generate iPSC colonies by three weeks (Fig. 1a). These colonies were picked to establish the iPSC line. In addition, we assayed for Nanog expression, a key pluripotency marker required for the acquisition of complete reprogramming as positive control. We did not observe detectable Nanog transcripts in hFibs at day 4, 7 or 14. Nanog was expressed by day 21 and in an established iPSC line (Fig. 1b) demonstrating that our reprogramming timelines were in agreement with that reported by other groups. iPSC colonies generated from hFibs exhibited typical ESC morphology and expressed the cell surface marker Tra 1-60 well as the pluripotency transcription factors Oct4 and Nanog (Supplementary Fig. 1a–c). H9-hESC obtained from WiCell Research Institute were used as a positive control for Tra 1-60, Oct4 and Nanog staining.

Transcript profiling of polycomb members over the same time course indicated an increase in Suz12 and Eed expression as early as day 4 post OSKM introduction, while Ezh2 upregulation was
noticed on day 7. Expression of all three polycomb members continued to increase until 21 days after addition of reprogramming factors (Fig. 1b). We also observed higher expression of Ezh2 in iPSCs compared to human fibroblasts (Fig. 1b). Ding et al.23 showed highest expression of three PRC2 components in established miPS and mES lines when measured against MEFs and MEFs undergoing reprogramming. However unlike mouse pluripotent cells, established hiPS and hES lines showed reduced expression of PRC2 components in comparison with advanced time courses in the reprogramming assay (Day 21) (Fig. 1c). While there are no reports comparing the amount of Ezh2 in mouse and human pluripotent cells, the differences between mouse and human pluripotent cells could be attributed to the naive vs. primed state and proliferation mode. Currently it is our speculation that Ezh2 expression is reduced once stable pluripotent lines are established from heterogeneous reprogramming cultures. Nonetheless, our studies revealed an increase in the kinetics of PRC2 expression during human fibroblasts reprogramming.

Ezh2 mediated H3K27me3 activity is critical in the early phase of reprogramming. To evaluate the role of Ezh2 in human iPSC generation we used a loss of function approach in the reprogramming assays. Validated shRNAs targeting the ORF (shEzh2 74) and 3’ UTR (shEzh2 75) of Ezh2 (Supplementary Fig. 2a–c) were transduced in hFibs prior to OSKM transduction (schema shown in Supplementary Fig. 2d). Using three biological replicates and three experimental replicates we observed a 0.05% frequency of iPSC generation from Wt and shCnt hFibs (Supplementary Fig. 1e) and no colony formation in hFibs transduced with shEzh2-74 or shEzh2-75. Investigations into the molecular details of shEzh2-transduced cells demonstrated an increased expression of p53, p21, and p19 both at the transcript and protein levels (Supplementary Fig. 2f,g) along with slower growth rate and cellular senescence (Supplementary Fig. 2h). Accordingly, OSKM transduced shEzh2 hFibs cultures displayed flat morphology of cells, strong reduction in proliferation and noticeable cell death compared to OSKM transduced Wt and shCnt starting at day 4 (data not shown).

While there are several reports indicating removal of Ezh2 from somatic cells leads to senescence by induction of p53, p21 and ARF proteins26,27, it is not clear if inhibition of its H3K27me3 activity would have the same senescence-inducing effect on hFibs. Thus we studied the effect of GSK-126 a small molecule inhibitor of Ezh2 on hFib cultures. Treatment of GSK126 inhibited H3K27me3 activity without altering Ezh2 protein levels in human fibroblasts (Fig. 2a). Similar to the effect of shEzh2, GSK increased the expression of p53 and p21, albeit at lower levels without affecting the cell shape or proliferation (Fig. 2b,c). Furthermore, GSK treated cells did not undergo cell senescence, which was detected by β-galactosidase staining (Fig. 2c). Collectively, these results suggest that the loss of Ezh2 versus abrogation of methyltransferase activity alone seems to have a differential effect on cell proliferation in somatic fibroblasts. This was particularly surprising since transduction of shRNA or treatment with inhibitors ultimately reduced overall H3K27me3 levels.

