The Role of PRDM14 in Regulating Self-Renewal in Human Embryonic Stem Cells

1. Introduction

Embryonic stem cells (ESCs) are self-renewing, pluripotent cells generated from the inner cell mass at the blastocyst stage. The "self-renewing" property of ESCs refers to their capability to proliferate indefinitely, producing unaltered daughter cells exhibiting the same properties as the parent cell. The "pluripotent" property of ESCs signifies their potential, under certain conditions, to exit the self-renewal state and differentiate into specialised cell types from all three germ layers (ectoderm, mesoderm, and endoderm)[1,2,3]. These characteristics of ESCs hold immense promise for the development of novel cell-replacement therapies to treat tissue/organ damage from injury, disease, or congenital defects[3]. In theory, scientists could isolate and cultivate hESCs in vitro (1) to produce an unlimited supply of hESCs, and (2) to generate an unlimited supply of specialised cells used in regenerative therapies. However, one major limitation of hESCs that remains to be addressed is the inability for hESCs to undergo long-term self-renewal in vitro. This makes it difficult to maintain hESCs in culture for long periods of time without differentiating into specialised cell types, limiting their use in clinical applications[4]. As a result, a better understanding of the mechanisms underlying the self-renewal and pluripotency of hESCs is necessary to expand on their potential clinical applications.

Previous studies have shown that the self-renewing and pluripotent properties of ESCs are maintained by three core transcription factors: Oct4, Nanog, and Sox2[4,5]. These transcription factors are regulated by various signalling pathways, including the Wnt pathway, insulin growth factor (IGF) pathway, Fgf pathway, and TGF-β pathway Wnt pathway, LIF/Stat3 pathway, Notch pathway, hedgehog pathway, etc[5]. Among these pathways, the Wnt pathway and TGF-β pathway are best understood for their roles in maintaining stem cell pluripotency and self-renewal (Figure 1)[5]. In the Wnt pathway, Wnt proteins initiate a signalling cascade by binding to Frizzled (FZD) and low-density lipoprotein receptor related protein (LRP) receptors. This leads to the inhibition of GSK-3β in the β-catenin degradation complex, which is made up of GSK-3β, Axin, and APC. As a result, β-catenin is stabilised, accumulates in the cytoplasm, and translocated to the nucleus where it interacts with the T-cell factor (TCF) and lymphoid enhancer factor (LEF) transcription factors (not shown), creating a transcriptional activation complex that activates transcription of target genes including Oct4, Nanog, and Sox2[5,6]. In the TGF-β pathway, signalling is initiated when TGF-β/Nodal/Activin binds to cell surface receptors that activate Smad proteins in the cytoplasm. This leads to the translocation of Smad proteins to the nucleus, where it activates transcription of target genes including Oct4, Nanog, and Sox2[5]. To develop a better understanding of the mechanisms underlying the self-renewal and pluripotency of hESCs, further exploration of other potential gene targets in the core transcriptional regulatory network is necessary.

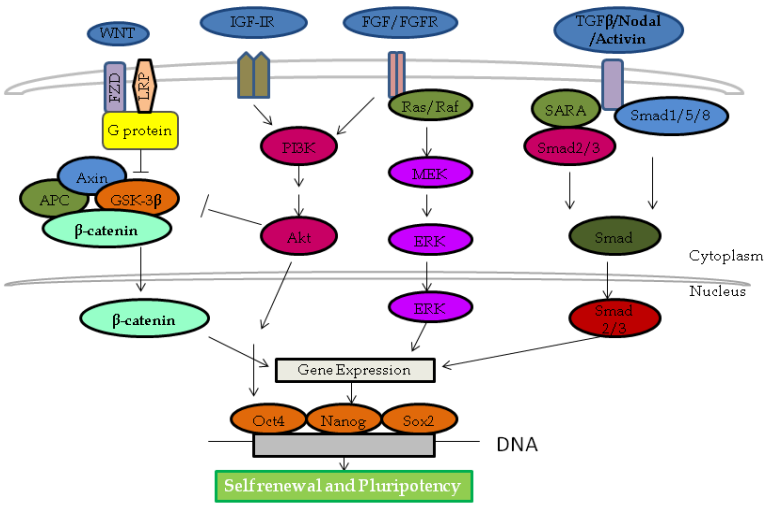


Figure 1. Signalling pathways implicated in the regulation of pluripotency and self-renewal in hESCs. From left to right, this pathway diagram illustrates four main pathways involved in the regulation of pluripotency and self-renewal in hESCs: Wnt pathway, insulin growth factor (IGF) pathway, Fgf pathway, and TGF-β pathway. Adapted from "Molecular Mechanisms Underlying Pluripotency," by E. Bieberich and G. Wang, 2013, *Pluripotent Stem Cells*. Copyright 2013 by IntechOpen.

