UNIVERSITY OF CRETE

SCHOOL OF SCIENCES AND ENGINEERING

DEPARTMENT OF BIOLOGY

Genetic and Epigenetic Regulatory Networks in mouse Embryonic Stem Cells

by

Christiana Hadjimichael

A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy (Ph.D)

SUPERVISORS

Prof. Papamatheakis Joseph

Dr. Androniki Kretsovali

Heraklion

February 2016

Table of Contents

Acknowledgements

The present study was carried out at the Institute of Molecular Biology and Biotechnology in the Foundation for Research and Technology, Heraklion, Crete.

I would like to take the opportunity to thank all the people who have helped, advised and supported me during this journey. Firstly, I would like to express my gratitude to my supervisor, Dr. Androniki Kretsovali for giving me the opportunity to be a member of her group. I appreciate her continuous support, helpful criticism and daily guidance in the lab. The day-to-day debates with her had an enormous influence on me both scientifically and personally. Our interaction helped me to know and exceed my limits. I am eternally grateful for everything you have taught me. I would also like to thank Prof. Joseph Papamatheakis for his scientific knowledge and excellent guidance which have been of great value to me. His enthusiasm and passion for science motivates and inspires everyone working with him, while his dedication into research is impressive.

During this exciting journey, I have met and collaborated with great people who became friends over the years. They all had a very significant and positive impact, both on my personal development as well as on my professional career. I am sure that I would not have succeeded without these supporters!!!

First of all, I extend my appreciation to Mr Takis Makatounakis, for providing me with direction, technical support at all levels of the research projects. He is one of the most generous, patient and helpful people I have ever met. He was always available or reachable when I had scientific questions and also had time and interest when I needed or felt like talking about non-scientific issues. I will never forget you Mr Takis and I will always love you. Thank you!! Appreciation also goes out to Mr George Vrentzos who trained me in cell culture sharing his advice, reagents, and protocols eagerly.

I owe my thanks to Eva Kokalaki. She was the first friendly face I met when I started my PhD and was always helpful no matter the task or circumstance. I also appreciate the efforts of the many undergraduate and graduate students- Nikos, Aggeliki, Dionisis, Gesthhmani, Sofia, Manolis S., Eva B., Amalia Z., Arguro, Giota Z., Dimitris K., Giannis, Ben - who implemented different experiments and assisted in

my research. You were all friendly, fun, responsible and bright and you deserve my respect and support! Thank you!!!

I could not have successfully completed my first exams ('qualifyings') for the PhD program without the support of Nikos Charbilas. I really appreciate his help and I will always remember our first year in the lab Niko.!! You deserve my respect and I wish you all the best!!!

Giotaki, Marigoula, Toskidiaki, Tsouroulaki, Petrouko, Eirini, Despoinaki. Having good friends is really important to my happiness. And you are great friends. Your love, support, and belief in me were a treasure. You were always there for me, gave me many valuable memories and you will have always a special place in my heart. Though miles may lie between us, I want you to know how much I love you and I am grateful to you for helping, supporting and loving me. I will never stop dreaming that one day we will be together, all of us laughing and talking, looking at the past and planning the future. I really miss you!!!

Next, I would like to thank KONSTANTINA and NIKOLETA, or as I call them, my little sisters. I cannot begin to express my gratitude and feelings for these gregarious women, with whom I shared moments of happiness and sadness. Entering into my life during this journey, they were involved in my dissertation process and provided me with friendship, great conversations, an instant social life, constant encouragement etc., etc. Need I say more? They have been sometimes tough on me but always supportive and caring. I know that I have two friends for a lifetime. For all these reasons and many, many more, I am really grateful. Thank you for being persistent and encouraging, for believing in me and for the many precious memories along the way. I love you so much!!!

I would like also to say special thanks to my sweet NATALAKI!! You were there for me every step of this way, and I wholeheartedly appreciate everything you've done for me. Your guidance and support has been amazing! Thank you for helping me improve. I really love you and I am sure you will have a great family and you will create meaningful moments with your son!!!

I would also like to express my gratitude to Amalia and Maria. Thank you for your support and your sympathy during the last year of this journey. I wish you all

the best in the lab and I am sure that one day your dream to get your PhD will come true. Believe me!!

Most importantly, none of these would have been possible without the love and patience of my family. I owe my deepest gratitude to Michael, Androulla, Irene and Philip, for their emotional and financial support all these years. They have been a constant source of love, concern, support and strength. I always knew that you believed in me and wanted the best for me. Without your empathy and support, this work would have never come to the end.

Last, but certainly not least, I owe my biggest thank to my husband, Dimitrios Konios. Our friendship began when we entered the Bachelor Program in Chemistry at the University of Athens. We became friends and have remained so ever since. With great surprise and joy, our close friendship evolved into a deep loving relationship and since then I have been happier, calmer, and more content. Without his love, patience, support and unwavering belief in me, I would have not been able to complete this long journey. He has taken care of whatever I needed without complaining, to be focused on completing my PhD. He was patient, waiting many, many long hours alone while I worked. At the same time, he has also given me so many happy and beautiful memories throughout this journey. I could not have completed this journey without having Dimitris by my side. He was the person who shared with me all the enthusiasm for many things, but he was also there with a smile every time I felt disappointed. Without his love and encouragement I would not have finished this thesis. Thank you for being part of my life!!!

Finally, I would like to apologize to everyone if I said or did something that hurt your feelings. I am really sorry if I did that!!

Thank you all!!!!!

I dedicate my PhD thesis to two of the most important women in my life who taught me that happiness is a decision… Thank you!!!

“For one minute walk outside,

Stand there in silence

Look up at the sky

And contemplate

How amazing life is”

Abbreviations

APL Acute promyelotic leukemia

BMPs Bone Morphogenetic Proteins

Cdh1 E-Cadherin

CSCs Cancer Stem Cells

EBs Embryoid Bodies

EMT Epithelial to Mesenchymal Transition

EpiSCs Epiblast Stem Cells

ESCs Embryonic Stem Cells

iPSCs Induced Pluripotent Stem Cells

LIF Leukemia Inhibitory Factor

MET Mesenchymal to Epithelial Transition

MiRNAs MicroRNAs

PML Promyelocytic Leukemia Protein

PML-NBs PML nuclear bodies

PSCs Pluripotent Stem Cells

RA Retinoic Acid

RARα Retinoic acid receptor-α

Rb Retinoblastoma Tumor Suppressor

SIM Sumo interacting motif

T T-Brachyury

TSA Trichostatin A

List of Figures

Chapter 1

Chapter 2

Chapter 3

Abstract

In the past decade, numerous studies demonstrated that both microRNAs (miRNAs) and Promyelocytic Leukemia Protein (PML) were central regulators of different biological processes including cell proliferation, apoptosis and tumorigenesis. Emerging research links miRNAs as well as PML with the regulation of stem cells maintenance. Several miRNAs were reported to sustain embryonic stem cells (ESCs) identity (miR-290-295), while others to induce ESC differentiation (miR-34a, miR-145). At the same time, recent reports using p19 embryonal carcinoma cells and ESCs showed that Nanog and Oct4 – the core pluripotent regulators - associate with PML protein. Moreover, PML was described as a regulator of metabolic pathways in stem cell compartments (hematopoietic stem cells-HSCs) and as an essential component in leukemia-initiating cells maintenance. In an effort to elucidate the molecular mechanism underlying the contribution of PML and miRNAs in stemness phenotype, we examined their role in mESCs.

In the first part of this work, we present four new miRNAs (miR-16-1, miR-191, miR-23a, miR-421), which are differentially expressed upon differentiation. miR-191 and miR-16-1 are highly expressed in ESCs and inhibits Activin-Nodal signaling, leading to the suppression of mesoderm and endoderm differentiation. miR-23a, which is also down-regulated in the differentiated state, represses differentiation towards the endoderm and ectoderm lineages. In contrast, miR-421 was characterized as a differentiation-associated regulator that targets the core pluripotency transcription factor *Oct4* and the BMP-signaling pathway to promote endoderm and ectoderm differentiation. Our results uncover a regulatory network between the studied miRNAs and both branches of TGF-β/BMP signaling pathways unveiling their importance for ESC lineage decisions.

In the second part of this thesis, we show that PML positively correlates with the undifferentiated state and is a vital regulator of ESC pluripotency. PML knockdown induces up-regulation of multi-lineage differentiation markers and produces enhanced differentiation towards mesodermal lineage, while ectopic expression of PML prevents ESC differentiation in vitro and maintains stem cell phenotypes. Through transcriptome analysis of ESCs WT and ESC PML KD, we

identified a large number of differentially expressed genes. Specifically, a group of deregulated genes is involved to the transition from naïve to primed pluripotent state, while a great number of genes associate with both signaling pathways essential for ESC identity as well as cell cycle. Additionally, reprogramming of *Pml*$^{-/-}$ mouse embryonic fibroblasts (MEFs) showed significantly lower efficiency compared to MEFs WT, highlighting PML as a pivotal mediator of somatic cell reprogramming. We speculate that the potential mechanism is based on regulation of EMT through TGF-β signaling. Our findings reveal that PML acts as an essential regulator in naïve and primed pluripotency as well as somatic cell reprogramming.

Περίληψη

Τα τελευταία χρόνια, πληθώρα ερευνητικών εργασιών κατέδειξε ότι τόσο τα μικρά-RNAs όσο και η πρωτεΐνη προμυελοκυταρικής λευχαιμίας (ΠΠΛ) διαδραματίζουν σημαντικό ρόλο σε πολλές κυτταρικές διαδικασίες, συμπεριλαμβανομένου του πολλαπλασιασμού, της απόπτωσης και της ογκογένεσης. Παράλληλα, ο ρόλος τους έχει συσχετιστεί με τη ρύθμιση των βλαστικών κυττάρων. Συγκεκριμένα, έχει δειχθεί ότι πολλά μικρά-RNAs συμβάλουν στη διατήρηση των εμβρυικών βλαστικών κυττάρων (ΕΒΚ) (miR-290-295), ενώ άλλα προάγουν τη διαφοροποίηση αυτών (miR-34a, miR-145). Επιπρόσθετα, έρευνες με τη χρήση κυττάρων εμβρυικού καρκινώματος και ΕΒΚ ανέδειξαν την αλληλεπίδραση δύο βασικών ρυθμιστών της πλειοδυναμίας -Oct4 και Nanog- με τη ΠΠΛ, η οποία με τη σειρά της έχει χαρακτηρισθεί ως ρυθμιστής των μονοπατιών μεταβολισμού σε βλαστικά συστήματα (αιμοποιητικά βλαστικά κύτταρα) και ως βασικό στοιχείο της διατήρησης των λευχαιμικών αρχέγονων κυττάρων. Η παρούσα διδακτορική διατριβή είχε ως στόχο τη κατανόηση του μοριακού μηχανισμού ρύθμισης των ΕΒΚ τόσο από τα μικρά-RNAs όσο και από τη ΠΠΛ.

Στο πρώτο μέρος της διατριβής, παρουσιάζονται 4 νέα μικρά-RNAs (miR-16-1, miR-191, miR-23a, miR-421), τα οποία εκφράζονται ποικιλοτρόπως κατά τη διαφοροποίηση. Συγκεκριμένα, τα miR-16-1 και miR-191 εκφράζονται σε υψηλά επίπεδα στα ΕΒΚ και καταστέλλουν το σηματοδοτικό μονοπάτι Activin-Nodal, παρεμποδίζοντας τη μεσοδερμική και ενδοδερμική διαφοροποίηση. Το miR-23a, του οποίου η έκφραση μειώνεται σημαντικά κατά τη διαφοροποίηση, μετριάζει τη κατεύθυνση προς την ενδοδερμική και εκτοδερμική στιβάδα. Αντίθετα, το miR-421 στοχεύει το βασικό παράγοντα πλειοδυναμίας, Oct4, καθώς και στοιχεία του BMP σηματοδοτικού μονοπατιού, *Smad5* and *Id2,* καθιστώντας το ως ρυθμιστή της διαφοροποίησης των ΕΒΚ. Τα αποτελέσματα αυτά υποδεικνύουν ένα νέο μηχανισμό αλληλεπίδρασης μεταξύ των παραπάνω μικρών-RNAs και των δύο κλάδων του TGF-β μονοπατιού, τονίζοντας τη σημαντικότητά τους σε αποφάσεις-κλειδιά για τη μοίρα των βλαστικών κυττάρων.

Στο δεύτερο μέρος της ερευνητικής εργασίας, μελετήθηκε και συσχετίστηκε ο ρόλος της ΠΠΛ με την αδιαφοροποίητη κατάσταση των ΕΒΚ. Συγκεκριμένα, η αποσιώπηση της ΠΠΛ οδηγεί στην επαγωγή παραγόντων διαφοροποίησης των τριών βλαστικών στιβάδων και ενισχύει τη μεσοδερμική κατεύθυνση, ενώ αντίθετα η υπερέκφρασή της παρεμποδίζει τη διαφοροποίηση και διατηρεί το φαινότυπο των ΕΒΚ. Με τη χρήση μικροσυστοιχιών έκφρασης σε ΕΒΚ παρουσία και απουσία της ΠΠΛ, βρέθηκαν εκτεταμένες αλλαγές στο πρότυπο γονιδιακής έκφρασης. Μια ομάδα αυτορυθμιζόμενων γονιδίων εμπλέκεται στη μετάβαση από την αρχέγονη πλειοδύναμη στην ενεργοποιημένη-όψιμη κατάσταση επιβλάστης, ενώ μια άλλη σχετίζεται με τον κυτταρικό κύκλο και τα σηματοδοτικά μονοπάτια που εμπλέκονται στα ΕΒΚ. Επιπρόσθετα, ο επαναπρογραμματισμός ινοβλαστών από έμβρυο ποντικού με αποσιωπημένη τη ΠΠΛ δεν ήτανε επιτυχής, επισημαίνοντας ότι η ΠΠΛ είναι αναγκαία για την ολοκλήρωση της διαδικασίας. Ο πιθανός προτεινόμενος μηχανισμός δράσης της ΠΠΛ στον επαναπρογραμματισμό βασίζεται στη ρύθμιση της επιθηλιακής-μεσεγχυματικής μετάβασης μέσω του TGF-β μονοπατιού. Τα στοιχεία αυτά αποκαλύπτουν ότι η ΠΠΛ διαδραματίζει καθοριστικό ρόλο τόσο στη πλειοδυναμία των ΕΒΚ όσο και στον επαναπρογραμματισμό σωματικών κυττάρων.

Chapter 1: General Introduction

1.1 Pluripotent Stem Cells

Pluripotent Stem Cells (PSCs) are characterized by both the ability to self-renew indefinitely and to differentiate into all of the three germ lineages, ectoderm, mesoderm, and endoderm, of the developing embryo. Due to their unique properties, PSCs hold the immense potential for use as a model for screening new drugs, or in regenerative medicine, as a source for cell-based therapeutics and a tool for studying and understanding the prevention and treatment for birth defects.