Ezh2 mediated H3K27me3 activity is implicated in the maintenance of bivalent chromatin domains in pluripotent cells. In addition
Ezh2 is abundantly expressed in both ESC and iPS cells. Therefore we determined the importance of Ezh2 and H3K27me3 activity in maintaining the pluripotent state. We transduced shEzh2 in Wt.iPSC and observed over 30% reduction of Ezh2 transcript (Supplementary Fig. 2i, j). Lowered levels of Ezh2 in iPSC had no appreciable difference in colony morphology and expression of pluripotency markers such as Tra 1-60 and Nanog (Supplementary Fig. 2j). Next we treated IMR iPSC and H9 hESC with DMSO or GSK. Similar to that seen in fibroblasts, GSK treatment reduced H3K27me3 methylation over 70% without influencing Ezh2 expression (Fig. 2d). Cultures were monitored for retaining the pluripotency marker Tra 1-60 at least three passages in presence of DMSO or GSK. Staining for pluripotency marker Tra 1-60 did not indicate loss of pluripotency in DMSO control and GSK treated hPSC cultures (IMR90 iPSC and H9 hESC) (Fig. 2e). Since GSK treatment did not affect hFib proliferation or pluripotency of hPSC we speculated that use of GSK in reprogramming assays would allow us to identify additional roles of H3K27me3 in iPSC generation.

Ezh2 mediated H3K27me3 activity inhibits Pro-EMT signaling.

Overcoming the blockade of TGF-β signaling is a hallmark of MET and is critical for pluripotent reprogramming. Accordingly, treatment of fibroblasts with TGF-β inhibitors improves iPSC formation. Based on our results suggesting the important link between H3K27me3 activity and MET, we speculated that Ezh2 negatively regulate TGF-β signaling and thus suppresses EMT. Accordingly, among all the polycomb members, upregulation of Ezh2 coincided with diminished TGF-βR2 expression on day 7 of reprogramming (Fig. 3a left). We also observed overexpression of the epithelial marker E-cadherin (CDH1) on the 7th day of reprogramming (Fig. 3a right). These results are in alignment with our findings where MET was reduced from GSK treated hFibs (Fig. 2g,i). To validate the inverse correlation between Ezh2 and TGF-βR2 that was observed during reprogramming, we performed loss and gain of function experiments in human fibroblasts. Towards this we experimentally verified expression of members of TGF-β and BMP pathway in shEzh2 hFibs. Compared to shCnt, we observed increased expression of TGF-β R1 and R2 at both the transcript and protein levels while TGF-β R3 or TGF-β ligands remain unchanged (Fig. 3b,c and Supplementary Fig. 3a). Results of Ezh2 knockdown were confirmed by inhibitor treatment wherein GSK treatment increased
expression of TGF-β receptor expression (Fig. 3d). If differential expression of TGR-β receptors is directly linked to knockdown of Ezh2, and its H3K27me3 activity then overexpression should reverse this trend. To test this hypothesis, hFibs were transduced with retrovirus encoding Ezh2 transgene (Ezh2 OE). Molecular investigation at transcript and protein level identified increased Ezh2 expression associated with reversed levels of TGF-β receptors (Supplementary Fig. 3b–3c). In contrast to knockdown, Ezh2 overexpression decreased the expression of TGF-βR1 and R2 suggesting that these receptors are direct targets of Ezh2 mediated H3K27me3 activity (Supplementary Fig. 3d–3e).

Activation of the TGF-β pathway involves phosphorylation of Smads that ultimately activate the mesenchymal transcription factor Snail. Accordingly, we also detected phosphorylation of Smad2 (Fig. 3e) along with increased mRNA expression of Twist and Snail in sh-Ezh2 transduced hFibs (Fig. 3f). Expression of epithelial E-cadherin (CDH1) is indicative of MET initiation during the reprogramming process and is negatively correlated with TGF-β activation. We observed downregulation of E-cadherin (CDH1) in shEzh2 hFibs (Fig. 3f). These results confirm the Ezh2 depletion releases the repression on TGF-β receptors thereby activating downstream TGF-β signaling. Overall our results demonstrate that Ezh2 mediated H3K27me3 activity restricts TGF-β signaling thereby dictating the reprogramming outcome.

Unlike TGF-β signaling, components of the BMP pathway such as BMP receptor I/II synergize with OSKM to promote iPS generation29. We observed reduced transcript levels of BMP receptor II and I in shEzh2 hFibs (Supplementary Fig. 3f). Taken together, our results demonstrate Ezh2 mediated H3K27me3 activity regulate fibroblast specific gene expression programme by restricting TGF-β signaling. Nonetheless, these results support our model that Ezh2 and its H3K27me3 promotes reprogramming by inhibiting EMT.