PRDM14, a gene belonging to a family of PR domain-containing (PRDM) transcriptional regulators, was previously suggested to have functional roles associated with regulating self-renewal and differentiation properties in hESCs. Previous studies demonstrated that knockdown of PRDM14 resulted in the downregulation of OCT4 and hESC differentiation. Furthermore, in genome-wide location profiling experiments, PRDM14 was found to be colocalized with other key transcription factors including OCT4, NANOG, and SOX2, indicating its connection to the core transcriptional regulatory network. In a gain-of-function experiment, PRDM4, in conjunction with OCT4, SOX2, and KLF4, increased the efficiency of reprogramming of human fibroblasts to a pluripotent state. Altogether, these results demonstrate the key role of PRDM14 in maintaining hESC identity[7,8]. However, while these studies demonstrate the functional potential of PRDM14, its specific connection to key transcription factors in the pluripotency circuitry in hESCs remain unclear. In this study, we perform differential gene expression analyses on a PRDM14-knockdown gene expression dataset to answer three main questions:

1. Does PRDM14 play a role in regulating hESC self-renewal?
2. Are there additional target genes of PRDM14 that may play a role in the regulation of hESC self-renewal?
3. How are these target genes related to the core transcriptional network of hESC self-renewal and pluripotency?

2. Materials and Methods

2.1. Data Collection

The dataset used in this study (DataSet_01_045.txt) was downloaded from the KnockTF2.0 database (https://bio.liclab.net/KnockTFv2/download.php) under the GEO Series accession number GSE22795. The purpose of this dataset was to investigate changes in gene expression in 16,670 genes following knockdown of PRDM14 in hESCs. To conduct the knockdown experiment, hESCs were transfected with PRDM14 shRNA (experimental group) or Luciferase shRNA (negative control group). 3 PRDM14 knockdown samples ((GSM563545, GSM563546, GSM563547) and 3 Luciferase knockdown samples (GSM563542, GSM563543, GSM563544) were harvested 3 days post-transfection. RNA was amplified and hybridized following standard Illumina protocols, and gene expression was measured using an Illumina microarray (HumanRef-8 v3.0 Expression BeadChips)[8].

2.2. Data Preprocessing

The DESeq2 library in R is used to calculate log2 fold changes, p-values, and adjusted p-values for each experimental sample in the dataset.

2.3. Differential Gene Analysis

First, to confirm that the knockdown of PRDM14 induced changes in gene expression, we plotted a correlation heatmap to determine the correlation of gene expression levels in each experimental condition compared to every other condition. Among the observed changes in gene expression levels, a scatter plot was used to compare levels of gene expression in PRDM14-knockdown experiments and negative controls. Genes with a log2FoldChange greater than 1 were upregulated after PRDM14 knockdown compared to normal controls. Genes with a log2FoldChange less than -1 were downregulated after PRDM14 knockdown compared to normal controls. Genes with a log2FoldChange between 1 and -1 showed no significant change in gene expression after PRDM14 knockdown compared to normal controls.

Second, to determine if PRDM14 plays a role in regulating hESC differentiation, we compared gene expression levels in 47 markers of undifferentiated hESCs, 12 markers of ectoderm cells, 18 markers of endoderm, and 21 markers of mesoderm cells after knockdown of PRDM14 compared to negative controls (Table 1). These markers were previously shown to be able to reliably distinguish between undifferentiated and differentiated cell states[9]. Furthermore, to determine if PRDM14 induces changes in the core transcriptional network of hESC pluripotency, we compared gene expression levels in the core-transcriptional factors NANOG, OCT3/4, and SOX2 after knockdown of PRDM14 compared to negative controls. Changes in gene expression were visualised using a volcano plot, in which the default cut-off for p-value was 10e-6 and the default cut-off for log2FC was >|1|.