The first pluripotent cells, the embryonal carcinoma cells (ECCs), were derived from murine and human germ cell tumors (Finch and Ephrussi 1967; Kahan and Ephrussi 1970). Subsequently, the isolation of mouse embryonic stem cells (mESCs) from normal, pre-implantation embryos (E3.5) was reported in 1981 (Evans and Kaufman 1981; Martin 1981) and they were the first normal pluripotent cells that could be cultured, maintained, and differentiated *in vitro*. Seventeen years later, the derivation of human embryonic stem cells (hESCs) (Thomson, Itskovitz-Eldor et al. 1998) was described, followed by the isolation of mouse epiblast stem cells (EpiSCs) from the post-implantation mouse epiblast (E6.5) (Brons, Smithers et al. 2007; Tesar, Chenoweth et al. 2007), representing a more differentiated state than mESCs. Therefore, hESCs/EpiSCs are termed as “primed” pluripotent state, whereas mESCs possess the “naïve” or “ground” pluripotent state (Nichols and Smith 2009) (**Figure 1.1**).

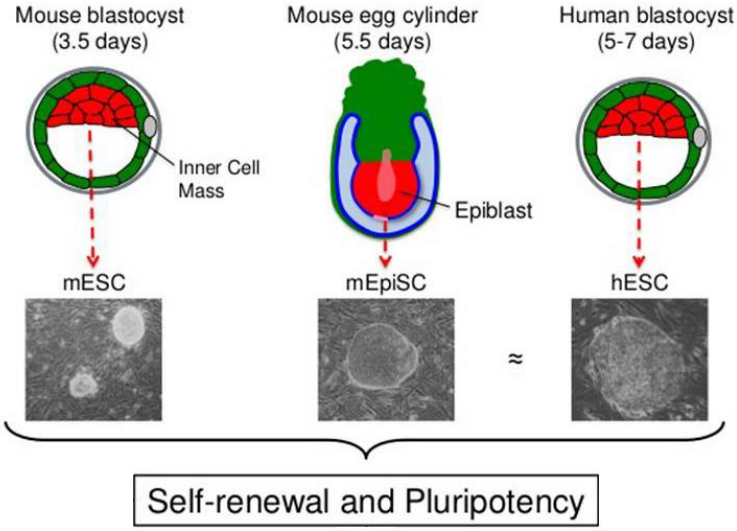


Figure 1.1: Isolation of Pluripotent Stem Cells. mESCs are isolated from an early stage blastocyst (Day 3.5), while EpiSCs are derived from post-implantation mouse embryo (Day 5.5), closely resemble hESCs obtained from inner cell mass of days 5-7 mammalian blastocyst.

Comparison of the two pluripotent states revealed that they differ in mRNA and microRNA signatures (Tesar, Chenoweth et al. 2007; Jouneau, Ciaudo et al. 2012), colony formation, X chromosome inactivation and signaling pathways (Nichols and Smith 2009; Greber, Wu et al. 2010). Additionally, EpiSCs do not contribute to chimera after blastocyst injection, highlighting an important distinction between the two states (**Figure 1.2**). Interestingly, mESCs and EpiSCs can be reciprocally converted one into another, using epigenetic and molecular factors (Chenoweth, McKay et al. 2010). Noteworthy, although the transition from the naive to the primed state can be easily achieved in culture, the opposite is more demanding considering the epigenetic factors (Bao, Tang et al. 2009; Guo, Yang et al. 2009).

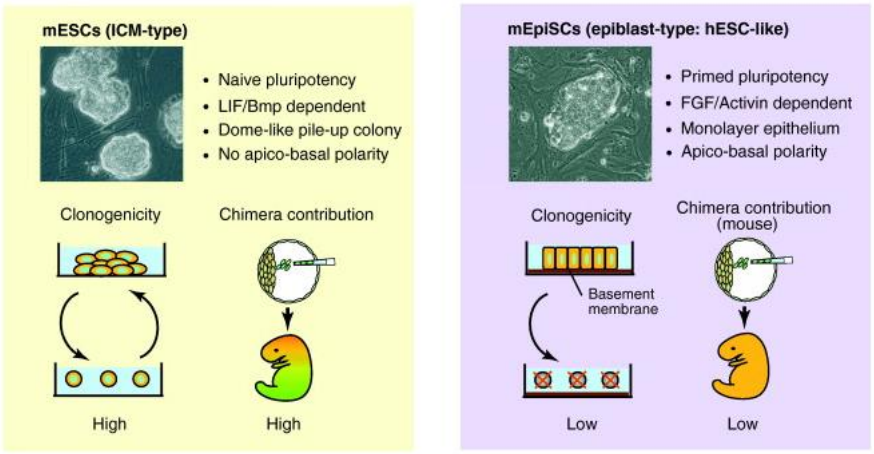


Figure 1.2: ESCs and EpiSCs show significantly different phenotypes. mESCs and EpiSCs exhibit different gene expression pattern and signaling pathways to sustain the pluripotency. Moreover, they differ in colony morphology, X-inactivation pattern and chimera contribution (Ohgushi and Sasai 2011).

1.2 The maintenance of Pluripotency

1.2.1 Transcriptional Control of PSCs the core transcriptional network

The maintenance of pluripotency has been initially attributed to three transcription factors, Oct4, Sox2 and Nanog, constituted the “core pluripotency network”.

Oct4 belongs to the POU family of homeodomain proteins and is encoded by the *Pou5f1* gene. Its expression has been identified in undifferentiated mouse and human ESCs, ECCs, EpiSCs and embryonic germ cells (EGCs) (Rosner, Vigano et al. 1990; Scholer, Dressler et al. 1990; Cauffman, Van de Velde et al. 2005). Nichols *et al* showed that *Oct4* is required for the formation of a pluripotent inner cell mass (ICM) and its expression is under strict control in mESCs (Nichols, Zevnik et al. 1998). Reduced expression of *Oct4* led to the up-regulation of trophectoderm genes (*Cdx2*), while its overexpression caused differentiation into primitive endoderm and mesoderm (Niwa, Miyazaki et al. 2000). Under serum free culture conditions *Oct4* overexpression in ESCs promoted neuroectoderm formation and subsequent neuronal differentiation (Shimozaki, Nakashima et al. 2003). These results indicated that the precise levels of *Oct4* determined the ESC fate and that Oct4 is a master player in sustaining stem cell self-renewal.

Sox2*,* the second essential factor of the network, is a member of the SOX (SRY related HMG box) family that consists of transcription factors with a single high mobility group box DNA binding domain (Kamachi, Uchikawa et al. 2000). *Sox2* is expressed in the ICM and extraembryonic ectoderm of pre-implantation blastocysts (Avilion et al., 2003). *Sox2*-deficient mESCs differentiated primarily into trophectoderm, while the *Oct4* overexpression rescued the pluripotency of *Sox2*- null mESCs (Masui et al., 2007). As a result, *Sox2* is critical for the maintenance of stem cell identity through regulation of *Oct4* expression. Strikingly, a synergistic function of Sox2 and Oct4 for the activation of Oct¬-Sox binary enhancers was identified, leading to the regulation of various transcription factors (Avilion, Nicolis et al. 2003). Moreover, Oct-Sox complexes could be also formed by a direct interaction between Oct4 and Sox2 family members (Lodato, Ng et al. 2013). This tightly correlation of Oct-Sox genes regulation is vital for controlling ESC self-renewal and pluripotency (Aksoy, Jauch et al. 2013; Lodato, Ng et al. 2013). For example, when the *Oct4* or *Sox17* expression levels increased, *Sox2* was replaced by *Sox17* and targeted genes that trigger the endodermal expression program (Aksoy, Jauch et al. 2013). Except for its function as a regulator of pluripotency induction, *Sox2* also orchestrates the cell fate decision. *Sox2* promotes differentiation towards neuroectoderm by suppressing key regulators of the other lineages, such as *T-Brachyury* (*T*) (Zhang, Cui

et al. 2014). Hence, Sox2 seems to be a central regulator in inducing neural initiation and maintaining neural progenitor stem cells self-renewal (Zhang, Cui et al. 2014).

Nanog is the third member of the core network and is required for pluripotency establishment (Chambers, Colby et al. 2003; Mitsui, Tokuzawa et al. 2003). It is a homeodomain containing transcription factor, which was discovered through a functional screening for pluripotency factors which revealed that *Nanog* expression is crucial to maintain ESC identity independently of the LIF/STAT3 signaling pathway (Chambers, Colby et al. 2003; Mitsui, Tokuzawa et al. 2003). Chambers and colleagues demonstrated that *Nanog* is high in *Oct4*-null embryos, whereas its overexpression does not counteract the differentiation program of ESCs prompted by *Oct4* deletion. In the absence of *Nanog*, embryos do not form a pluripotent ICM (Mitsui, Tokuzawa et al. 2003; Silva, Nichols et al. 2009), although *Nanog*-null mESCs can be established (Mitsui, Tokuzawa et al. 2003; Chambers, Silva et al. 2007). Intriguingly, these *Nanog*$^{-/-}$ mESCs although disposed to differentiation, could still be maintained in the undifferentiated state. In addition, in mouse embryonic development it is reduced during implantation and then is increased again at E11.5 in the genital ridges (Chambers, Colby et al. 2003). This observation is also reflected *in vitro*, as *Nanog* expression decreases during the transition from ESCs to EpiSCs (Guo, Yang et al. 2009).

Oct4, Sox2 and Nanog control pluripotency and self-renewal in all PSCs by forming the central transcriptional regulatory network. They bind together to their own promoter/enhancer elements establishing an auto-regulatory circuit, which maintains the entire pluripotency network (Boyer, Lee et al. 2005; Loh, Wu et al. 2006). It was found that several of their target genes are co-occupied, whose expression is increased and decreased in undifferentiated or differentiated state, respectively. Additionally, Nanog, Sox2 and Oct4 co-occupy differentiation genes promoter, suggesting their dominant function in suppressing genes involved in lineage specification (Boyer, Lee et al. 2005; Loh, Wu et al. 2006). Moreover, Nanog, Oct4 and Sox2 cooperate with other transcription factors (e.g signaling pathways mediators), resulting in the activation or the repression of the core factors regulated genes. Their ability to regulate gene expression relies also on the epigenetic interaction including histone methyltransferases and remodeling complexes (Morey,

Santanach et al. 2015). Sites co-occupied by the three core regulators generally have enhancer activity, while the transcription of the respective genes requires the recruitment of at least one of the trio (Chen, Xu et al. 2008).

1.2.2 The expanded ESC pluripotency network

Several studies expanded the transcriptional network controlling the naïve pluripotent state beyond the three core factors. Downstream components of signaling pathways (*Stat3, Smads, Ids, β-catenin*) were characterized as potent regulators of self-renewal maintenance, providing a direct correlation between extracellular cues and ESC properties (Boeuf, Hauss et al. 1997; Niwa, Burdon et al. 1998; Ying, Nichols et al. 2003; Sato, Meijer et al. 2004; ten Berge, Kurek et al. 2011). Chromatin modifiers (e.g Wdr5, Jarid2) (Hadjimichael, Chanoumidou et al. 2015), microRNAS (miR-290-295 cluster) (Marson, Levine et al. 2008) and long-non coding RNAs (long intergenic RNA, lincRNA) are also occupied by the core factors, resulting in the maintenance of ESC properties. Alternative transcription factors including Esrrb, *Tbx3, Sall4, Krüppel-like factors* (*Klfs*), were also implicated in pluripotency maintenance by forming a regulatory circuitry with the three master regulators (**Figure 1.3**).

Esrrb is critical for mESC identity as its knockdown led to a rapid differentiation (Ivanova, Dobrin et al. 2006). Supporting the idea that Esrrb is tightly associated with the extended transcription network, it was shown that it directly interacts with OCT4, SOX2 and NANOG and enhanced *Esrrb* expression maintains mESC self-renewal without the presence of LIF (Martello, Sugimoto et al. 2012). Several studies also revealed that *Esrrb* is a downstream target of Wnt signaling. Both *Tcf3* and *β-catenin* are required for the induction of *Esrrb* expression to maintain mESC self-renewal (Martello, Sugimoto et al. 2012). The loss of pluripotency is also resulted upon the *Tbx3* inhibition, whereas its forced expression is sufficient to sustain the naive state in the absence of LIF (Niwa, Ogawa et al. 2009). *Tbx3* was partially regulated by phosphatidylinositol-3 kinase (PI3K), as it is a direct target of P13K-Akt signaling, but repressed by MAP-kinase (Niwa, Ogawa et al. 2009). Moreover, *Nanog* and *Tcf3* bind to its promoter, implying its regulation by Wnt signal (Han, Zhang et al. 2012). Sall4 has also been classified as a stemness factor. It is a

multifunctional protein that contributes to pluripotency maintenance through various mechanisms including up-regulation of the three master regulators (Zhang, Tam et al. 2006; Jiang, Chan et al. 2008), positive correlation to Lif/Stat3 pathway (Bourillot, Aksoy et al. 2009) and association with *Esrrb*, *Dax1* (van den Berg, Snoek et al. 2010) and *CyclinD1 (CcnD1)* (Bohm, Kaiser et al. 2007) transcription factors.

A subgroup of Klf family – Klf2, Klf4 and Klf5 - is also critical for ESC pluripotency. They are highly expressed in ESCs and their expression decreases strongly upon differentiation (Jiang, Chan et al. 2008). Ectopic expression of *Klf4* promotes self-renewal through up-regulation of *Oct4* expression and postpones differentiation. In line with the above, *Klf4* deletion prompts cells to committed state (Li, McClintick et al. 2005). Similarly, *Klf5*$^{-/-}$ ESCs provokes differentiation induction, while *Klf5* overexpression maintains the ground state without LIF (Ema, Mori et al. 2008). *Klf4* binds directly to the promoter of *Nanog* and in conjunction with *Oct4* and *Sox2* drives its expression (Jiang, Chan et al. 2008) as well as the expression of other pluripotency genes such as *Lefty1* and *Sox2* (Nakatake, Fukui et al. 2006).