Inhibition of TGF-β signaling rescues the H3K27me3 mediated initiation impairment during reprogramming. After establishing that H3K27me3 activity is required to suppress TGF-β signaling and thus pro-EMT signaling, we tested if inhibition of TGF-β signaling is sufficient to rescue the initiation defect from shEzh2 or GSK treated hFibs. To test this hypothesis, reprogramming experiments were performed in the presence or absence of TGF-β inhibitor SB431542 (SB) as indicated in the schema (Supplementary Fig. 4a). While control hFibs showed the appearance of E-cadherin positive colonies around day 7, shEzh2 hFibs or shEzh2 hFibs treated with SB did not show changes in-vitro (Supplementary Fig. 4b). Molecular analysis indicated that shEzh2 transduced hFibs continued to show reduced Ezh2 and increased TGF-βR1/R2 transcript irrespective of SB treatment (Supplementary Fig. 4c). Moreover we observed enhanced CDH1 mRNA in SB treated shEzh2 treated hFibs indicating that the programme associated with MET was set in place upon inhibition of TGF-β signaling (Supplementary Fig. 4c). Further probing into reasons for the lack of MET in shEzh2 treated with SB indicated transient recovery of cell senescence (Supplementary Fig. 4d) and eventual growth crises due to enhanced p21 levels (Supplementary Fig. 4e).

Since we demonstrated removal of Ezh2 protein vs. inhibition of PRC2 activity has differential effects in terms of induction of cell senescence in human fibroblasts (Fig. 2 and Supplementary Fig. 2), we tested whether SB can reverse the impaired MET from GSK treated hFibs. Towards this we added SB and GSK to OSKM transduced cultures as indicated in the schema (Fig. 4a). Interestingly GSK + SB treated fibroblasts showed similar number of E-cadherin

Figure 4 | Inhibition of TGF-β signaling rescues the H3K27me3 mediated MET defect. (a) Schematic representation for OSKM transduction, GSK and SB treatment during hFib reprogramming. (b) E-Cadherin DAB staining of OSKM transduced cultures treated with indicated inhibitors. (c) Total number of E-cadherin positive colonies from OSKM, OSKM+GSK and OSKM+GSK+SB cultures. (d) Relative expression of indicated mRNAs from OSKM, OSKM+GSK and OSKM+GSK+SB reprogramming cultures.
positive colonies as untreated hFibs (Fig. 4b,c), indicating that MET defects due to H3K27me3 inhibition could be rescued by inhibition of downstream TGF-β signaling. Molecular analysis of fibroblast specific markers such as Twist and Snail showed elevated expression upon GSK treatment and their levels went down in GSB1SB treated cells (Fig. 4d). In alignment with our rescue data upon SB treatment, we also observed elevated levels of epithelial marker Occludin in GSK1SB treated cultures (Fig. 4d). Cumulatively our studies have established that H3K27me3 activity is required to suppress the fibroblast specific programme setup by TGF-β signaling in order to facilitate a mesenchymal to epithelial transition.

Ezh2 interacts with c-Myc to suppress TGF-β Signaling. To dissect the molecular mechanism by which Ezh2 controls iPSC generation, we studied its interaction with pluripotency factors. Protein lysates from OSKM transduced hFibs were collected and subjected to Ezh2 immunoprecipitation [IP]. Western blot analysis followed by Ezh2 IP showed the presence of c-Myc but not Oct4 and Sox2 (Fig. 5a). Such interactions were not seen in control IP IgG reactions indicating the specificity of the immunoprecipitation (Fig. 5a). Next we investigated if Ezh2 associates with OSM factors in pluripotent cells. To address this we performed immunoprecipitation using control, Ezh1 or Ezh2 antibodies using H9 hESC cell lysate. Our results displayed no interaction between Ezh2 and Sox2, however there was weak but detectable amount of Oct4 and c-Myc immunoprecipitated with Ezh2 in pluripotent cells (Fig. 5b). These results suggest that Ezh2 interacts with c-Myc in human fibroblasts undergoing reprogramming and in pluripotent cells. Because Ezh2 trimethylates lysine 27 on histone3 and transcriptionally represses gene expression, we tested if OSM factors forms a complex with Ezh2 on the TGF-βR2 promoter. We did not observe binding of Oct4 but surprisingly Sox2 was recruited on the TGF-βR2 promoter in day 4 cultures. Sox2 binding was independent of c-Myc-Ezh2 occupancy, which was noticed exclusive at day7 of reprogramming. This result indicates that Sox2 precedes c-Myc and Ezh2 binding on TGF-βR2 locus (Fig. 5c). Amplification of control CDH1 from OSM and Ezh2 immunoprecipitates did not reveal Ezh2 associated repressive complex on the CDH1 promoter at any time point studied (Fig. 5d). Assessment of histone modification status confirmed the presence of H3K27me3 modification on TGF-βR2 but not on CDH1 promoter at day 7 of reprogramming (Fig. 5e). Heavy enrichment of K27me3 mark coincided with downregulation of receptor activity in early time course of reprogramming (Fig. 3). Together, our results demonstrate that Ezh2 transcriptionally represses TGF-βR2 promoter and suppress EMT.