Table 1. Marker Gene Names[9]

Condition	Marker Genes
Undifferentiated hESCs	LIN28, DNMT3B, CLDN6, IFITM1, POU5F1, ITGB1, SFRP2, GJA1, SOX2, CD9, GAL, LIN41, IMP-2, LECT1, ZNF206, GABRB3, CYLTLIB, UTF1, NR6A1, LEFTY1, SCGB3A2, KIT, CKMT1, TDGF1, FOXD3, DIAPH2, NUMB, CER1, PMAIP1, CDH1, LEFTY2, NANOG, SMAD2, BRIX, REST, ZFP42, EDNRB, PTEN, NOG, GDF3, GBX2, TFCP2L1, COMMD3, TERT, PODXL, FGF4, NR5A2, IFITM2, GRB7, DPPA5, NODAL, LCK, NTS, ITGBIBP3, CHST4
Ectoderm	VIM, CRABP2, SEMA3A, MSI1, MAP2, GFAP, OLIG2, SOX1, NES, NEUROD1, TH, TUBB3
Mesoderm	COLIA1, HAND1, MSX1, ACTC, GATA6, COL2A1, HBZ, T, WT1, MYF5, DES, NPPA, HBB, RUNX2, IGF2, EOMES, CDX2, GCM1, KRT1, SYCP3, DDX4, IFITM1, IFITM2
Endoderm	FN1, DCN, H19, AFP, LAMB1, LAMC1, BMP2, SERPINA1, FLT1, ACVRIB, GATA4, GCG, INS, PECAM1, FABP2, HNF4A, FGF8, HGF, FOXA2

2.4. Identification of additional target genes of PRDM14

To determine if there are additional target genes of PRDM14 that may play a role in the regulation of hESC self-renewal, the top 50 genes with the smallest adjusted p-value for changes in gene expression were ranked. The following genes were identified: PLAU, IGFBP5, SERPINE1, FOXD1, EMP1, IGFBP7, IER3, PRSS23, FZD2, KCNF1, NOX4, COL5A1, GDF15, F2RL1, FAM43A, CADM1, TGFBI, DKK3, GREM1, VGF, ITGA5, PRICKLE2, TNC, ANKRD1, AXL, UBE2J1, KLF6, SPP1, CST1, ACTC1, CD44, MSX1, EDN1, CCDC92, S100A6, RBP1, PAPPA, GLIPR1, NPPB, PNMA2, IL11, COL1A1, PALLD, NLRP7, S100A16, COL8A1, COL7A1, PHLDA1, STC2, RGS10. A smaller adjusted p-value represents a greater statistical significance in the change in gene expression. Next, the z-score for the top 50 genes were calculated and visualised using a heatmap. The z-score represents a gene's expression level compared to the mean expression level. A positive z-score indicates that the gene expression is upregulated, and a negative z-score indicates that the gene expression is downregulated.

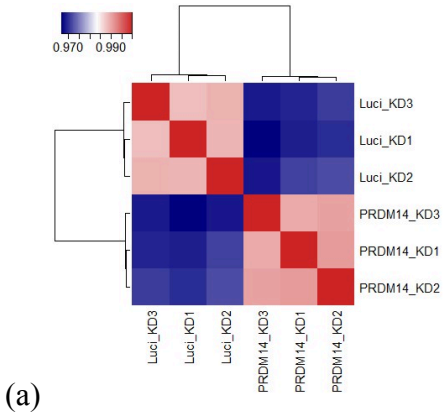
2.5. GO Analysis using PANTHER

To determine the functional mechanisms of the top 50 target genes and their potential integration in the core transcriptional network, Gene Ontology (GO) analyses on the pathways of these genes were performed using the PANTHER classification system (www.pantherdb.org). The results of the GO analysis were visualised using bar plots. Genes with unclassified pathways were filtered.