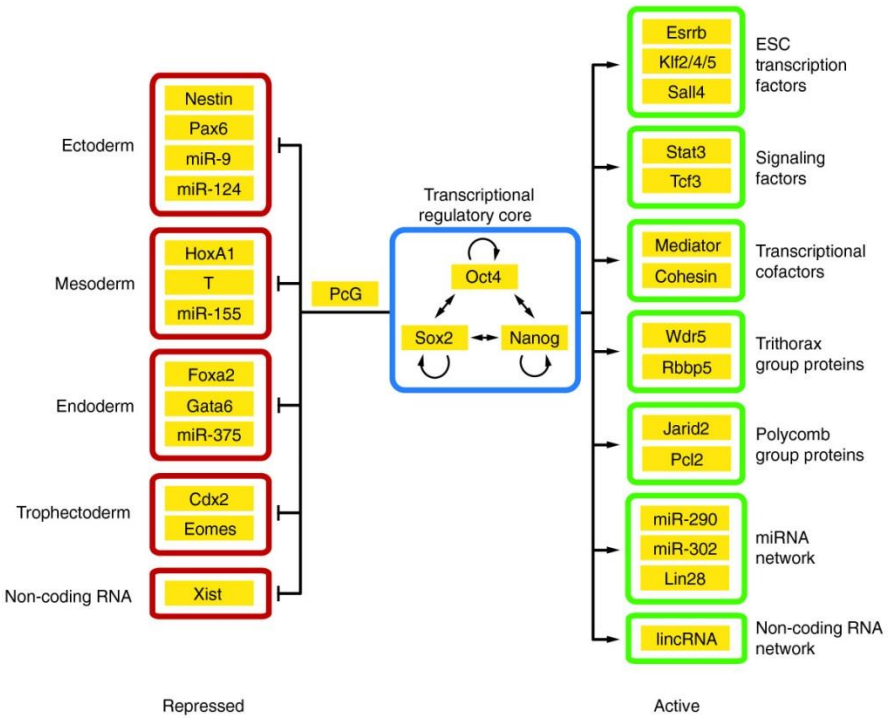


Figure 1.3: The expanded transcriptional regulatory network. Oct4, Sox2 and Nanog form an inter-connected regulatory network by binding their own promoters and promoters of other ESC members. The model of the extended pluripotency network is created by the core factors target genes that encode signaling and ESC transcription factors as well as chromatin regulators (Yeo and Ng 2011).

An additional target of *Klf4* and *Klf5* is *Esrrb* (Feng, Jiang et al. 2009) strengthening the notion that Klf4 and Klf5 are part of the regulatory circuitry for maintaining pluripotency. Recently, it was described that *Klf4* reduction induces differentiation towards visceral and definitive endoderm, while *Klf5* inhibition enhances mesodermal differentiation (Aksoy, Giudice et al. 2014). Moreover, both *Klf4* and *Klf5* have direct link to Jak/Stat3 signaling (downstream targets) and are regulated by *Stat3* and *Nanog* (Bourillot, Aksoy et al. 2009; Aksoy, Giudice et al. 2014). Interestingly, it was revealed that both *Klf4* and *Klf2* are able to drive conversion from EpiSCs to the ground pluripotent state (Guo, Yang et al. 2009). Furthermore, Klfs repress directly the characteristic epiblast marker, *Fgf5*, (Jiang, Chan et al. 2008), underlining the importance of their reduction during the transition from the naïve to the primed state.

Another transcription factor Dax1 (Nr0b1), is emerging as an essential member of the expanded transcriptional network. Its silencing induces up-regulation of differentiation genes and reduces ESCs viability (Clipsham, Niakan et al. 2004). Noteworthy, it has been shown that ectopic expression of *Dax1* leads also to ESC differentiation, proposing a bi-functional role *Dax1* as both co-activator and co-repressor. Dax1 expression is regulated by *Stat3*, *Oct4*, *Lrh-1*, *Nanog* and *Esrrb* (Sun, Nakatake et al. 2008; Kelly, Ng et al. 2011; Uranishi, Akagi et al. 2013) and it is known to bind directly to *Oct4*, resulting in the inhibition of *Oct4* transcriptional activity (Sun et al., 2009). Consequently, *Dax1* acts as a negative regulator of *Oct3/4* to sustain ESC self-renewal (Sun, Nakatake et al. 2009), underlying the importance of *Oct4* levels regulation to avoid differentiation of ESCs (Niwa, Miyazaki et al. 2000).

Besides the core regulatory network, three groups revealed that the maintenance of ESC self-renewal and pluripotency necessitates another extended network centered on *c-myc* (Chen, Chen et al. 2008; Kim, Chu et al. 2008; Hu, Kim et al. 2009). Several genome-wide analyses were performed to determine how c-myc regulates ESCs pluripotency and unveiled that it binds to and possibly regulates the transcription of at least 8000 genes in ESCs (e.g *Oct4, Sox2, Stat3 N-myc*) (Chen, Chen et al. 2008; Kim, Chu et al. 2008; Sridharan, Tchieu et al. 2009). *c-myc* has a well-established role in cell growth, proliferation, differentiation and apoptosis and it is crucial for stem cell pluripotency and proliferation (Cole et al., 2006, Laurenti et

al., 2009). It has been shown that *c-myc*-null mice develop well past the blastocyst stage of development and ESCs derived from these mice have their ability to self-renew and remain pluripotent (Charron, Malynn et al. 1990; Baudino, McKay et al. 2002). *c-Myc* is a direct target of both *Stat3* and *β-catenin* and sustained *c-myc* activity renders ESC self-renewal in the absence of LIF, while the overexpression of a dominant negative *c-myc* induces differentiation (Cartwright, McLean et al. 2005). Although inactivation of *c-Myc* has no effect on pluripotency, the simultaneous deletion of *c-myc* and *n-myc* destabilizes the pluripotent state leading to primitive endoderm and mesoderm differentiation (Smith, Singh et al. 2010). Moreover, overexpression of *c-Myc* restores pluripotency of ESCs (Smith, Singh et al. 2010), supporting the idea that *c-Myc* performs redundant roles in maintaining PSC identity. Recently, it has been indicated that c-myc in conjunction with BMP4 pathway represses MAPK signaling and subsequently inhibits differentiation (Chappell, Sun et al. 2013).

Collectively, it is obvious that the stability of naïve pluripotent state is due to the existence of a highly regulated circuit. A number of central transcription factors act concurrently to ensure the maintenance of pluripotency.

1.2.3 Signaling Pathways regulating naïve pluripotent state

1.2.3.1 LIF/STAT3 signaling

The maintenance of mESC pluripotency in culture is succeeded by Leukemia Inhibitory Factor (LIF), a member of Interleukin-6 (IL6) family. LIF binds to its high-affinity receptor LIF-R, which is heterodimerized with the membrane gp-130 protein to trigger the activation of Janus kinases (JAKs) (Lutticken, Wegenka et al. 1994; Stahl, Boulton et al. 1994). Subsequently, activated JAKs phosphorylate LIF-R and gp130 on tyrosine residues (Boulton, Stahl et al. 1994)and in turn, recruit STAT3 to the receptor complex where it is phosphorylated, resulting in its translocation into the nucleus to regulate gene transcription (Darnell 1997). Forced expression of *Stat3* sustains ESC properties in the absence of LIF (Matsuda, Nakamura et al. 1999), whereas *Stat3* deletion in LIF-maintained ESCs promotes differentiation(Niwa, Burdon et al. 1998). Interestingly, LIF cannot maintain mESC pluripotency and

induces neural differentiation in a serum-free culture conditions (Wilson, Rydstrom et al. 2001; Ying and Smith 2003), denoting the presence of alternatives pathways that cooperates with LIF to control ESC characteristics. Stat3 targets and activates several pluripotency regulators, including *Tfcp2l1, Gbx2, Klf4, Klf5, Pim1, Pim3, Pramel7*, and *c-Myc* (Cartwright, McLean et al. 2005; Aksoy, Sakabedoyan et al. 2007; Casanova, Shakhova et al. 2011; Martello, Bertone et al. 2013; Tai and Ying 2013). These lines of evidence reinforce the observation that Stat3 is required for the maintenance of ESC identity.

1.2.3.2 TGF-β signaling

The TGF-β pathway can be classified in two branches, Smad1/5/8 and Smad2/3, depending on the downstream effector molecules. Secreted TGF-β ligands bind to the extracellular domain of Type I and Type II TGF-β trans-membrane receptors (TGFβR) thereby phosphorylating and activating latent cytoplasmic SMAD transcription factors (Derynck 1994). Bone Morphogenetic Proteins (BMPs) activate Smad1/5/8, while Activin and Nodal trigger phosphorylation of Smad2/3 through TGFβR I/II. Activated Smads form a higher-order protein complex with Smad4, which then translocates to the nucleus to activate target genes. Both Smad1/5/8 and Smad2/3 branches are involved in pluripotency and differentiation of ESCs. BMP/Smad1/5/8 signaling promotes self-renewal in mESCs (Ying, Nichols et al. 2003), whereas Activin/Nodal/Smad2/3 signaling is important for proper differentiation toward the mesendoderm lineage (Fei, Zhu et al. 2010). BMP signaling strengthens the undifferentiated status of mESCs cooperatively with LIF. BMP/Smad1/5/8 signaling induces inhibitor of differentiation proteins (Ids) leading to the suppression of neuroectodermal specification and the induction of mesendoderm (Pera, Andrade et al. 2004). On the contrary, LIF/Stat3 signaling blocks mesendoderm and facilitates neural differentiation (Ying, Nichols et al. 2003). The synergistic effect of the two signaling postpones differentiation and maintains mESC self-renewal. An alternative function of concerted LIF/BMP signaling, which confers their combined effect is that BMP represses two major differentiation pathways - extracellular receptor kinase (ERK) and p38 mitogen activated protein kinase (MAPK) -, while LIF induces MAPK activation.

1.2.3.3 Wnt/β-catenin signaling

Canonical Wnt/β-catenin signaling plays also a key role in ESC identity (Sato, Meijer et al. 2004). There are more than 30 extracellular Wnt ligands, which bind to the receptor complex Frizzled and LRP5/6 (member of the LDL receptor family) (Logan and Nusse 2004). In the absence of Wnt signals, Axin/Adenomatous Polyposis Coli (APC)/Glycogen activated kinase-3 (GSK-3) complex is inactivated, resulting in β-catenin stabilization and accumulation in the nucleus (Li, Gao et al. 2012). Its interaction with the T-cell factor/Lymphocyte enhancer binding factor (Tcf/Lef) family leads to transcription of downstream target genes such as c-Myc (He et al., 1998). Studies using small molecule inhibitors have highlighted GSK-3 as a critical regulator of pluripotency for mESCs (Sato, Meijer et al. 2004). β-catenin is dispensable for mESC maintenance, however, in its absence, the positive effect of GSK3 inhibition on self-renewal is abolished (Wray, Kalkan et al. 2011). Double mutants of GSK3 and b-catenin promote exit from pluripotency and induction of neuroectoderm differentiation(Holland, Klaus et al. 2013). From the mechanistic point of view accumulating evidence indicates that the key pathway effector Tcf3 is acting as a repressor of Oct3/4, Sox2 and Nanog, whereas β-catenin inhibits this repression by converting Tcf3 into activator(Niwa 2011).

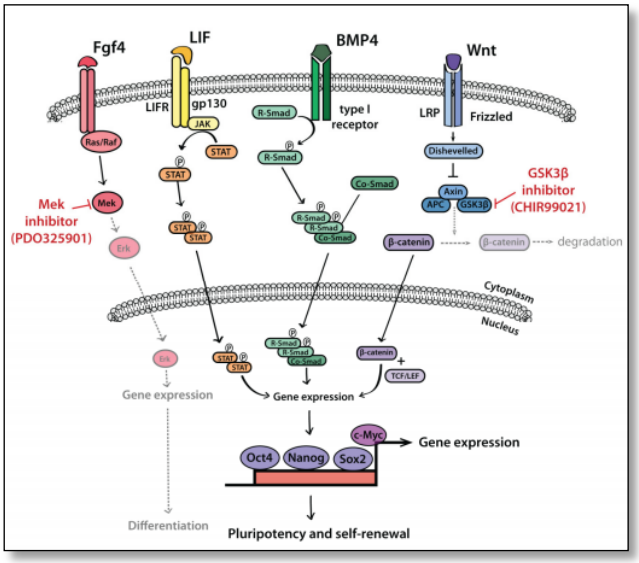


Figure 1.4: Core signaling pathways in naïve pluripotent state. mESCs require concerted LIF/BMP signaling as well as Wnt sgnaling to sustain self-renewal and pluripotency, whereas pro-differentiation FGF pathway is inhibited by Mek inhibitor (PDO325901) (Muller et al., 2013)

Consistent with Tcf3 function to suppress pluripotency, Tcf3 deletion in mESCs confers resistance to differentiation and raises self-renewal ability even on LIF withdrawal (Tam, Lim et al. 2008). Furthermore, active β-catenin interacts with Oct4 and enhances its activity in a Tcf-3 independent manner (Kelly, Ng et al. 2011). **Figure 1.4** illustrates the core signaling pathways in mESCs that are responsible for pluripotency maintenance.

1.2.4 Transcriptional control of primed pluripotent state-EpiSCs

Primed pluripotent state is deficient in many regulators that drive naive pluripotency. *Nanog, Tbx3, Klf4, Klf5, Esrrb, Dax1,* and *Myc* are low or absent in EpiSCs (Tesar, Chenoweth et al. 2007; Festuccia, Osorno et al. 2012), whereas markers such as *Otx2, Fgf5, Lefty1, Lefty2* are among the many lineage-specific genes that are elevated (Tesar, Chenoweth et al. 2007). At the same time, *Oct4*, *Sox2*, and *Nanog* activate a different subgroup of genes in EpiSCs, suggesting an alteration in their regulatory behaviour (Tesar, Chenoweth et al. 2007). Although functional pluripotency factors are not expressed in EpiSCs, primed pluripotent state is stable and is dependent on Activin/Nodal/Smad2 and FGF/ERK signaling (Vallier, Mendjan et al. 2009; Vallier, Touboul et al. 2009). Nodal, is the ligand that induces EpiSCs pluripotency by promoting the expression of *Oct4*, *Nanog* and other pluripotency genes (Camus, Perea-Gomez et al. 2006). Activin/Nodal signaling directly targets *Nanog*, resulting in the maintenance of primed state by inhibition of neuroectoderm differentiation (Vallier, Mendjan et al. 2009; Vallier, Touboul et al. 2009). FGF/ERK signaling supports primed pluripotency by preventing neural specification independent of Nanog regulation (Greber, Wu et al. 2010).

It is clear that EpiSCs are characterized by a simple network of pluripotency-related transcription factors that is also essential for cell fate decision in response to extrinsic signals.

1.3 Discovery of induced PSCs

In 2006, Yamanaka and Takahashi established a revolutionary technology in nuclear reprogramming field. They uncovered a set of transcription factors -Oct4, Sox2, Klf4, c-myc (OSKM)- that can reprogram somatic cells to pluripotent state (Takahashi and Yamanaka 2006). This was accomplished by combinatorial screening of 24 stem cell regulated genes, overexpressed in mouse embryonic fibroblasts (MEFs) by retroviruses transduction and followed by selection correlated with Oct4 target gene, first-generation F-box containing protein 15 (Fbx15) locus (Takahashi and Yamanaka 2006). The resulting Fbx15 positive colonies were called induced PSCs (iPSCs). These iPSCs showed significant expression of pluripotency markers (e.g SSEA-1 and Nanog), formed teratoma and gave rise to the three germ layers of developing embryos upon blastocyst injection in immunocompromised mice. Although some principles of ESCs were fulfilled, they expressed lower levels of many pluripotency markers compared to ESCs, showed imperfect Oct4 and Nanog promoter demethylation and failed to generate chimeras (Takahashi and Yamanaka 2006), implying that they represented an incomplete state of reprogramming. A year later, Yamanaka's and several other groups announced the generation of fully reprogrammed iPSCs that were epigenetically and molecularly resembled to ESCs, by using the Oct4 or Nanog selection method (Maherali, Sridharan et al. 2007; Okita, Ichisaka et al. 2007). Meanwhile, iPSCs have been derived from different species (human, rat, monkey) (Yu, Vodyanik et al. 2007) and other somatic cells (keratinocytes, melanocytes, stomach/ liver cells, neural cells) (Maherali and Hochedlinger 2008), by OSKM ectopic expression, demonstrating that pluripotency transcriptional network governed by Yamanaka factors is maintained in both human and mouse cells.