Ezh2 mediated H3K27me3 activity regulates miR-27a expression during the early phase of reprogramming. MicroRNAs play crucial roles in MET of somatic reprogramming and are extensively regulated by PRC2 complex members31,32. Therefore, we reasoned that depletion of Ezh2 in hFibs Ezh2 might alter miRNA expression thereby influencing the reprogramming process. In order to gain further insights into this we performed small RNA deep sequencing analysis, using biological replicates of shCnt and shEzh2 transduced hFibs. The Pearson coefficient of R > 0.96 shown in Supplementary Fig. 5a–c demonstrated the strong correlation between the biological duplicates. The statistical significance of miRNA expression and fold change was calculated using DESeq and EdgeR (Fig. 6a and Supplementary Table 1 and 2) programmes. miRNAs with p < 0.05 in both DESeq and EdgeR
analysis were considered for pathway analysis using the Diana miRPath pathway analysis programme. This interrogation revealed a list of signaling pathways that changed significantly upon Ezh2 depletion (Supplementary Table 3). Specifically, genes implicated in cell cycle, p53 and TGF-β signaling were targeted by miRNAs upon Ezh2 depletion (Fig. 6b). This is of particular interest since representative pathways are strongly implicated in pluripotent cells or in iPSC generation (Fig. 6b) and were upregulated upon depletion of Ezh2 in our studies.

Further analysis revealed widespread differences in the abundance of several microRNAs including those that have been previously associated with regulating EMT and MET (Supplementary Fig. 5d). Among those identified miR-23a and miR-27a that belong to miR-23 cluster were overrepresented following Ezh2 knockdown (Fig. 6b). Interestingly, miR-23a and miR-27a are known to promote EMT in cancers33,34. Since Ezh2 depletion upregulated TGF-β signaling thereby activating pro-EMT genes (Snail and Twist), it was intriguing to study if Ezh2 regulated the pro-EMT miR-23 cluster directly.

Taqman qRT-PCR assays demonstrated increased expression of miR-27a and miR-23a in Ezh2-deficient hFibs confirming our sequencing data (Fig. 6c Upper and Lower panel). If differential expression of miR-23a and miR-27a is directly linked to the knockdown of Ezh2, then overexpression should reverse this trend. As expected, overexpression of Ezh2 reversed the transcript profile of miR-27a and miR-23a (Fig. 6c Upper and Lower panel).

To address the differential activity of depletion of Ezh2 vs inhibition of H3K27me3 activity on expression of miR-23a and miR-27a miRNAs, we performed qPCRs in DMSO and GSK treated cells. Our results indicate enhanced expression of miR-23a and miR-27a in GSK treated cells (Fig. 6d Upper and Lower panel). These results are consistent with our findings demonstrating that the miR-23 cluster is regulated by H3K27me3 activity of Ezh2 in human fibroblasts.