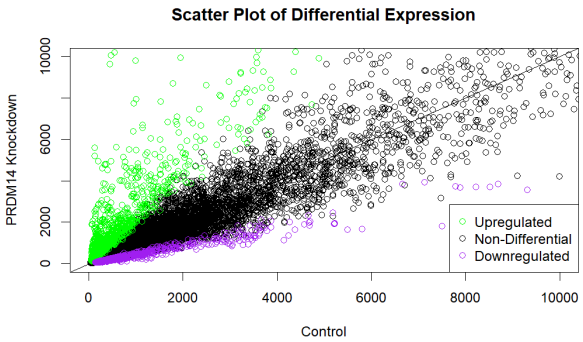
3. Results

3.1. Knockdown of PRDM14 induced changes in gene expression

Following the knockdown of PRDM14 in hESCs, changes in gene expression levels were observed in comparison to negative controls. As shown in the correlation heatmap, gene expression levels in PRDM14-knockdown samples were less correlated with gene expression levels in negative controls compared to other PRDM14-knockdown samples (Figure 2A). Among the observed changes in gene expression levels, 542 gene samples were upregulated, 209 gene samples were down regulated, and 15920 gene samples observed no significant changes in gene expression (Figure 2B).



(a)



(b)

Figure 2. Differential gene expression in PRDM14-knockdown versus control conditions. **(a)** The correlation heatmap illustrates the pairwise correlation of gene expression levels in each experimental condition compared to every other condition. Red squares illustrate a stronger correlation and blue squares illustrate a weaker correlation in gene expression patterns. Clusters in the heatmap reveal differential gene expression between PRDM14-knockdown experiments (PRDM14_KD1, PRDM14_KD2, and PRDM14_KD3) and negative controls (Luci_KD1, Luci_KD2, and Luci_KD3). **(b)** The scatter plot compares levels of gene expression in PRDM14-knockdown experiments (y-axis) and negative controls (x-axis). Genes that were upregulated (log2FoldChange > 1) after PRDM14 knockdown are shown in green, genes that showed no significant change in expression ($-1 \leq \log2FoldChange \leq 1$) after PRDM14

knockdown are shown in black, and genes that were downregulated after PRDM14 knockdown (log2FoldChange < -1) are shown in purple.

3.2. Knockdown of PRDM14 induced the downregulation of markers associated with undifferentiated hESCs and the upregulation of markers associated with differentiated cells

Following the knockdown of PRDM14 in hESCs, 10 out of 47 markers of undifferentiated hESCs were downregulated ($p < 10e-6$, $log2FC<-1|$), including DIAPH2, NANOG, IFITM2, CHST4, LECT1, DNMT3B, SOX2, IFITM1, DPPA5, GDF3. Interestingly, 4 out of 47 markers of undifferentiated hESCs were upregulated ($p < 10e-6$, $log2FC>1$), including GAL, CER1, ITGB1, LEFTY2. The remaining 33 out of 47 markers of undifferentiated hESCs observed no significant changes in gene expression (Figure 3A). These results demonstrate that PRDM14 may play a role in suppressing differentiation marker genes.

Following the knockdown of PRDM14 in hESCs, 4 out of 12 markers of ectoderm cells were upregulated ($p < 10e-6$, $log2FC>1$), including VIM, CRABP2, TUBB3, and NES. In contrast, 1 out of 12 markers of ectoderm cells were downregulated ($p < 10e-6$, $log2FC<-1|$), including SEMA3A. The remaining 7 out of 12 markers of ectoderm cells observed no significant changes in gene expression (Figure 3B). These results demonstrate that PRDM14 may play a role in upregulating or downregulating ectoderm marker genes.

Following the knockdown of PRDM14 in hESCs, 1 out of 18 markers of endoderm cells were upregulated ($p < 10e-6$, $log2FC>1$), namely LAMC1. The remaining 17 out of 18 markers of endoderm cells observed no significant changes in gene expression (Figure 3C). These results demonstrate that PRDM14 may play a role in upregulating endoderm marker genes.

Following the knockdown of PRDM14 in hESCs, 1 out of 21 markers of mesoderm cells were upregulated ($p < 10e-6$, $log2FC>1$), namely MSX1. In contrast, 2 out of 21 markers of mesoderm cells were downregulated ($p < 10e-6$, $log2FC<-1|$), including IFITM1 and IFITM2. The remaining 18 out of 21 markers observed no significant changes in gene expression (Figure 3D). These results demonstrate that PRDM14 may play a role in upregulating or downregulating endoderm marker genes.

Altogether, the changes in gene expression observed in markers of undifferentiated hESCs and markers of endoderm, mesoderm, and ectoderm cells suggest that PRDM14 may play a role in regulating the self-renewal mechanisms of hESCs and discourage differentiation into specialised cells.