To date, iPSCs derivation was performed using different delivery methods including viral (retrovirus, lentivirus and adenovirus) and non-viral plasmids (e.g. episomes, transposons, nanoparticle carriers). Furthermore, different efforts facilitated the discrimination of substitute reprogramming factors. Regardless of c-myc ability to effectively reprogram somatic cells into iPSCs, its overexpression leads to tumorigenicity, proposing a possible risk for iPSCs. Thus, researchers showed

several factors (*Utf1, Wnt3a, Tbx3,* and *Zscan4*) that can substitute for *c-myc* and improve the reprogramming efficiency (Zhao and Daley 2008; Han, Yuan et al. 2010; Jang, Seo et al. 2013). *Esrrb, Sall4, E-cadherin (Cdh1)* and *Bmi-1* are additional factors that can replace OSKM and mediate the reprogramming process (Feng, Jiang et al. 2009; Moon, Heo et al. 2011; Redmer, Diecke et al. 2011). Other approaches developed for the improvement of pluripotency induction are the usage of small chemical molecules (VPA), nutritional supplements (Vitamin C) and antioxidants (NAC) (Rony, Baten et al. 2015). Recently, iPSCs were derived through direct transfection of mature microRNAs (miRNAs) (Miyoshi, Ishii et al. 2011) or by using modified RNA encoding OSKM (Mandal and Rossi 2013) (**Figure 1.5**). Despite these methods increase hope for cell-based therapeutic applications, they require further optimization before being used in clinical trials due to the lower reprogramming efficiency and lower kinetics.

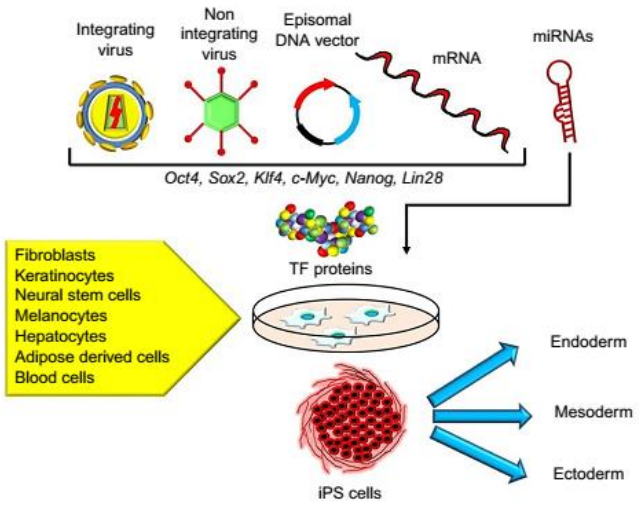


Figure 1.5: Methods of iPSCs generation and different somatic cells sources. iPSCs isolation was achieved with different methods such as viral and non-viral vector, miRNAs and modified mRNA (Sohn et al., 2012).

1.3.1 Phases of Reprogramming

Several studies proposed that reprogramming is required a stepwise transition through key sequential events in order to be successful. Researchers revealed three different reprogramming phases called initiation, maturation and stabilization (**Figure 1.6**).

Initiation step is characterized by a mesenchymal-epithelia transition (MET) (Li, Liang et al. 2010), since it is known that ESCs and iPSCs have an epithelial character, whereas fibroblasts are mesenchymal cells and it is essential to gain epithelial characteristics during this process (Li, Liang et al. 2010). Signaling pathways that influenced MET affect the reprogramming process. For instance, TGF-β signaling suppression enhances reprogramming efficiency through up-regulation and down-regulation of epithelial and mesenchymal markers, respectively (Li, Liang et al. 2010). On the other hand, BMP signaling rises reprogramming by regulating the expression of miRNAs which promote MET progression (Samavarchi-Tehrani, Golipour et al. 2010).

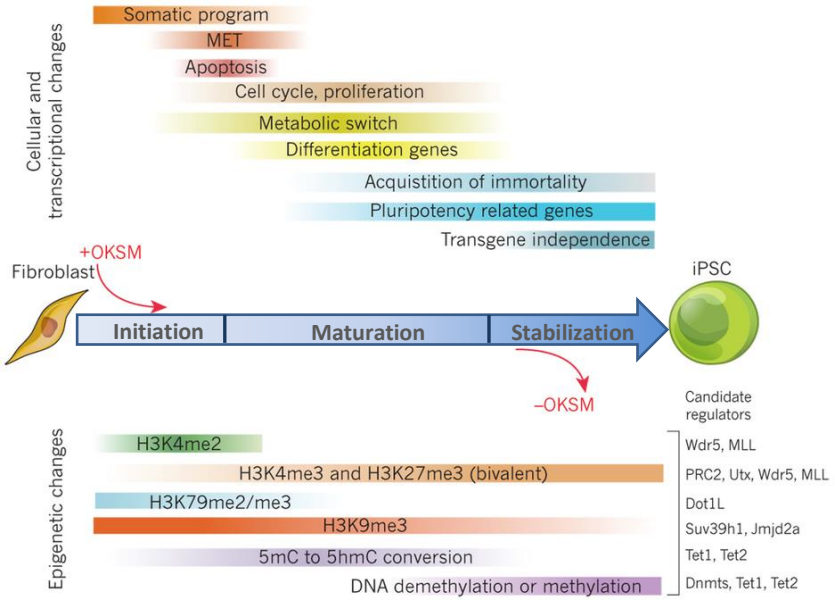


Figure 1.6: Main steps of reprogramming process. An overview of transcriptional and epigenetic alterations during the three steps of iPSC generation (Apostolou and Hochedlinger 2013)

At the same time, genes associated with proliferation, metabolism, cytoskeleton organization are activated, while development markers are down-regulated (Polo, Anderssen et al. 2012). Moreover, in this phase cells undergo alterations in histone modifications and activate DNA repair and RNA processing. Maturation step is characterized as the second wave of gene activity and is a more extended phase in comparison with the initiation step. In this stage, a stochastic activation of genes related to the embryonic development and pluripotency (Polo, Anderssen et al. 2012) is performed. The first genes that are activated are *Fbx15*, *Sall4*, *Oct4* and

subsequently *Esrrb* and *Nanog*. Furthermore, genes linked to signaling pathways and glycolysis (metabolic switch) are also up-regulated. In some cases, the pluripotency transcription factors *Utf1, Dppa2, Lin28* and *Sox2* are up-regulated, prompting the last phase of reprograming process. Sox2 activation causes deterministic events that finally result in the generation of an iPSC (Polo, Anderssen et al. 2012). In the stabilization step, the transgenes are silenced and cells adopt the full pluripotency character. In addition, during this phase, several epigenetic rearrangements are allocated such as reactivation of X chromosome (Stadtfeld, Maherali et al. 2008), DNA methylation (TETs and AID) as well as DMNTs (Polo, Anderssen et al. 2012).

Taken together, reprogramming process requires extensive transcriptional and epigenetic changes and its growing understanding raises our hope that in the future somatic cells will reach a bona fide iPSC state that will be used in regenerative medicine and stem cell therapies.

Chapter 2:

MicroRNAs for fine-tuning of mouse Embryonic Stem Cell fate decision through regulation of TGF-β signaling

2.1 Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators of gene expression through targeting the 3' or 5' untranslated regions (UTR) or the coding sequences of messenger RNAs (mRNAs) leading to translational repression and/or mRNA destabilization and degradation (Bhaskaran and Mohan 2014). The first miRNA -lin-4- was discovered in C. elegans in 1993 to control its larval development, while the second one, let-7, was found to be crucial for C.elegans in later developmental stage in 2000 (Lee, Feinbaum et al. 1993; Reinhart, Slack et al. 2000). Thereafter, thousands miRNAs have been discovered in different species and it is believed that they regulate up to 30% of the human genome. Researchers proposed that miRNAs are encoded in three different genomic regions: (a) introns of protein-coding genes, (b) introns/exons of non-coding RNA genes and (c) intergenic regions. It was also suggested that a single miRNA can control the expression of many mRNAs while several miRNAs can target a single mRNA (Bhaskaran and Mohan 2014).

Many studies provided evidence about the importance of miRNAs in the regulation of several biological processes including embryonic development as well as cancer progression and it is thought that they may be involved in therapeutic approaches for many diseases (Bhaskaran and Mohan 2014).

2.1.1 miRNA Canonical Biogenesis Pathway

Nuclear Processing

Mammalian miRNA genes are transcribed by RNA polymerase II (Pol II) or RNA polymerase III (Pol III) to generate transcripts of several kilobases, known as primary-miRNAs (pri-miRNAs), which contain one or more characteristic hairpin stem-loop structures (Cai, Hagedorn et al. 2004; Monteys, Spengler et al. 2010). Usually, the double stranded hairpin stem is approximately 33 base pairs (bp) in length, with terminal loop and two single stranded flanking regions located at the 5' and 3' ends of the transcript, with a 5' 7-methyl guanocine cap and a 3' poly-A tail (Cai, Hagedorn et al. 2004; Lee, Kim et al. 2004). Firstly, the microprocessor complex is formed by the RNase III endonuclease, Drosha and its cofactor, the DiGeorge syndrome critical

region gene 8 (DGCR8) protein. The double stranded hairpin is recognized by the complex and cleaved at the base of the stem into a smaller hairpin, the precursor-miRNA (pre-miRNA). The 70-100nt long pre-miRNAs are then recognized by Exportin 5 and exported to the cytoplasm through a Ran-GTP dependent mechanism (Bohnsack, Czaplinski et al. 2004).

Cytoplasmic Processing

Following nuclear export, pre-miRNAs are recognized by another RNase endonuclease, known as Dicer, which cooperates with a double strand RNA (dsRNA) binding protein, TRBP, to cleave the stem loop releasing the miRNA duplex. After Dicer processing, the ~22 nt RNA duplex is loaded in an Argonaut protein, Ago (1-4), to generate the RNA induced silencing complex (RISC). One of the RNA strands, termed “guide strand” is retained, while the other strand, called “passenger strand”, is released and degraded by endonucleolytic enzymatic activity of Ago protein depending on its relative thermodynamic stability (Schwarz, Hutvagner et al. 2003). In some cases, both strands have an equal propensity to retain to Ago (1-4), suggesting that guide and passenger strands may have a functional role (Ro, Park et al. 2007). Finally, miRNA guides RISC to silence messenger RNA (mRNA) targets by inhibition of mRNA translation or induction of mRNA degradation (**Figure 2.1**).

2.1.2 miRNA Non–Canonical Biogenesis Pathways

Small-RNA profiling studies from Dgcr8, Drosha or Dicer-null cells revealed unusual miRNAs that can be generated in a Drosha/DGCR8 or Dicer-independent manner (**Figure 2.2**) (Babiarz, Ruby et al. 2008; Chong, Zhang et al. 2010).

2.1.2.1 Drosha/DGCR8 independent pathway

Non-canonical intronic miRNAs are generated during “mirtron” production (Berezikov, Chung et al. 2007; Ruby, Jan et al. 2007), in which the Drosha cleavage step is bypassed (**Figure 2.2A**). These short introns are spliced from mRNAs and debranched into a short stem-loop hairpin, resembling pre-miRNAs. Debranched mirtrons are then entered the canonical pathway, cleaved by Dicer and loaded into

Ago (1-4) protein. Some mirtrons have extra tails at the 5' or 3' end and they need to be trimmed by exonucleases (Flynt, Thatcher et al. 2009).

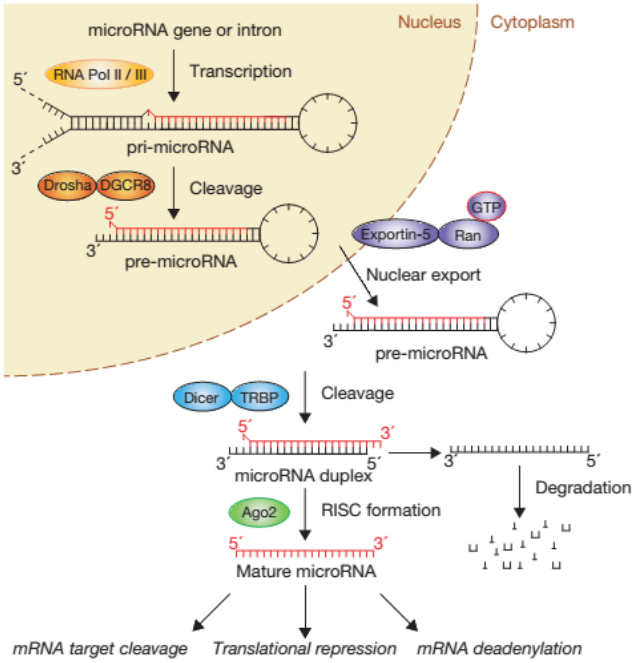


Figure 2.1: microRNA canonical biogenesis pathway. The steps of miRNAs maturation include the pri-miRNA transcript production by RNA Pol II or III and its cleavage by the microprocessor complex Drosha/DGCR8 in the nucleus. The resulting pre-miRNA is then exported to the cytoplasm by Exportin-5–Ran-GTP, where the RNase cleaves the pre-miRNA to its mature length. The guide miRNA strand is loaded together with Ago (1-4) protein into the RISC, where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand is degraded (Winter, Jung et al. 2009).

2.1.2.2 Dicer-independent pathway

Several studies have uncovered an unusual pathway for miR-451 maturation (**Figure 2.2B**). In Drosha null MEFs, miR-451 expression levels were decreased approximately 20 fold compared to wild type levels, while its expression remained constant in Dicer knockout cells, indicating that the maturation pathway of pre-miR-451 is independent of Dicer. Additionally, it has been shown that pre-miR-451 interacts with Ago2 protein and its maturation depends on Ago2 catalytic activity.

Together, these findings propose a Dicer independent-pathway in which Drosha/DGCR8 cleavage produces a short pre-miRNA (~42nt), that is exported to the cytoplasm and loaded directly to Ago2. Instead of Dicer, Ago2 slices the 42nt hairpin

resulting in an Ago2-cleaved pre-miR-451 (~30nt long), that is further trimmed by an exonuclease PARN to mature as ~22–26 nt miRNA (Yoda, Cifuentes et al. 2013).