To verify if miR-23a and miR-27a miRNAs are suppressed during the initiation of iPSC generation, we studied their expression pattern during reprogramming. Specifically, we focused on the day 7 time-point due to molecular changes in Ezh2 expression and initiation of MET as evidenced by emergence of epithelial cells in culture. We detected reduced expression of miR-27a and miR-23a at day 7, which remained downregulated in iPSC cells (Fig. 6e Upper and Lower panel). Downregulation of miR-23a and miR-27a is in alignment with suppression of EMT and acquisition of an epithelial state in iPSC generation. To further confirm that Ezh2 regulates miR-23a and miR-27a expression in reprogramming, we monitored Ezh2 binding and enrichment of H3K27me3 marks on the miR-23 locus in hFbs, OSKM transduced hFibs at day7 and iPSCs. Chromatin IP for Ezh2 and H3K27me3 was performed in hFbs, OSKM transduced hFibs at day7 and iPSC. Two Kilobase upstream sequence of miR27a was amplified from immunoprecipitated material.

To test the functional role of Ezh2 regulated miR-27a in cellular reprogramming, miR-27a was overexpressed in human fibroblasts (Supplementary Fig. 5e). RT-PCR analysis indicated a four-fold increase in miR27a levels in miR-27a overexpressed hFibs compared to control hFibs. This is of particular interest since these miRNAs are strongly implicated in pluripotent cells or in iPSC generation and were upregulated upon depletion of Ezh2 in our studies.

Figure 6 | Ezh2 regulates miR-27a expression during human fibroblasts reprogramming. (a) Differentially expressed miRNAs in sh-Ezh2 hFibs calculated by Edge R and DESeq programmes. (b) Histogram depicting the number of miRNA regulating indicated pathways in shEzh2 hFibs. (c) qPCR results of indicated miRNA expression levels in shCnt, shEzh2 and Ezh2 OE hFibs. (d) qPCR results of indicated miRNA expression levels in DMSO control and GSK treated hFibs. (e) Expression profile of indicated miRNA in hFibs, OSKM transduced hFibs on day7 and iPSCs. (f) Chromatin IP for Ezh2 and H3K27me3 was performed in hFbs, OSKM transduced hFibs at day7 and iPSC. Two Kilobase upstream sequence of miR27a was amplified from immunoprecipitated material. (g) Schematic representation of miR-27 plasmid overexpression and OSKM transduction. E-cadherin DAB staining was performed 10 days post introduction of reprogramming factors. (h) E-cad DAB staining in H9 hESC, control and miR-27a overexpressing hFibs.
to control or untransfected hFib (Supplementary Fig. 5f). We also observed increased expression of Snail and reduced levels of CDH1 in miR-27a overexpressing cells (Supplementary Fig. 5f) compared to controls, indicative of alleviated pro-EMT signaling. Next we directly assayed the role of miR-27a in initiation of reprogramming. Control or miR27a transfected cultures were transduced with OSKM and stained with E-cadherin antibody as shown in the schema (Fig. 6g). Phenotypic analysis combined with E-cadherin staining indicated emergence of E-cadherin positive cells with an epithelial morphology in control cultures while no such changes were noticed in miR-27a overexpressing cultures (Fig 6h). Molecular analysis using transcript profiling confirmed enhanced expression of miR-27a and Snail combined with diminished CDH1 transcripts in miR27a overexpressing reprogramming cultures compared to control (Supplementary Fig. 5g). These results demonstrate that increased miR-27a expression favors the EMT signaling during initiation of reprogramming and acts as an inhibitory microRNA. Taken together our findings confirm that Ezh2 mediated H3K27 activity negatively regulates miR-27a in favor of a mesenchymal to epithelial transition.

Discussion

Maintenance of cellular identity is subjected to stringent regulatory mechanisms and safeguarded at multiple checkpoints. However, the successful generation of iPSCs has demonstrated that such strict integrity can be altered1–3. Recently, detailed molecular characterization of the reprogramming process has revealed distinct steps/waves involved in successful iPSC generation25. Among them, activation of mesenchymal to epithelial transition and inhibition of p53-mediated cell senescence are immediate responses following overexpression of OSKM25,26. Therefore, to initiate reprogramming, the ectopically expressed pluripotency factors and chromatin modulators must tackle the inhibitory pro-EMT signaling and suppress the stress-response induced by fibroblast via p53, p21 and Ink4/Arf pathways. Global occupancy of these factors revealed similar number of targets of OSK in both the waves, while Myc targeted genes were biased and mostly unregulated in the first wave26.