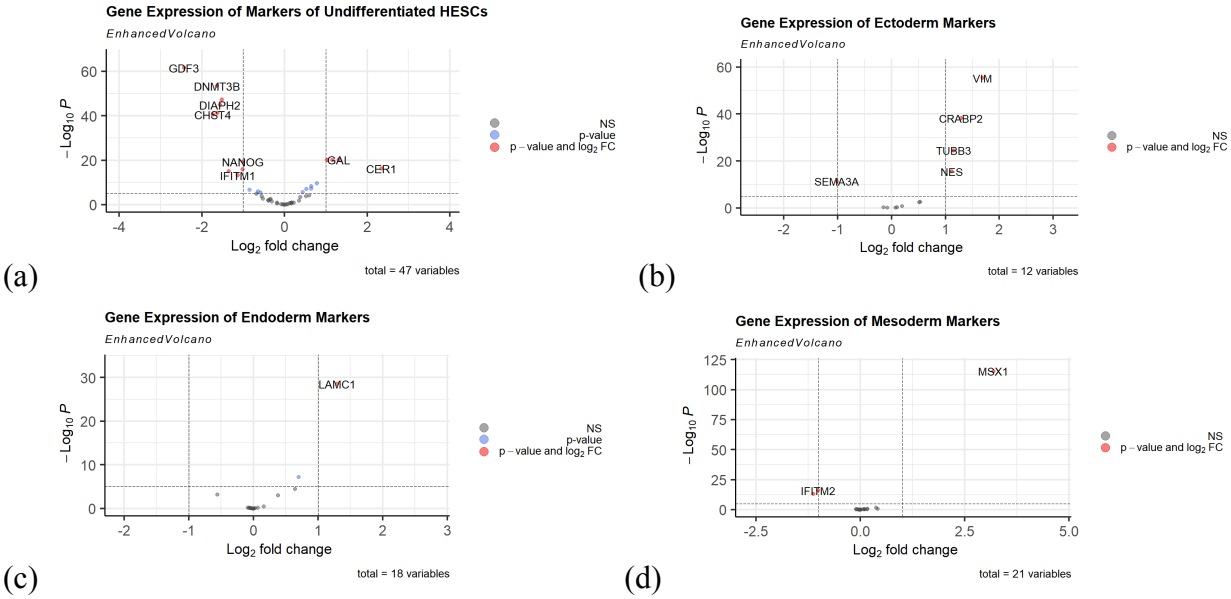


Figure 3. Comparison of gene expression changes in differentiation marker genes for hESCs after knockdown of PRDM14. Markers of (a) undifferentiated hESCs, (b) ectoderm, (c) endoderm, and (d) mesoderm were assessed for changes in gene expression after knockdown of PRDM14. The default cut-off for p-value is 10e-6 and the default cut-off for log2FC is >|1|. Red points denote significantly upregulated or downregulated genes (i.e. significant p-value and Log2 fold change), blue points denote genes with no change in gene expression (i.e. significant p-value and non-significant Log2 fold change), and grey points denote genes that had no significant p-value and Log2 fold change.

3.3. Knockdown of PRDM14 induced the downregulation of core transcription factors NANOG and SOX2

Following the knockdown of PRDM14 in hESCs, SOX2 and NANOG were downregulated ($p < 10e-6$, log2FC<-1|), and OCT4 observed no significant changes in gene expression (Figure 4). Altogether, the changes in gene expression observed in these core transcription factors suggest that PRDM14 may be integrated in the core transcriptional network for regulating hESC self-renewal in some manner. In particular, PRDM14 may have functional properties that lead to the upregulation of NANOG and SOX2, thereby promoting self-renewal of hESCs and discouraging differentiation into specialised cells.