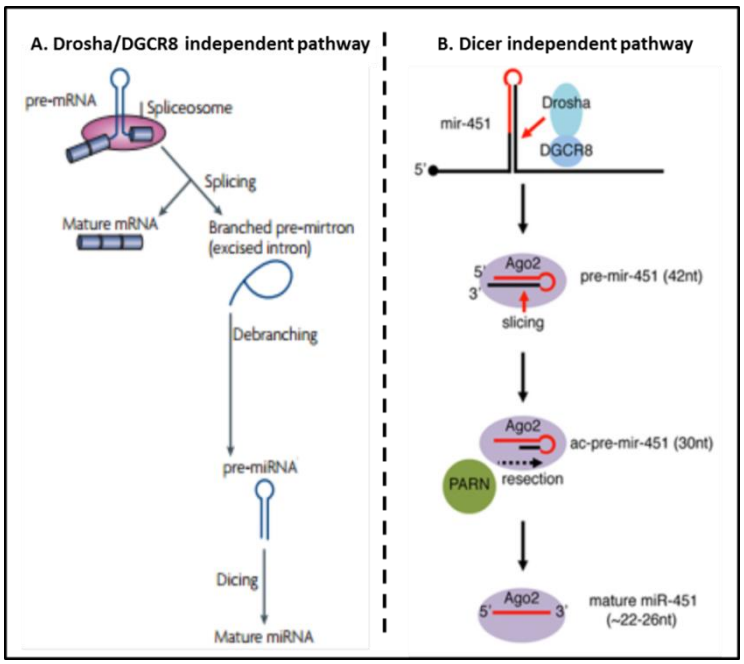


Figure 2.2: microRNA non-canonical biogenesis pathways. A. Drosha/DGCR8 independent pathway: miRNA precursors are produced from spliced introns and debranching. Then, pre-miRNAs enter the canonical biogenesis pathway for miRNA maturation (Kim et al., 2009). **B. Dicer-independent pathway:** Mir-451 precursor is generated by Drosha and is loaded directly to Ago2 and is further cleaved by PARN exonuclease to mature (Chak and Okamura 2014).

2.1.3 miRNAs and ESCs

Accumulating evidence reveals that miRNAs are crucial in controlling of pluripotent stem cell state (**Figure 2.3**). Their important regulatory role in mouse and human ESCs has been identified using Dicer and DGCR8 knockout mice. *Dicer* and *Dgcr8* deletion resulted in embryonic lethality (Bernstein, Kim et al. 2003), while *Dgcr8* or *Dicer*-deficient mESCs were viable but defective in proliferation (Wang, Medvid et al. 2007). Additionally, these cells cannot differentiate into all the germ lineages and fail to form teratomas as well as to contribute to chimeras (Kanellopoulou, Muljo et al. 2005; Murchison, Partridge et al. 2005) suggesting the importance of miRNAs for the viability and pluripotency of mESCs. Among the first families of microRNAs identified were the miR-290-295 (miR-371 family, human homologous) and miR-302-367

clusters, which include the majority of miRNAs molecules presented in mouse and human ESCs. They share a common seed sequence and regulate ESC cell cycle. Therefore, they have been characterized as ESC cell cycle regulating miRNAs (ESCC miRNAs)(Wang, Baskerville et al. 2008). Common characteristics of the two clusters include the binding of their promoters by the core pluripotency transcription factors (*Oct4*, *Sox2* and *Nanog*) (Marson, Levine et al. 2008) and their decline expression during differentiation (Babiarz, Ruby et al. 2008). The members of these families demonstrate important roles in proliferation and normal ESC self-renewal rescuing. These functions were identified after reintroducing these miRNAs to *Dicer*-knockout (Sinkkonen, Hugenschmidt et al. 2008), and *Dgcr8*-knockout (Wang, Baskerville et al. 2008) mice ESCs, respectively. It was found that these two clusters maintain the self-renewal by targeting retinoblastoma like 2 (*Rbl2*), a repressor of DNA methyltransferases (Dnmt3a and Dnmt3b). The latter methylate CpG islands and epigenetically silence *Oct4* (Benetti, Gonzalo et al. 2008; Sinkkonen, Hugenschmidt et al. 2008). In addition, these miRNAs were shown to regulate the G1/S transition of the ESC cell cycle, by repressing directly or indirectly the expression of the G1/S transition inhibitors (*p21, Lats2, Rb1, Rbl2* and *Rbl1*) (Wang, Baskerville et al. 2008). Interestingly, the *CyclinD1* and *D2* are also targeted by the miR-302 cluster in human ESCs (Card, Hebbar et al. 2008).

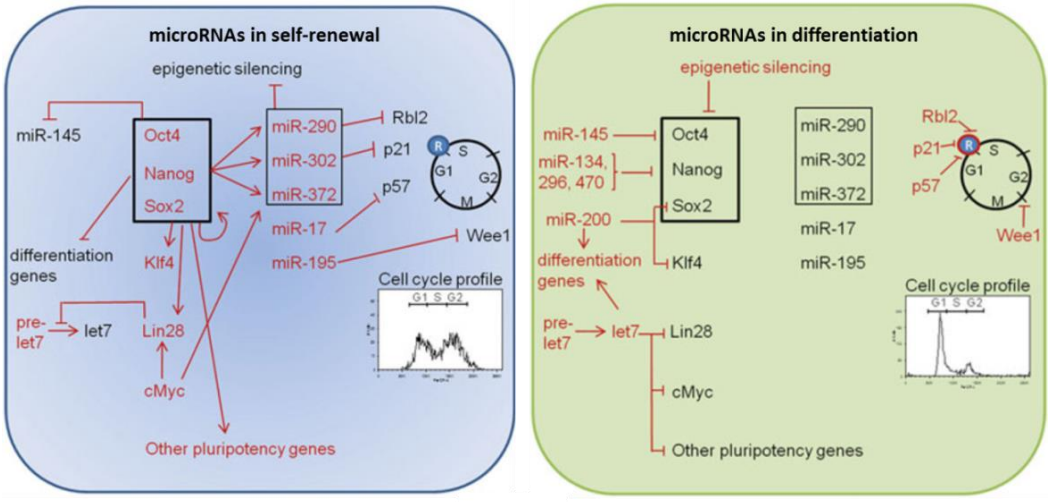


Figure 2.3: miRNAs in ESC self-renewal and differentiation. ESCs express a number of unique miRNA clusters (miR-290-95, miR-17-93, miR-302) at high levels, which control the ESC cell cycle by targeting

regulatory genes involved in G1/S and G2/M transitions and maintain ESC self-renewal(right panel). On the other hand, miRNAs such as miR-145, miR-134 and let-7 are elevated during differentiation, target pluripotency factors and activate differentiation genes (left panel) (Mathieu and Ruohola-Baker 2013).

In hESCs additional miRNAs have been implicated in cell cyle regulation. In more details, Qi and colleagues have shown that miR-195 and miR-372 promote the transition of G2/M and G1/S, by suppressing the G2/M checkpoint kinase *Wee1* and *Cdkn1a* respectively (Qi, Yu et al. 2009). Furthermore, miR-92a and 92b have been identified to target the *Cdkn1c* gene and *Cdkn2b* (known as *p57*), promoting in this way the G1/S transition (Sengupta, Nie et al. 2009).

Beside their function in maintaining pluripotency, miRNAs play important role in the differentiation capacity of ESCs. The study of Tay et al. demonstrated that miR-134, miR-296 and miR-470 bind to the coding regions of *Oct4*, *Sox2* and *Nanog* and suppress their expression and thus the self-renewal state (Tay, Zhang et al. 2008; Tay, Tam et al. 2008). Similarly, miR-200c, miR-203 and miR-183 target *Sox2* and *Klf4* (Wellner, Schubert et al. 2009), while miR-145 was shown to repress human *OCT4*, *SOX2* and *KLF4* by binding to their 3'UTR, suggesting their role to regulate pluripotency (Xu, Papagiannakopoulos et al. 2009). In another study, induction of differentiation caused the rise of miR-21 expression levels revealing its crucial role in stem cells differentiation by targeting *Nanog*, *Sox2* and *Oct4* (Singh, Kagalwala et al. 2008). Moreover, the expression of miR-22 has been also identified in high levels during ESC differentiation (Gangaraju and Lin 2009). Landgraf et al. have demonstrated that miR-26a, miR-99b, miR-193, miR-199a-5p, and miR-218 are able to suppress the self-renewal of ESC but the mechanism remains unclear (Landgraf, Rusu et al. 2007). Another example of miRNAs that regulate the differentiation of ESC is the miR-125 and miR-181 clusters. A recent study showed that Cbx7, which is the primary polycomb ortholog of the polycomb repressive complex 1 (PRC1) and is crucial for the pluripotency of ESCs, is regulated by these clusters that are induced during ESC differentiation. Their overexpression leads to the differentiation of ESCs via regulation of *Cbx7* (O'Loghlen, Munoz-Cabello et al. 2012). Furthermore, it was found that miR-34a, miR-100, and miR-137 are required for the differentiation of

ESCs, and that they function in part by targeting *Sirt1*, *Smarca5*, and *Jarid1b* mRNAs (Tarantino, Paolella et al. 2010).

Let-7 is another miRNA family which has been widely associated with the differentiated cell state. Melton et al. have identified that the silencing of pluripotency and self-renewal of ESCs can be caused by the introduction of let-7 into *Dgcr8*-knockout ESCs. In addition, let-7 binds to 3'UTR of several stemness factors (*c-Myc*, *Sall4*, *Lin28*), inhibiting their expressions and inducing ESCs differentiation (Melton, Judson et al. 2010). Interestingly, *Lin28* forms a negative feedback loop with let-7, resulting from its blocking function during let-7 biogenesis at the Dicer processing step (Newman, Thomson et al. 2008; Viswanathan, Daley et al. 2008). Let-7 can also target the G1/S transition activators (*cdc25a*, *cdk6*, *ccnD1* and *ccnD2*), improving the susceptibility of G1 phase cells to pro-differentiation signaling cascades and providing the differentiation of ESCs (Newman, Thomson et al. 2008; Viswanathan, Daley et al. 2008).

2.1.4 miR-16-1

miR-15/16 family consists of six highly conserved miRNAs (miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, miR-497), which are clustered on three different chromosomes (**Figure 2.4A**). miR-15a and miR-16-1 are located on human chromosome 13 in the first intron of deleted in leukemia 2 gene (*DLEU2*), miR-15b and miR-16-2 reside in intron 5 of the structural maintenance of chromosomes 4 gene (*SMC4)* on chromosome 3 and miR-195 as well as miR-497 are sited on chromosome 17 (Sampath, Liu et al. 2012) (**Figure 2.4B**). The first study describing a connection between miRNAs and human disease was reported in Chronic Lymphocytic Leukemia (CLL) (Calin, Dumitru et al. 2002). Deletions of 13q14 chromosomal region are always found in CLL, proposing the existence of a tumor suppressor gene in this region.

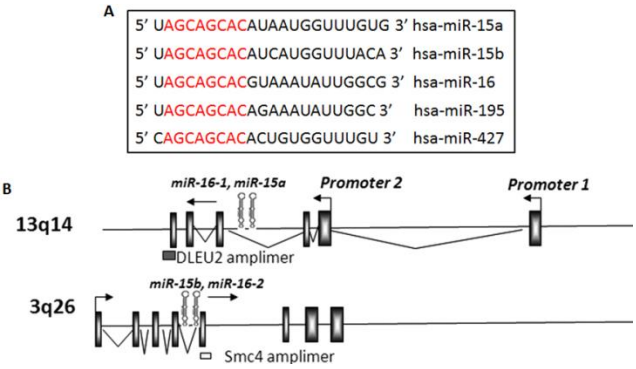


Figure 2.4: mir-15a/miR-16 family. A. Sequence of human/mouse miR-15 family members (miRBase Release 2.1). **B.** Structure and location of dleu2-miR-15a-16-1 and smc4-miR-15b-16-2 genes (Sampath, Liu et al. 2012).

Indeed, this region includes *DLEU2* gene and the intronic miR-15a/miR-16-1 cluster, whose expression levels showed significant reduction or deletion in the two/third of CLL patients (Calin, Dumitru et al. 2002). miR-15a/miR-16-1 family acts as a tumor-suppressor by targeting *BCL-2* and *WNT3A*, resulting in the induction of apoptosis and inhibition of cancer cell survival, proliferation and invasion (Calin, Cimmino et al. 2008). Other studies revealed that miR-15a/miR-16-1 cluster controls cell cycle and induces G1 arrest in many types of cancer by repressing the expression of several cell cycle related genes (*CCND1, CCND3, CCNE1, CDK6*) (Liu, Fu et al. 2008; Bandi, Zbinden et al. 2009; Aqeilan, Calin et al. 2010) (**Figure 2.5**).

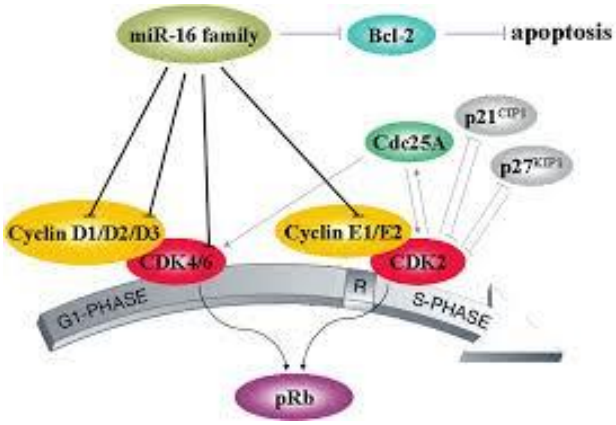


Figure 2.5: miR-15a/16-1 target genes. miR-16 family regulates G1/S transition through targeting Cdk4/6-cyclin D and Cdk2-cyclin E complexes. miR-16 cluster also promotes apoptosis by regulating Bcl2 (Liu, Fu et al. 2008).

Moreover, it was also shown that this family regulates early embryogenesis by controlling components (*Acvr2a*) of fundamental signaling pathways (TGF-β signaling) (Martello, Zacchigna et al. 2007). Additional work unveiled that miR-15a/16-1 family regulates angiogenic response. Their angiogenic effect is exerted by targeting *VEGFR2* and *FGFR1* in endothelial cells (Caporali and Emanueli 2011). During differentiation of monocytes to macrophages, it has been reported that miR-15a and miR-16-1 are repressed and lead to an elevated and reduced activity of non-canonical and canonical NF-κB, respectively, due to *IKKα* de-repression (Li et al., 2010). As stated above, miR-15a/miR-16-1 family is implicated in many human diseases. Researchers support that the investigation of its functional role may provide new tools for miRNA-based therapy.