Multiple groups have documented the critical role of the chromatin regulator Ezh2 in mouse and human fibroblast reprogramming25,26, however the exact mechanism by which Ezh2 alters the epigenome is not clear and therefore an area of great interest. In this regard a recent study by Ding et al.24 documented that Ezh2 regulates iPSC generation in part by repressing the Ink4A/Arf locus. Our results are in agreement with Ding et al.’s observation wherein depletion of Ezh2 resulted in impaired iPSC generation by induction of p53/Arf pathways [Supplementary Fig. 2]. In the current study, we demonstrate an additional role for Ezh2 in human fibroblast reprogramming and provide mechanistic details by which c-Myc and Ezh2 restrain the somatic program and promote iPSC generation.

Enhanced expression of PRC2 throughout the reprogramming process was of particular interest since it provided us with the hint regarding the contributions that repressive H3K27me3 might have in distinct phases of iPSC generation. MET of fibroblast, a key step in reprogramming is subjected to strict intrinsic barriers pro-EMT signaling posed by fibroblast specific transcription factors such as Twist and Snail. Experiments using a small molecule inhibitor of H3K27me3 allowed us to identify the epigenetic basis of regulation of EMT signaling in favor of MET during initiation of reprogramming. Our combined results from Ezh2 perturbation and early fibroblast reprogramming studies demonstrate a negative correlation between Ezh2 and TGF-β receptors and its downstream signaling in reprogramming time courses. Soufi et al. (2012)29 have demonstrated binding of OSM to the TGF-β promoter during the early hours of reprogramming. Consistent with this finding, we provide evidence of H3K27me3 marks along with localization of an Ezh2, c-Myc and Sox2 repressor complex on the TGF-β2 promoter. Further we demonstrate that Ezh2 modulates Snail expression via TGF-β signaling. Enhanced Snail expression in shEzh2 hFibs seems to repress CDH1 transcription. This assumption is supported by previous studies where Snail is shown to bind to CDH1 promoter and repress its transcription30. Our experiment demonstrating the rescue of MET defects upon inhibition of TGF-β signaling convincingly demonstrates the role of Ezh2 in inhibiting the fibroblast specific programme. Overall our work provides the mechanism for the initial action of c-Myc and Ezh2 to target chromatin sites to disrupt the existing somatic programme and to facilitate the somatic exit. Most importantly we identified previously unreported microRNAs miR-23a and miR-27a, as barriers in human iPSC generation. Binding of Ezh2 and localization of H3K27me3 marks on miR-23a locus further supports our claim that H3K27me3 activity is critical to negate miRNA mediated pro-EMT signaling. In conclusion, our data demonstrates that Ezh2 favors iPSC generation by directly repressing fibroblasts specific genes and also by miRNA mediated trans-regulatory mechanisms to keep these signaling pathways under control (Fig. 7).

Our observations are consistent with Onder et al.25; Buganim et al.26; Ding et al.27 studies demonstrating inactivation of Ezh2 inhibited cellular reprogramming. In contrast Fraga et al.31 did not observe impaired reprogramming upon genetic inactivation of Ezh2 and its catalytic activity. In addition they fail to notice the cellular senescence defects in Ezh2 deficient MEFs. Such observations may have been unnoticed due to lack of efficient Ezh2 depletion in early phase of reprogramming. As such we demonstrated the requirement of H3K27me3 activity in the inhibition of pro-EMT signaling. In the future, it would be important to determine how multiple epigenetic regulators collaborate with OSKM and work in concert to establish the epigenomic networks to govern differential patterns of permissive and non-permissiveness on gene loci during iPSC generation.

Experimental Procedures

Antibodies and Inhibitors. We used antibodies specific to the Ezh2 (Millipore #17-662 and #CS203195), c-Myc (9E10) (abcam ab32), Sox2 (abcam ab59776), H3K27me3 (abcam ab24604), Oct3/4 (c-10) (Santa Cruz Sc3279), Nanog (abcam ab21624), Pan H3 (ab791), GAPDH (abcam ab9485), CDKN2A/p14ARF (abcam ab470), TGF-β RI (C-4) (Santa Cruz Sc7791), TGF-β RI Ribonucleic Acid (C-16) (Santa Cruz Sc220), Normal Rabbit IgG (Millipore #12-370), Normal Mouse IgG (Millipore #12-371), p21 (F-5) (Santa Cruz sc-6246), Phospho-Smad2 (Ser465/467) (138D4) (Cell Signaling 3108), Smad2 (D3438) (Cell Signaling 5339), E-Cadherin (Abcam#4146), TGF-β1 inhibitor SB431542 was purchased from Stemgent (04-0010), H3K27me3 inhibitor GSK-126 was purchased from Xcesbio.