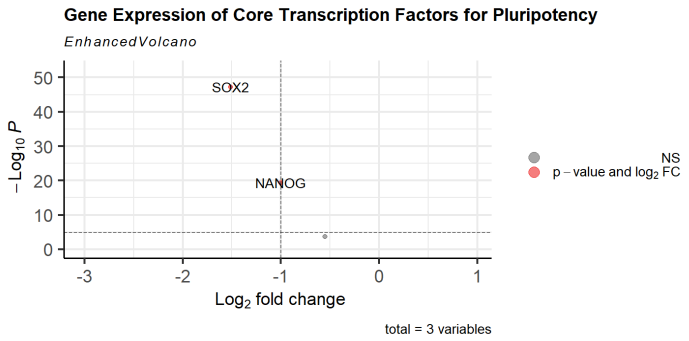
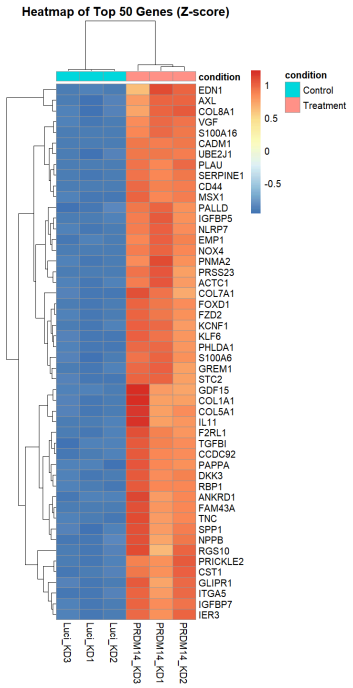


Figure 4. Gene expression changes in core transcription factors for hESC pluripotency after knockdown of PRDM14. Using the DESeq2 library in R, the default cut-off for p-value is 10e-6 and the default cut-off for log2FC is >|2|. Red points denote significantly upregulated or downregulated genes (i.e. significant p-value and Log2 fold change).

3.4. Knockdown of PRDM14 induced changes in gene expression in genes associated with Wnt and TGF-β signalling pathways

Following the knockdown of PRDM14, the top 50 genes with the highest statistical significance in change in gene expression were all upregulated compared to normal controls (Figure 5A). According to the results from the PANTHER GO analysis, these genes are found in 16 different pathways. In particular, knockdown of PRDM14 resulted in the upregulation of frizzled-2 (FZD2), actin alpha cardiac muscle 1 (ACTC1), endothelin-1 (EDN1), which are associated with the Wnt signalling pathway. Furthermore, knockdown of PRDM14 resulted in the upregulation of growth differentiation factor 15 (GDF15), which is associated with the TGF-β signalling pathway (GDF15) (Figure 5B). Altogether, these results suggest that FZD2, ACTC1, EDN1, and GDF15 are potential target genes of PRDM14 that play a role in regulating the self-renewal mechanisms of hESCs via the Wnt and TGF-β signalling pathways.

(a)



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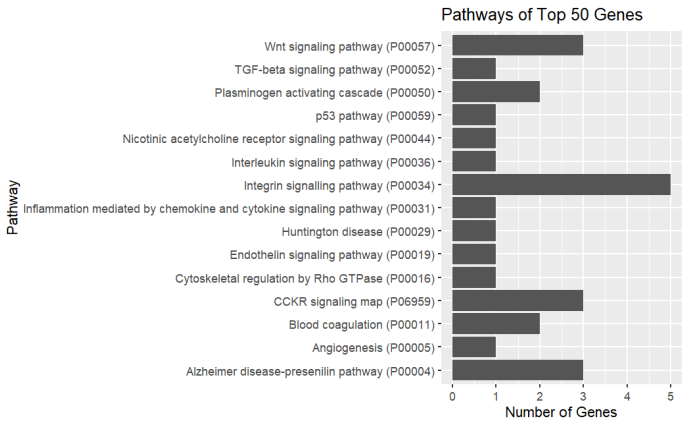

Figure 5. Functional analysis of the top 50 genes with the most statistically significant changes in gene expression following knockdown of PRDM14. (a) The heatmap shows the expression changes (z-score) in genes with the top 50 smallest adjusted p-values compared to the control group following knockdown of PRDM14. Positive z-scores, shown in red, indicate that the gene expression is upregulated. Negative z-scores, shown in blue, indicate that the gene expression is downregulated. (b) GO analysis results summarising pathways from top 50 genes with the most statistically significant changes in gene expression.