2.1.5 miR-191

miR-191/425 cluster is highly conserved in several species and contains four mature miRNAs - miR-191-5p, miR-191-3p, miR-425-5p, miR-425-3p. This family is located on human chromosome 3, in DALR Anticodon Binding Domain Containing 3 (*DALRD3*) gene first intron (**Figure 2.6**) (Griffiths-Jones 2006).

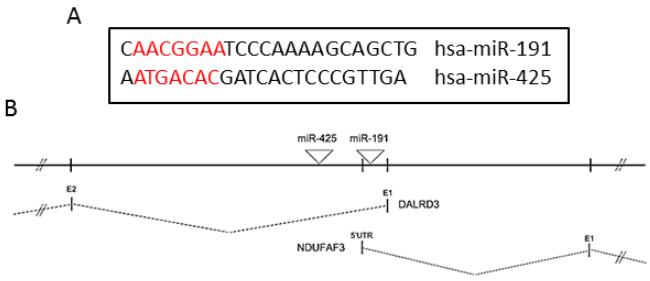


Figure 2.6: miR-191/425 family. A. Sequence of human/mouse miR-191 family members (miRBase Release 2.1). **B.** Structure and location of dalrd3-miR-191/425 (Griffiths-Jones 2006).

Previous studies have demonstrated that miR-191 connects with twenty different types of cancer, proposing that miR-191 is a famous player in cancer. Its expression is up-regulated in 16 distinct cancer types (e.g breast, prostate, pancreas, colon, lung, liver, stomach, ovarian cancer), characterizing it as an oncogenic miRNA. Recently, it was proposed that its expression is greatly down-regulated in six other types including retinoblastoma and melanoma (Nagpal and Kulshreshtha 2014). miR-

191 exerts its function through targeting several important genes ranged from chromatin remodelers to transcription factors and cell cycle regulators. For instance, in breast cancer miR-191 acts as an estrogen inducible oncomiR, and regulates cell proliferation and migration through *SATB1* targeting (Nagpal, Ahmad et al. 2013). In addition, *CDK6* is characterized as bona fide target of miR-191 in breast cancer and thyroid follicular carcinoma, resulting in the regulation of cell cycle progression and migration (Nagpal, Ahmad et al. 2013). Moreover, It was stated that mir-191 induces the EMT in hepatocellular carcinoma through directly repressing the expression of TIMP metallopeptidase inhibitor 3 (*TIMP3*) (Qin, Zhu et al. 2014). Additionally, *MDM4* gene, a p53 negative regulator, was detected as a risk factor to breast cancer and esophageal squamous cell carcinoma (Zhou, Zhang et al. 2013). Mir-191 modulates its expression and regulates cancer progression. Hence, miR-191 function is crucial for cancer biology and it is thought that regulation of its expression levels in patients (in reliable manner) might help to fight different types of cancer.

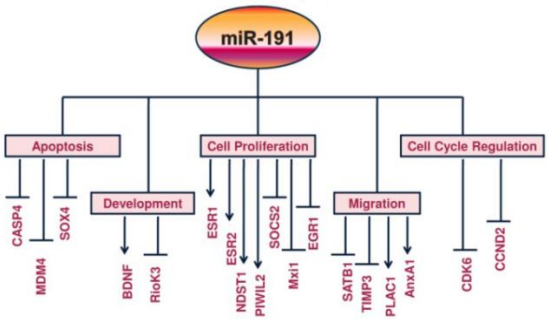


Figure 2.7: miR-191 target genes. Genes of cell cycle, development, migration, apoptosis and proliferation are regulated by miR-191 in several cancer types (Nagpal and Kulshreshtha 2014).

2.1.6 miR-23a

miR-23a, is a member of an intergenic cluster, miR-23a/miR-27a/miR-24-2, which is located on chromosome 19 and 8 in human and mouse, respectively. Interestingly, it was found that miR-23a has modified expression levels and distinct target genes in a disease dependent manner (Chhabra, Dubey et al. 2010). It is well-known that miR-23a involves in osteogenesis. miR-23a is negatively regulated by *RUNX2*, whereas it represses *SATB2* (characteristic osteogenic marker), that interacts with *RUNX2* and enhances its activity. In the final stage of osteoblastic differentiation, *RUNX2* and

SATB2 are down-regulated while miR-23a expression is induced (Hassan, Gordon et al. 2010). Recently, it was proposed that miR-23a targets *LRP5* and prevents osteogenic differentiation of mesenchymal stem cells, reinforcing its effect in osteogenesis (Li, Li et al. 2016). Furthermore, miR-23a has also a central role in muscle development and cardiac pathophysiology. The nuclear factor of activated T cells (*NFATc3*) directly regulates miR-23a expression, while muscle specific ring finger protein 1 (*MURF1*), an anti-hypertrophic protein, is targeted by miR-23a, conveying the cardiac hypertrophy. On the other hand, miR-23a is characterized as an anti-atrophic miRNA in muscle atrophy, since its overexpression protects myofibers from muscle atrophy by repressing MAFbx/atrogin-1 translation (Wada, Kato et al. 2011) (**Figure 2.8**). Concerning cancer, miR-23a is characterized either as an oncomiR (Davies, Newnham et al. 2015) or tumor suppressor (Mitchell, Guglielmo et al. 2015). Through regulating molecular targets including *PTEN, DAPP* and *CDH1* (Chandran, Keller et al. 2014), miR-23a affects apoptosis, migration and invasion in cancer cells, while TGF-β/BMP controls its expression in several human cancers(Rahman, Akhtar et al. 2015).

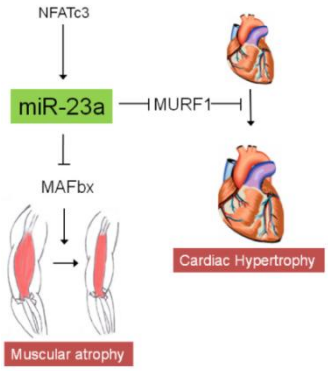


Figure 2.8: miR-23a in pathological conditions. NFATc3 activates miR-23a which targets MURF1, resulting in cardiac hypertrophy. miR-23a down-regulates MAFbx (an atrophic factor), and provides resistance to muscle atrophy (Chhabra, Dubey et al. 2010).

2.1.7 miR-421

miR-421 belongs to miR-421/374b,c family which is located intergenically on X chromosome in bouth mouse and human. A number of works demonstrated that miR-421 is significantly up-regulated in many cancer types, such as pancreatic,

gastric, hepatocellular, and nasopharyngeal carcinoma, proposing an oncogenic role for miR-421 (Zhang, Cui et al. 2012). In gastric cancer, miR-421 targets three different genes - RNA binding motif protein, X-linked-like 1 (*RBMXL1*), contactin 1 (*CNTN-1*) and chromobox homolog (*CBX7*) – and its expression levels are significantly up-regulated (Zhang, Cui et al. 2012). In addition, miR-421 promotes apoptosis resistance and cell growth in human nasopharyngeal carcinoma and neuroblastoma through *FOXO4* and menin (*MEN1*) down-regulation, respectively (Li, Li et al. 2014). Moreover, new indications showed that miR-421 is related to tumor associated nuclear receptors, like estrogen and progesterone receptor (Zhang, Gong et al. 2012). Zhang et al. revealed that farnesoid X receptor (*FXR*) is directly regulated by miR-421 in hepatocellular carcinoma cells, promoting cell proliferation and migration (Zhang, Gong et al. 2012). Another study showed that miR-421 also targets *FXR* in biliary tract cancer and induces cell proliferation and colony formation (Zhong, Yu et al. 2012). miR-421 is a central player in cancer regulation and it may be a valuable marker for specific cancer types (Zhou, Wang et al. 2016).

2.2 Study Objectives

ESC self-renewal is regulated by a complex network of transcription factors and signaling pathways (Ng and Surani 2011). On top of that, accumulating evidence reveals that miRNAs are crucial in controlling pluripotent stem cell state. Several studies reported about miRNAs involvement in ESC identity (Wang, Baskerville et al. 2008), whereas others about miRNAs promoting differentiation (Tay, Zhang et al. 2008; Tay, Tam et al. 2008). Although there is no doubt that miRNAs regulate ESC self-renewal and lineage commitment, their role in ESC related signaling pathways that determine ESC function, remains unclear. Hence, the aim of this study is to identify new miRNAs implicated in ESC identity and understand their functions. The novel identified miRNAs could be targets for miRNA-based cancer therapies since stem and cancer cells share common properties and signaling pathways. The specific aims of this project are:

- Performing miRNA expression profile of ESCs and differentiated EBs D8 using the ION-TORRENT PGM platform
- Identifying and validating differentially expressed miRNAs that were not previously involved in ESC properties
- Examining the effect of the selected miRNAs upon their inhibition or overexpression using inhibitors or mimics, respectively
- Identifying miRNA target genes using bioinformatic prediction tools and analyzing their mRNA and protein expression levels combined with luciferase reporter assay

2.3 Materials and Methods

2.3.1 Cell Culture

The murine feeder-independent ESC line CGR8 was cultured in gelatin-coated flasks in Glasgow minimal essential medium (GMEM; Gibco) supplemented with 100U/ml LIF (ESGRO-Millipore), 2mM L-Glutamine (Gibco), 100μM beta-mercaptoethanol (Gibco) and 15% heat-inactivated HyClone fetal bovine serum (FBS; GE Healthcare Life Sciences). For EB formation, cells were trypsinized and diluted in Iscove's Modified Dulbecco's Medium (IMDM; Gibco) supplemented with the above components, to a final concentration of 1000 cells/20μl. EBs were cultured without LIF as hanging drops for 2 days and then were collected and cultured in suspension for 6 more days.

2.3.2 Library Preparation-Sequencing-Analysis

Small RNAs (smRNAs) were extracted from mESCs and EBs D8 using mirVana™ miRNA Isolation Kit (Ambion). The concentrations of smRNAs were determined using NanoDrop ND-1000 Spectrophotometer and the size and purity were assessed using the Bioanalyzer 2100 (Agilent Technologies). Approximately 100ng of miRNA was used for library constructions following the protocol for the Ion Torrent Total RNA-Seq Kit according to the manufactures' instructions (ThermoFisher Scientific). Final libraries were used for sequencing analysis on the Ion Torrent PGM instrument using 316 chips. Sequencing was performed for 18–30-nt small RNA libraries using the Ion Torrent PGM platform and produced a total of more than 3Mi reads after QC and annotation was performed against miRBase (Release 21). Downstream analysis of relative miRNA abundance was conducted with the use of the CLC Workbench Suite (CLC Bio, Qiagen, http://www.clcbio.com). Samples were normalized using quantile normalization and subsequently transformed to log2 values to normalize variation across orders of magnitude. Differential expression was calculated on the basis of a paired t-test and corrected for multiple hypotheses under an FDR of 5%.

2.3.3 Reverse transcription and Real-time PCR for miRNAs and mRNA analysis

Total RNA was extracted from mESCs and differentiating EBs D8 with TRIzol Reagent (Invitrogen). Reverse transcription was done using the Universal cDNA synthesis kit (Exiqon) with 20 ng total RNA per 10 µl reaction and real-time PCR was performed with microRNA LNA PCR primer sets (Exiqon), according to the manufacturer's protocol. The miRNA expression was normalized against small nuclear RNA, U6.

Total RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was subsequently reverse-transcribed from mRNA by M-MLV Reverse Transcriptase (Takara). Target genes expression was normalized against Actin. The primers are shown below:

Actin FOR: 5' GTGTGACGTTGACATCCGTA 3', REV: 5' GTAACAGTCCGCCTAGAAGC 3', Afp FOR: 5' AAGCTGCGCTCTCTACCACCAGA 3', REV: 5' ACCACAGCCGGACCATT 3', Bmp4 FOR: 5' TTCCTGGTAACCGAATGCT 3', REV: 5' AAGTGTCGCCTCGAAGTC 3', Eomes FOR: 5' GCTTCCGGGACAACTACGA 3', REV: 5' GAGAGGAGGCCGTTGGTCT 3', Fgf5 FOR: 5' GCAGAAGTAGCGCGACGTTT 3', REV: 5' TTGACTTTGCCATCCGGGTAG 3' , Flk1 FOR: 5' GGATGGAGGCCTCTACACC 3', REV: 5' TGCCGACGAGGATAATGAC 3', Gata4 FOR: 5' GCCAACTGCCAACTACCAC 3', REV: 5' GACCTGCTGGCGTCTTAGA 3', Gata6 FOR: 5' GCCACTGTGGAGACGAGA 3', REV: 5' CATATAGAGCCCCGAAGCA 3', Gsc FOR: 5' TGCTGCCCTACATGAACGTG 3', REV: 5' CTCCAGGGCTTCGAGCTG 3', Id1 FOR: 5' GACTACATCAGGGACCTGCAGC 3', REV: 5' GGCCGCCAAGGCACTGATCTCG 3', Id2 FOR: 5' ATCCCCCAGAACAACAAGGT 3', REV: 5' ACCTTCTTGTTCTGGGGGAT 3', Id3 FOR: 5' CCAGGTGGAAATCCTGCACC 3', REV: 5' CTCTTGTCCTTGGAGATCACAA 3', Islet1 FOR: 5' GCAGCAGCAACCCAACGA 3', REV:5' TTTGCAAGGCGAAGTCAC 3' , Nanog FOR: 5' CGCTGCTCCGCTCCATAACT 3', REV: 5' GCGCATGGCTTTCCCTAGTG 3', Nr0b1 FOR: 5' CTGGTGTGCAGCGTCTGA 3', REV: 5' GTGTTGGTCTCCGGATCTC 3', Pax6 FOR: 5' GGTGCTGGACAATGAAAACA 3', REV: 5' GGTACAGACCCCCTCGGATAA 3', Oct4 FOR: 5' CCCTGGGCGTTCTCTTTGGA 3', REV: 5' ACCAGGGTCTCCGATTTGCAT 3', Sox1 FOR: 5' GAAGCGGCCGTTCATC 3', REV: 5' TCCTTCTTGAGCAGCGTCT 3',

Sox17 FOR: 5' CTCTGCCCTGCCGGGATGG 3', REV: 5' AATGTCGGGGTAGTTGCAATA 3',

T FOR: 5' GTTCCCGGTGCTGAAGGTAAAT 3', REV: 5' GCGAGTCTGGGTGGATGTAGA 3'

2.3.4 Transfection

mESCs were plated to 60-70% confluence in medium one day before transfection. Mimics and inhibitors (QIAGEN) were transfected into the cells at a final concentration of 50nM, using Lipofectamine 2000 transfection reagent (Invitrogen). Negative controls (inhibitor or mimic with no homology to any known mammalian mRNA or miRNA) were also transfected. Cells were incubated at 37°C, 5% CO2 for 72 h and harvested for protein extraction or total RNA (TRIzol; Invitrogen). For long-term experiments, cells were transfected every two days.