Cell Culture. Fetal human dermal fibroblasts were purchased from ScienCell and maintained in fibroblast medium (DMEM supplemented with 10% FBS, L-glutamine, NEAA). HEK-293, 293-LX and 293-AMPo cells were cultured in DMEM medium supplemented with 10% FBS, L-glutamine, NEAA and sodium pyruvate. DIEP-iPSC generated from fetal lung fibroblasts and H9 hESCs were purchased from WiCell and maintained in mTESR media on hESC grade matrigel. For generation of stable cell lines expressing Ezh2 shRNA (shEzh2), 1 μg of vector DNA was transfected into 293
cells using λ- lipofectamine 2000 reagent (Invitrogen). Forty-eight hours post transfections clones were selected for stable integration of plasmids using 1.5 μg/ml puromycin.

Viruses. Sendai virus encoding human Oct4, Sox2, c-Myc and Klf4 was purchased from Invitrogen (Cytotune- A1378001). Lentiviral vectors pLKO.1 NTs Control shRNA (SHRNA-00216), pLKO.1 EZh2 shRNA (SHRNA-100074), plKO.1 Ezh2-75 shRNA (TRCR000100475), were purchased from Sigma. These vectors were co-transfected with psPAX2, pMDG2 in 293-LX packaging cell line using lipofectamine LTX (Invitrogen). Viral supernatants were harvested 48 h post transfection and concentrated using Amicon filters (Millipore). Human fibroblasts were transduced with control and EzH2 shRNA at MOI of 3 in presence of 8-9 ng/ml polybrene. shEzh2-75 referred as shEzh2 in the manuscript was used in almost all the experiments unless otherwise mentioned.

Retroviral MSCV-Ezh2 vector was purchased from Addgene. This vector was transfected in AmphiPack-293 cell line (Clontech). Viral supernatants were harvested 48 h post transfection. 2 ml of viral supernatant was used to transduce hFibs.

Transfections. For Ezh2 depletion or overexpression experiments, 20,000 hFibs were transduced. Forty-eight hours post viral infection cells were washed off with 1X PBS and DME and supplemented with fresh complete medium. Transduced fibs were cultured for another 48 hrs and analyzed in various experiments including PCRs, western blotting and senescence assays. In case of TGF-β signaling inhibition experiments, 2 μM SB431542 was added to the hFibs post 48hs of transduction with shRNAs. Cells were cultured for additional 48 h prior to analysis.

Inhibitor treatment. Equal number (20,000) of hFibs was seeded on 12 - well dishes. Twenty-four hours post seeding, H3K27me3 inhibitor GSK was added at 0.5 μM concentration. Treated cells were analyzed by RT- PCRs four days post treatment.

Induction of reprogramming. For reprogramming experiments 50,000 hFibs were seeded on matrigel coated wells. For reprogramming experiments hFibs were pre-transduced with NTS-control shRNA referred as shCnt, Ezh2 shRNA referred as shEzh2 and untransduced referred as Wh hFibs. Twenty-four hours post shRNA transduction hFibs were washed with knockout DMEM and transduced with OSKM (Cytotune kit Invitrogen) in complete hFib medium. OSKM transduced hFibs were switched to iPSC culture medium containing Knockout DMEM, 10% FBS, 1% Glutamine and 1% Penicillin-Streptomycin for 48 hrs. In case of TGF-β signaling inhibition experiments 2 μM SB431542 was added to the iPSC medium. Colonies were picked manually three weeks post reprogramming to establish iPSC lines. Later iPSC cultures were maintained in E8 and/or mTESR medium. In case of inhibition of H3K27me3 experiments 0.5 μM GSK was added to the iPSC medium.

Cloning of mir-27a. mir-27 was amplified from human genomic DNA with primers containing the appropriate restriction enzyme sites (Fw mir-27a-AgeI- 5'-GATCAGCAAGCTTTCACTCATTGACGG-3'; Bw-mir27a-HindIII - 5'-GATCAGCAACGGGCGAGGAGGGACA-3' ). The amplified product was cloned into the pre-mir-27 flank sequences and was cloned down stream to CB promoter with CMV enhancer in the plasmid containing AAV inverted terminal repeats.