4. Discussion & Conclusion

In this study, we demonstrated that knockdown of PRDM14 induced the downregulation of markers associated with undifferentiated hESCs and the upregulation of markers associated with differentiated cells in the three germ layers (endoderm, mesoderm, ectoderm), suggesting that PRDM14 may play a role in regulating the self-renewal mechanisms of hESCs and discourage differentiation into specialised cells. Furthermore, we showed that knockdown of PRDM14 induced the downregulation of core transcription factors NANOG and SOX2, suggesting that PRDM14 may be integrated in the core transcriptional network for regulating hESC self-renewal in some manner. In particular, the knockdown of PRDM14 upregulated FZD2, ACTC1, EDN1, and GDF15, which are genes associated with the Wnt and TGF-β signalling pathways. In the Wnt signalling pathway, Frizzled-2 (FZD2) is a transmembrane signal receptor that initiates a signalling cascade following binding with Wnt[5]. Altogether, these results suggest that FZD2, ACTC1, EDN1, and GDF15 are potential target genes of PRDM14 that play a role in regulating the self-renewal mechanisms of hESCs via the Wnt and TGF-β signalling pathways.

These findings unravel potential mechanisms by which PRDM14 interacts with the core transcriptional network to regulate hESC self-renewal and differentiation. However, further exploration of the roles of FZD2, ACTC1, EDN1, and GDF15 individually and in combination with PRDM14 are required to determine the specific mechanisms by which they regulate the self-renewal mechanisms of hESCs via the Wnt and TGF-β signalling pathways. It would be beneficial to investigate the relationship between these genes in future in vitro studies, such as using CRISPR/Cas9 technology to manipulate the expression of these target genes individually or in combination with PRDM14, and examining changes in self-renewal and differentiation of hESCs. These results would offer valuable insights in the development of novel cell-replacement therapies to treat tissue/organ damage, particularly in developing new mechanisms to allow hESCs to undergo long-term self-renewal in vitro.

Works Cited

1. Young, R. A. (2011). Control of Embryonic Stem Cell State. *Cell*, *144*(6), 940–954. https://doi.org/10.1016/j.cell.2011.01.032
2. Vallier, L., Touboul, T., Brown, S., Cho, C., Bilican, B., Alexander, M., Cedervall, J., Chandran, S., Ährlund-Richter, L., Weber, A., & Pedersen, R. A. (2009). Signaling Pathways Controlling Pluripotency and Early Cell Fate Decisions of Human Induced Pluripotent Stem Cells. *Stem Cells*, *27*(11), 2655–2666. https://doi.org/10.1002/stem.199
3. Romito, A., & Cobellis, G. (2016). Pluripotent Stem Cells: Current Understanding and Future Directions. *Stem Cells International*, *2016*, 9451492. https://doi.org/10.1155/2016/9451492
4. Mountford, J. C. (2008). Human embryonic stem cells: Origins, characteristics and potential for regenerative therapy. *Transfusion Medicine*, *18*(1), 1–12. https://doi.org/10.1111/j.1365-3148.2007.00807.x
5. Bieberich, E., & Wang, G. (2013). Molecular Mechanisms Underlying Pluripotency. InTech. doi: 10.5772/55596
6. Sokol, S. Y. (2011). Maintaining embryonic stem cell pluripotency with Wnt signaling. *Development*, *138*(20), 4341–4350. https://doi.org/10.1242/dev.066209
7. Nakaki, F., & Saitou, M. (2014). PRDM14: A unique regulator for pluripotency and epigenetic reprogramming. *Trends in Biochemical Sciences*, *39*(6), 289–298. https://doi.org/10.1016/j.tibs.2014.04.003
8. Chia, N.-Y., Chan, Y.-S., Feng, B., Lu, X., Orlov, Y. L., Moreau, D., Kumar, P., Yang, L., Jiang, J., Lau, M.-S., Huss, M., Soh, B.-S., Kraus, P., Li, P., Lufkin, T., Lim, B., Clarke, N. D., Bard, F., & Ng, H.-H. (2010). A genome-wide RNAi screen reveals determinants

of human embryonic stem cell identity. *Nature*, *468*(7321), Article 7321. https://doi.org/10.1038/nature09531

9. Cai, J., Chen, J., Liu, Y., Miura, T., Luo, Y., Loring, J. F., Freed, W. J., Rao, M. S., & Zeng, X. (2006). Assessing Self-Renewal and Differentiation in Human Embryonic Stem Cell Lines. *Stem Cells*, *24*(3), 516–530. https://doi.org/10.1634/stemcells.2005-0143