2.3.5 Luciferase Assay

For the 3' UTR luciferase assay, at least 300bp of the 3'UTR of the target genes was inserted downstream of the firefly luciferase gene in the pGL3 vector in XhoI and BamH1 restriction sites. Mutation in the miRNA binding sites of targets was obtained by replacing the miRNA binding site sequence with miRNA seed sequences using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). HEK293T cells were transfected with the wild type or mutant 3'UTR of target genes (1µg) in the presence of miRNAs mimcs or controls. Cells were harvested 48h post-transfection and luciferase activity was measured using dual-luciferase reporter assay system (Promega). CGR8 cells were transfected with ARE-Luc and BRE-Luc reporter plasmids using Lipofectamine 2000 following the manufacture's protocol. Cells were stimulated with 25ng/ml Activin A (R&D Systems) or 10µM SB431542 (Sigma-Aldrich) and 80ng/ml BMP4 (R&D Systems) respectively, 16h before harvesting for luciferase assay. pARE-lux was a gift from Joan Massague & Jeff Wrana (Addgene plasmid # 11768).

2.3.6 Antibodies

Proteins were detected by primary antibodies against Oct3/4 (sc-5279, Santa Cruz), Islet-1 (sc-23590, Santa Cruz), Sox17 (sc-17356, Santa Cruz), Afp (2137S, Cell Signaling), Nanog (8600S, Cell Signaling), NrOB1 (sc-13034X, Santa Cruz), p-Smad2/3 (3101, Cell Signaling), Actin (sc-47778, Santa Cruz), Smad2 (sc-8332, Santa Cruz) and Smad5 (sc-7443, Santa Cruz).

2.3.7 Flow Cytometry

For cell cycle distribution, 100.000 cells from each sample were trypsinized, washed with PBS, treated with RNAse A for 20 min at 37 0C and stained with propidium iodide (PI-Sigma) according to the manufacturer's protocol. The intensity of fluorescence was measure with flow cytometer.

2.3.8 Statistical analyses

Student's t-test was used for all statistical analyses. Statistical significance was defined as follows: * means $p < 0.05$; ** means $p < 0.01$. Values were presented as the mean ± SD.

2.4 Results and Discussion

2.4.1 Global miRNA analysis of mESCs and EBs Day 8 (D8)

To identify miRNAs pivotal for ESC function and biology, we performed a global miRNA analysis from mESCs and EBs D8. Although the analysis revealed a large number (442) of differentially expressed miRNAs, we restricted it by narrowing down on highly-abundant miRNAs. Thus, a total of 61 miRNAs with high abundance at either time point (D0 or D8) was further analyzed in terms of relative expression, relationship to the developmental process and expression of their target genes. Out of the 61 differentially expressed miRNAs, 32 were down- and 29 were up-regulated at D8, with this behavior being fairly consistent between replicates (**Figure 2.9A**). Among them, well-studied miRNAs crucial for pluripotent state were identified, such as miR-290-295 and miR-302 clusters (Gangaraju and Lin 2009; Melton, Judson et al. 2010). Additionally, those 61 miRNAs overlapped with previous published data for ESCs and EBs D5 or D7 (Lewis, Burge et al. 2005; Lee, Lim et al. 2011).

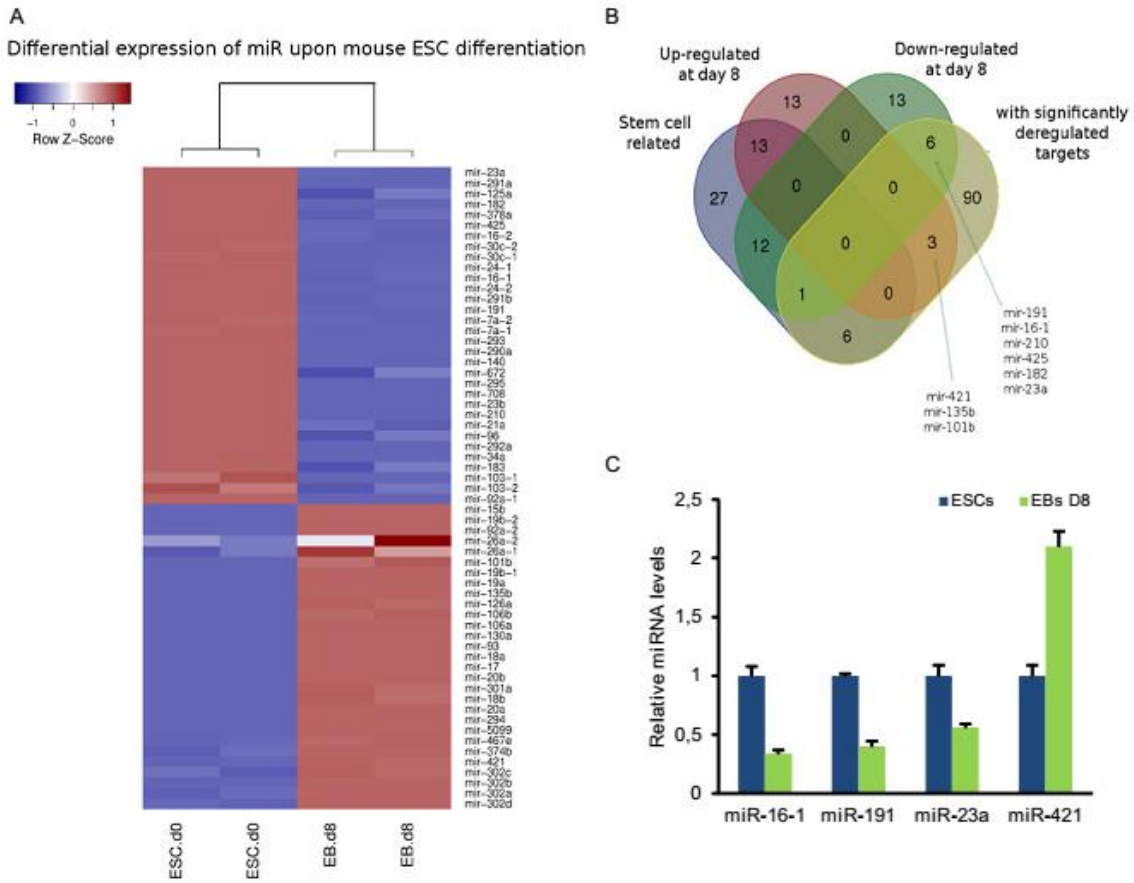


Figure 2.9: miRNA profiling of mESCs and differentiated cells (EBs D8). A. Heatmap of log2-transformed miRNA abundances in replicate samples of undifferentiated ESCs and differentiated EBs D8. **B** Venn diagram showing common miRNAs between 59 stem-cell related miRNA based on literature compiled from miRbase, our 32 down- and 29 up-regulated miRNA and 107 miRNA whose mRNA targets were found to be significantly deregulated between day 0 and day 9 in an independent study. Short lists of primary miRNA candidates for down- and up-regulated species are included. **C**. RT-PCR verification of the four selected miRNAs levels. Data shown as mean +SD of three independent experiments (n=3).

Screening the literature for mouse miRNAs (miRBase, Rel. 21), a list of 59 miRNAs reported to be implicated in the ESC differentiation process (Muheim, Sjoberg et al. 2016) was obtained. Comparing the above list with our deregulated miRNAs, 43% of them were identical (26/61). However, we focused on the rest miRNAs (57%), which have not been previously involved in ESC identity.

Following a different approach, we performed a combination of in silico target analysis coupled with gene expression data. Predicted miRanda (Gunning, Murray et al. 2016) and TargerScan (Lewis, Burge et al. 2005) miRNA targets were gathered to form a concise table of genes targeted by our differentially expressed miRNAs. We obtained expression values of mRNA genes from a genome wide expression profiling of mESC differentiation (Pellegrini, Schiff et al. 2015). Scanning the list of all miRNA measured in our study, we obtained the mean log (fold-change) of mRNA expression between D9 and D0 in the aforementioned study. By comparing this value for each of the miRNAs targets to the overall mean of expression change, we pinned down 106 miRNAs whose targets were significantly deregulated during differentiation. The intersection of these 106 miRNAs to our deregulated ones, not reported to be related to stem cell differentiation, led to two short lists containing three up-regulated and six down-regulated miRNAs (**Figure 2.9B**). After further searching the literature for the predicted targets of selected miRNAs and following validation of the expression level changes, we ended up with four miRNAs. miR-16-1, miR-191 and miR-23a are down-regulated upon differentiation, whereas miR-421 is up-regulated in EBs D8 (**Figure 2.9C**).

2.4.2 miR-16-1 and miR-191 inhibit mesendoderm differentiation via targeting Activin/Smad2 signaling pathway

To evaluate the functional role of miR-16-1 and miR-191 in mESCs, miR-16-1, miR-191 inhibitors or miR-16-1, miR-191 mimics (**Figure 2.10A**) were used, and their impact on self-renewal and pluripotency was examined. Neither the inhibition nor the overexpression of these miRNAs caused any changes at the expression levels of *Oct4* and *Nanog* (Appendix A1). Additionally, no effect on mESC morphology (data not shown) and cell-cycle (Appendix A1) was observed. This data suggests that miR-191 and miR-16-1 do not play a crucial role in mESC self-renewal.

We next examined the potential effect of miR-191 and miR-16-1 on the induction of differentiation markers in the undifferentiated state. We found that after 72h of their inhibition, characteristic endodermal (*Gata4, Gata6*) and mesodermal (*T, Gsc, Lhx1, Bmp4*) markers were slightly up-regulated, while ectodermal (*Pax6, Sox1*) markers did not seem to be affected (Appendix A1). Conversely, miR-16-1 and miR-191 overexpression did not exert changes on lineages markers compared to negative control mimic (data not shown).

To study the mechanism by which these miRNAs regulate mESC differentiation, we focused on their targets. *Smad2* mRNA is predicted to have binding sites for miR-16-1 and miR-191 (**Figure 2.10B**). Since it is known that Activin/Smad2 signaling is crucial for mesoderm and endoderm *in vivo* development (Moustakas and Heldin 2009) and *in vitro* mESC differentiation (Fei, Zhu et al. 2010), we hypothesized that miR-16-1 and miR-191 may compete with Activin/Smad2 signaling. To analyze if *Smad2* is a direct target of these miRNAs, luciferase reporter assays were performed using constructs that harbor wild type (WT) or mutant (MUT) 3' UTR of *Smad2*. We found that either miR-191 or miR-16-1 suppressed the WT but not MUT 3' UTR reporter activity and a combination of both miRNAs led to higher levels of suppression (**Figure 2.10C**).

To examine if miR-16-1 and miR-191 interfere with Activin/Smad2 signaling, we employed the Activin Response Element reporter (pARE-Lux) in mESCs and analyzed the effect of a mixture of miR-16-1/miR-191 mimics on the activity upon stimulation with 25ng/ml Activin A. Whereas Activin A enhanced the reporter

activity, simultaneous addition of 10μM SB431542 (inhibitor of Activin receptors) abolished the effect. Interestingly, the combined miR-16-1/miR-191 mimics inhibited the activation of the reporter by 47% (**Figure 2.10D**). To further confirm that miR-16-1 and miR-191 influenced Activin/Smad2 signaling, we examined the effect on SMAD2 and p-SMAD2 protein levels. miR-16-1/miR-191 knocked down mESCs had higher levels of SMAD2 and p-SMAD2, while mESCs transfected with miR-16-1/miR-191 mimics exhibited lower levels compared to controls (**Figure 2.10E**). This data reinforced the hypothesis that miR-16-1 and miR-191 diminish the activity of Activin/Smad2 signaling through *Smad2* down-regulation.

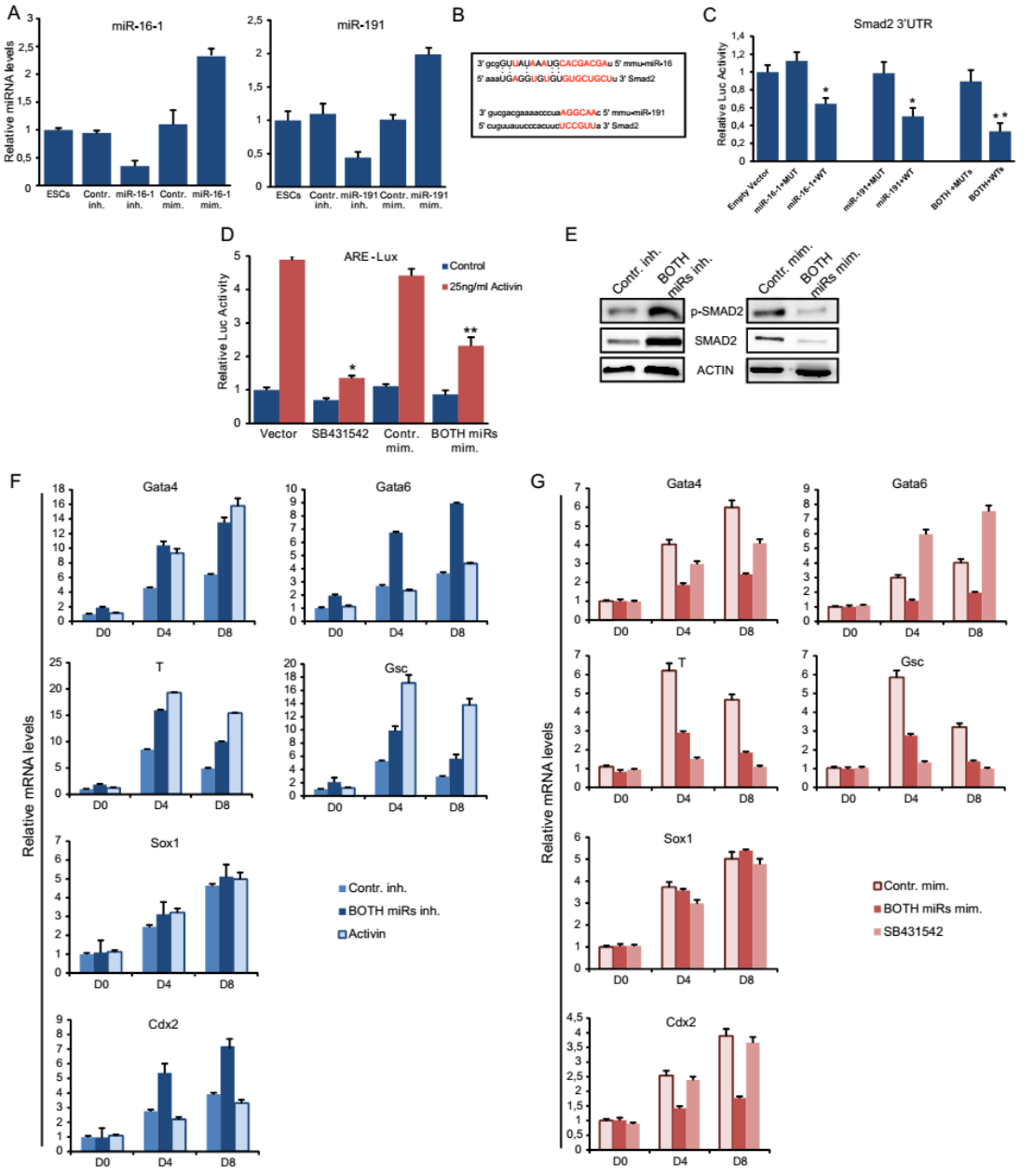


Figure 2.10: miR-16-1 and miR-191 antagonize Activin/Smad2 signaling in mESCs and repress mesendoderm differentiation. A. Measurement of miR-16-1 and miR-191 levels by RT-PCR after transient transfection with miR-mimics or inhibitors. Error bars indicate +SD (n=3). **B.** miR-16-1 and miR-191 target sites in the 3' UTR of Smad2. **C**. miR-16-1 and miR-191 specifically repress their target in the luciferase assay. Data are shown as mean + SD (n=4), *p<0.05 **p<0.001. **D**. Ectopic expression of miR-16-1/miR-191 inhibits ARE-Luc activity. Data are shown as mean + SD (n=4), *p<0,05 **p<0,001. **E**. Total SMAD2 and p-SMAD2 protein levels were detected. **F, G.** Relative mRNA levels of genes associated with the three germ layers at EBs D0, D4 and D8 in response to miR-16-1/miR-191 repression (**F**) or overexpression (**G**). Error bars indicate +SD (n=3).