Transfection of mir-27a. Following manufacturers instructions magnetic assisted transfection (Magnetofection Kit) of control or mir-27a was performed in human dermal fibroblast instructions (Osbiosciences Cat # NM51000). Transfection efficiency of 40% - 50% was standardized GFP expression using control pAAV-Vector.

Small RNA library Preparation and Sequencing. Small RNA libraries were prepared using the Illumina TruSeq Small RNA kit as described by the manufacturer (Illumina). 250 ng of total RNA was used from each sample for the library preparation. 5' and 3' Small RNA adaptors were ligated to the RNA and the ligated products were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). The reverse transcribed products were then amplified using various primer sets by PCR to the PCR product was concentrated using Amicon filters (Millipore). Human fibroblasts were transduced with shRNA encoding viruses. Cells were washed with PBS, fixed and stained using the Senescence Kit (Sigma). Stained cells were visualized using a Nikon Eclipse TE 2000-S microscope under bright field, and images were acquired using the Q Capture software.

Immunocytochemistry. hiPSC colonies were fixed in paraformaldehyde and permeabilized in Triton X-100 prior to Oct4 and Nanog staining. Cells were then stained with secondary antibody Alexa Fluor 488 anti-IgG (Molecular Probes). DAPI staining was performed to visualize the nucleus (Molecular probes). Cells were visualized using the Olympus IX81 fluorescence microscope and images were captured with the Q Capture software.

Western blotting and Immunoprecipitation. Cell extracts were prepared in RIPA buffer. Equal amount of protein was loaded for western blotting with indicated antibodies. For immunoprecipitation studies, protein A or protein G Dynabeads were used. Antibodies used: 1 μg of indicated antibodies. Approximately 400 μg of total extract was lysate from shCnt or shEzh2 stable lines overexpressing OSKM were subjected to immunoprecipitation. Beads were then washed three times with buffer containing 300 mM KCl and 0.1% (v/v) Nonidet P-40, and proteins were eluted in SDS loading dye and then subjected to western blotting.

Chromatin Immunoprecipitation. Chromatin IP was performed as described previously42. In brief cells were crosslinked using 1% formaldehyde and chromatin was digested in buffer containing 0.1% SDS to obtain fragments of approximately of 400 bp length. Sonicated DNA was subjected to immunoprecipitation using ChIP grade protein G Dynabeads (Invitrogen) and antibodies as described above. Chromatin immunoprecipitated DNA was further crosslinked, pooled and subjected to qPCR analysis using Platinum Syber Green-UDP mix. To calculate relative binding, we subtracted the signal observed in the control (IgG control) immunoprecipitation experiment from that observed with the specific antibody. Then we divided the resulting difference (IP specific – IP IgG) by the signal observed in the immunoprecipitation experiment from that observed with the specific antibody. For validation of miRNAs, 50 ng of total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). cDNA was synthesized by using specific primers for, miR -27a (002445), miR -23a (000399) or control U6 SmoRNA (001973) and amplified according to manufacturers instructions (Applied Biosystems). Subsequently, qPCR reactions were performed using 2X PCR Master Mix (Fermentas). Products were resolved on 1.2% agarose gels. Quantitative PCRs were performed using Platinum SYBR Green – UDP mix (Invitrogen). To calculate the relative expression, GAPDH mRNA was amplified as internal control. Primer sequences are provided in supplementary table 2 (Supplementary Table 4).

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Statistical Analysis. Results are presented as the mean ± SEM of at least 3 independent experiments unless stated otherwise. Significance levels were determined using 2-tailed Student’s t-tests where p value is indicated in the figure.

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Author contributions
S.R. conceptualized the project and designed the experiments. N.D. and R.R. performed experiments on lenti, retrovirus production, shRNA transduction, PCRs, immunostaining, immunoprecipitation and ChIP studies. S.R. and R.R. are responsible for iPSC generation and maintenance of iPSC lines. N.D. and A.K. performed the Ezh2 inhibitor studies. S.C. and J.G. cloned mir-27a for overexpression studies. D.P. is responsible for generating small RNA libraries and bioinformatics analysis of small RNA sequencing. S.R. and D.P. wrote the paper, discussed and commented on the manuscript.

Additional information
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