To examine if the aforementioned miRNAs affect mESC differentiation program, mESCs were transfected with a mixture of miR-inhibitors or mimics, and induced to differentiate. As a control, mESCs treated with Activin A or SB431542 was used. The efficiency of miR-16-1, miR-191 knockdown or overexpression (Appendix A1), as well as the expression of several lineage markers was measured at EBs D0, D4 and D8. The induction of mesodermal (*T, Gsc*) and endodermal (*Gata4, Gata6*) markers were up-regulated upon the inhibition of miR-16-1 and miR-191 (Figure 2.10F). Activin A caused an increase of mesodermal markers (*T, Gsc*) and the endodermal marker *Gata4*, while *Gata6* was not affected, in line with previously published data (Lee, Lim et al. 2011). The significant increase of *Gata6* induction by the addition of miR-inhibitors may be attributed to *Smad2* up-regulation (Fei, Zhu et al. 2010). In contrast, the *Sox1* ectodermal marker showed no significant changes (**Figure 2.10F**). Conversely, miR-mimics reduced endoderm and mesoderm induction similarly to the activity of SB431542 (**Figure 2.10G**). Contrary to miR-mimics, SB431542 increases *Gata6* induction (Lee, Lim et al. 2011). Interestingly, due to the alteration of *Smad2* expression levels, the induction of trophectoderm marker (*Cdx2*) was significantly elevated by the miR-inhibitors and declined by the miR-mimics (Figures 2.10F, 2.10G), while it remained unaffected by Activin A and SB431542 (Fei, Zhu et al. 2010; Lee, Lim et al. 2011).

Based on the above data, we conclude that miR-191 and miR-16-1 repress mesendoderm differentiation of mESCs through direct targeting of *Smad2* and subsequent post-transcriptional control of Activin/Nodal signaling. In different settings, miR-16-1 and miR-191 are reported to regulate cell proliferation and/or cell

cycle. In detail, miR-191 acts mainly as an oncomiR, but it can also serve as a tumor suppressor (Dhillon, Fairlie et al. 2015). miR-16-1 has a well-defined tumor suppressor and cell cycle arresting role in leukemia (Mann 2015). Our data revealed that these miRNAs did not affect ESC cell cycle and this difference may be attributed to the peculiar ESC cell cycle profile. It would be interesting to investigate if Activin signaling is also involved in the tumor-regulatory functions of these miRNAs.

2.4.3 miR-23a represses ectoderm and endoderm differentiation of mESCs

To gain insights into the potential role of miR-23a in mESCs, a miR-23a inhibitor and a miR-23a mimic were used (**Figure 2.11A**). To assess the influence of miR-23a on mESC self-renewal, the expression levels of stemness markers (*Oct4, Nanog, Nr0B1*) were analyzed in mESCs transfected with miR-23a inhibitor or mimic, but no difference compared to the controls was observed (Appendix A2). Furthermore, miR-23a inhibition or overexpression did not cause any changes on mESC morphology (data not shown) or cell cycle (Appendix A2).

Following in silico research, we identified three differentiation markers, *Afp, Sox17* and *Islet1* that were predicted to be targets of miR-23a (**Figure 2.11B**). Indeed, compared to controls, their protein and mRNA expression levels were induced in mESCs transfected with miR-23a inhibitor (**Figures 2.11C, 2.11D**), while remained constant in overexpressing miR-23a mESCs (Appendix A2). Next, through a luciferase reporter assay, we verified the direct link between miR-23a and the three differentiation markers. To verify the specificity of miR-23a binding to *Sox17*, a mutated 3'UTR was used (**Figure 2.11E**). Additionally, two endodermal (*Gata6, Gata4*) and three ectodermal (*Pax6, Sox1, Fgf5*) markers were up-regulated 72h after miR-23a inhibition (Appendix A2), whereas no effect was detected on their levels in miR-23a mimic-transfected mESCs (Appendix A2).

Sox17 has been previously reported to drive the up-regulation of the primitive endoderm-associated program, giving rise to endodermal progenitors (Niakan, Ji et al. 2010). The suppression of *Sox17* and *Afp*, another endoderm marker gene, by miR-23a is reinforcing the hypothesis that miR-23a inhibits differentiation towards this lineage.

To test this assumption, mESCs transfected by miR-23a inhibitor or mimic were allowed to differentiate as EBs. miR-23a inhibition or overexpression was verified on EBs D0, D4 and D8 (Appendix A2). A significant increase in the induction of endodermal (*Afp, Sox17, Gata6, Gata4*) and ectodermal (*Islet1, Fgf5, Sox1*) genes was observed (**Figure 2.11F**) upon miR-23a inhibition, whereas trophectoderm and mesoderm lineage markers were not affected (Appendix A2). Interestingly, in miR-23a overexpressing mESCs, the differentiation towards these lineages is suppressed, suggesting that expression level of miR-23a is critical for pluripotency maintenance (**Figure 2.11F**).

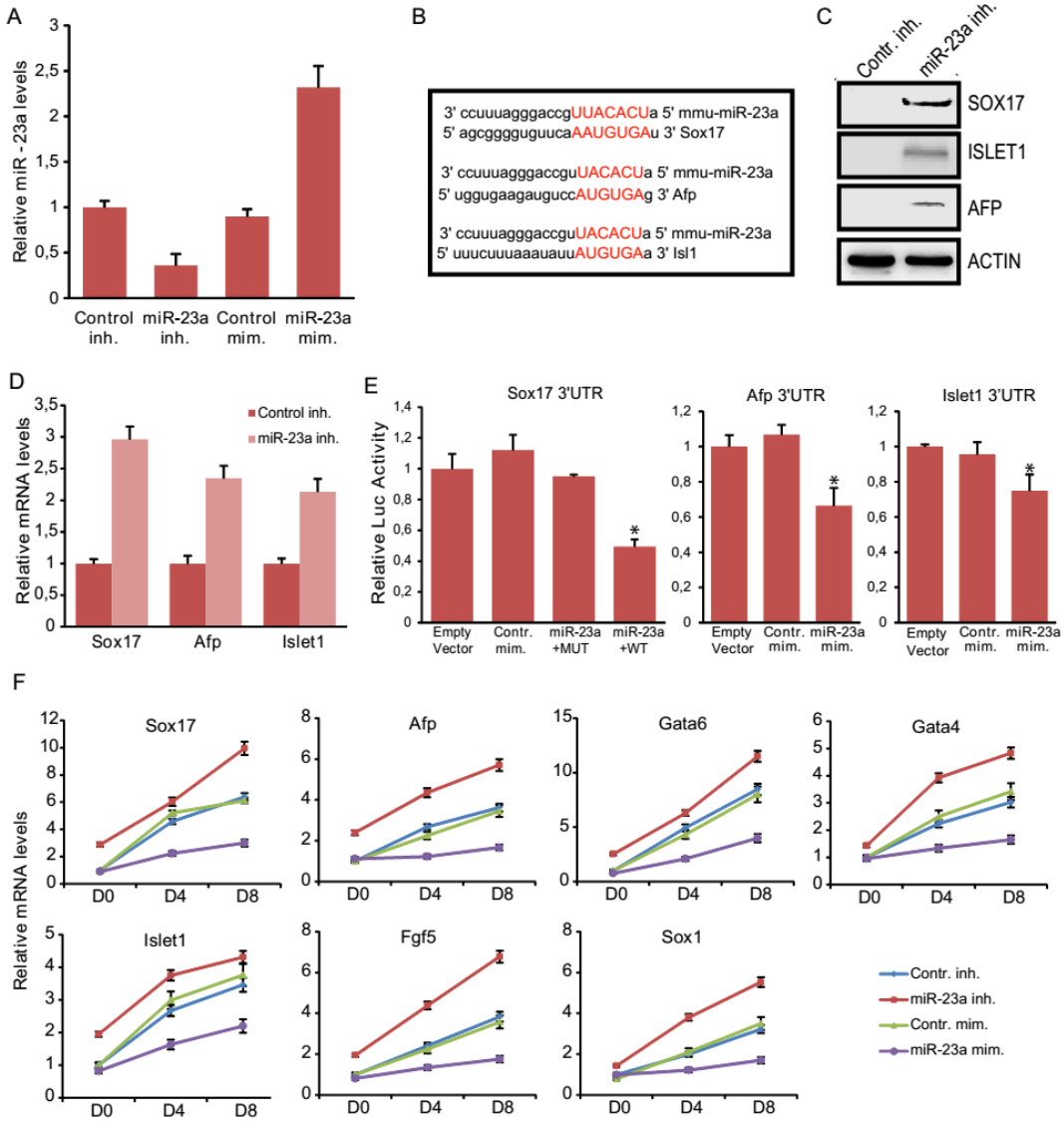


Figure 2.11: miR-23a represses endoderm and ectoderm differentiation. A. miRNA expression levels in miR-23a mimic or inhibitor transfected mESCs. Error bars indicate +SD (n=3). **B**. Prediction of the binding sites of miR-23a on the 3'UTR of the indicated differentiation associated genes. **C, D**. miR-23a inhibition led to induction of SOX17, AFP and ISLET1 protein (**C**) and mRNA (**D**) levels in mESCs. **E**. Relative luciferase activity of the wild-type 3'UTR reporter co-transfected with mir-23a mimic. Data are shown as mean +SD (n = 4), *p < 0.05. **F**. Relative mRNA levels of differentiation genes after EBs formation in response to miR-23a inhibition or overexpression. Error bars indicate +/-SD (n=3).

The above results clearly show that miR-23a is an additional regulator of ESC differentiation. Recently, miR-23a/24-2/27a cluster has been reported to be regulated by BMP4 and target *Smad5* to protect mESCs from apoptosis during the transition to EpiSCs (Musto, Navarra et al. 2014). Additionally, miR-23a inhibits the osteoblast differentiation via targeting *Runx2* (Matheson and Sakata 2015). In line with the above observations, our results strongly support that miR-23a is a pivotal regulator of differentiation and controls ESC specific germ layer commitment and subsequent lineage decisions.

With respect to cancer, miR-23a has been considered either as an oncomiR (Davies, Newnham et al. 2015) or tumor suppressor (Mitchell, Guglielmo et al. 2015). Apoptosis, migration and invasion are some of its effect in cancer through regulating molecular targets (*PTEN, DAPP*) (Chandran, Keller et al. 2014), while TGF-β/BMP has been implicated to control the expression of miR-23a in human cancers (Rahman, Akhtar et al. 2015).

In conclusion, miR-23a has a role in both tumor progression and mESC function and the cross regulatory relationship with the TGF-β/BMP signalling awaits further investigation.

2.4.4. miR-421 regulates distinct fate choices of ESCs through Oct4 repression and competition with BMP signaling

In contrast to the above miRNAs, miR-421 was identified as a differentiation-associated regulator and its expression level was up-regulated during EB formation.

To study whether miR-421 is a crucial player in controlling differentiation, we ectopically expressed miR-421 in mESCs by using its mimic (**Figure 2.12A**). Compared

to the control, miR-421 mimic had no effect on cell morphology (data not shown) and cell-cycle progression (Appendix A3), but its addition significantly reduced the *Oct4* expression levels (**Figure 2.12B**), while other pluripotency genes remained constant. Using bioinformatics tools (Miranda, TargetScan), miR-421 was predicted to bind to the *Oct4* 3'UTR and the direct link between the two was further confirmed by luciferase reporter assay (**Figure 2.12C**).

To test the effect of miR-421 overexpression on differentiation, the expression levels of several lineage markers were analyzed. Interestingly, the trophectoderm marker *Cdx2* was up-regulated (**Figure 2.12D**), in agreement with previous studies showing the repression of trophectoderm by *Oct4* (Beck, Erler et al. 1995; Velkey and O'Shea 2003). Moreover, miR-421 overexpression was accompanied by a slight induction of primitive endoderm markers (*Gata4, Gata6, Afp*), which is consistent with previously published data analyzing the changes of gene expression upon inhibition of *Oct4* (Hay, Sutherland et al. 2004; Strumpf, Mao et al. 2005). Interestingly, ectoderm associated markers (*Pax6, Sox1*) were also up-regulated (**Figure 2.12D**), indicating that miR-421 might exert its action through additional mechanism.

Due to the fact that several components of BMP-signaling were predicted as candidate targets of miR-421 (*Bmpr1, Smad5, Id2*) (**Figure 2.12E**), we hypothesized that miR-421 may regulate this signaling and thereby, lineage specification. Since BMP pathway plays important role in maintaining mESCs in the pluripotent state (Ying, Nichols et al. 2003), through the activation of Id proteins acting as neuronal differentiation inhibitors (Ying, Nichols et al. 2003; Zhang, Li et al. 2010), the effect of miR-421 on the BMP activity was investigated. Firstly, we confirmed that miR-421 overexpression significantly repressed the luciferase activity of the BRE-Luc reporter gene in response to BMP4 treatment, compared to control (**Figure 2.12F**). Overexpression of miR-421 also reduced the mRNA expression levels of the endogenous targets of BMP signaling *Id1, Id2* and *Id3* (**Figure 2.12G**). Moreover, a luciferase reporter assay confirmed that miR-421 targeted directly the *Id2* 3'UTR (**Figure 2.12H**). Interestingly, SMAD5 protein levels were decreased in mESCs expressing miR-421 mimic (**Figure 2.12I**), while *Smad5* 3' UTR reporter assays verified the direct regulation of *Smad5* by miR-421 (**Figure 2.12J**). To further analyze

the function of miR-421 in differentiation, mESCs transfected with miR-421 mimic or

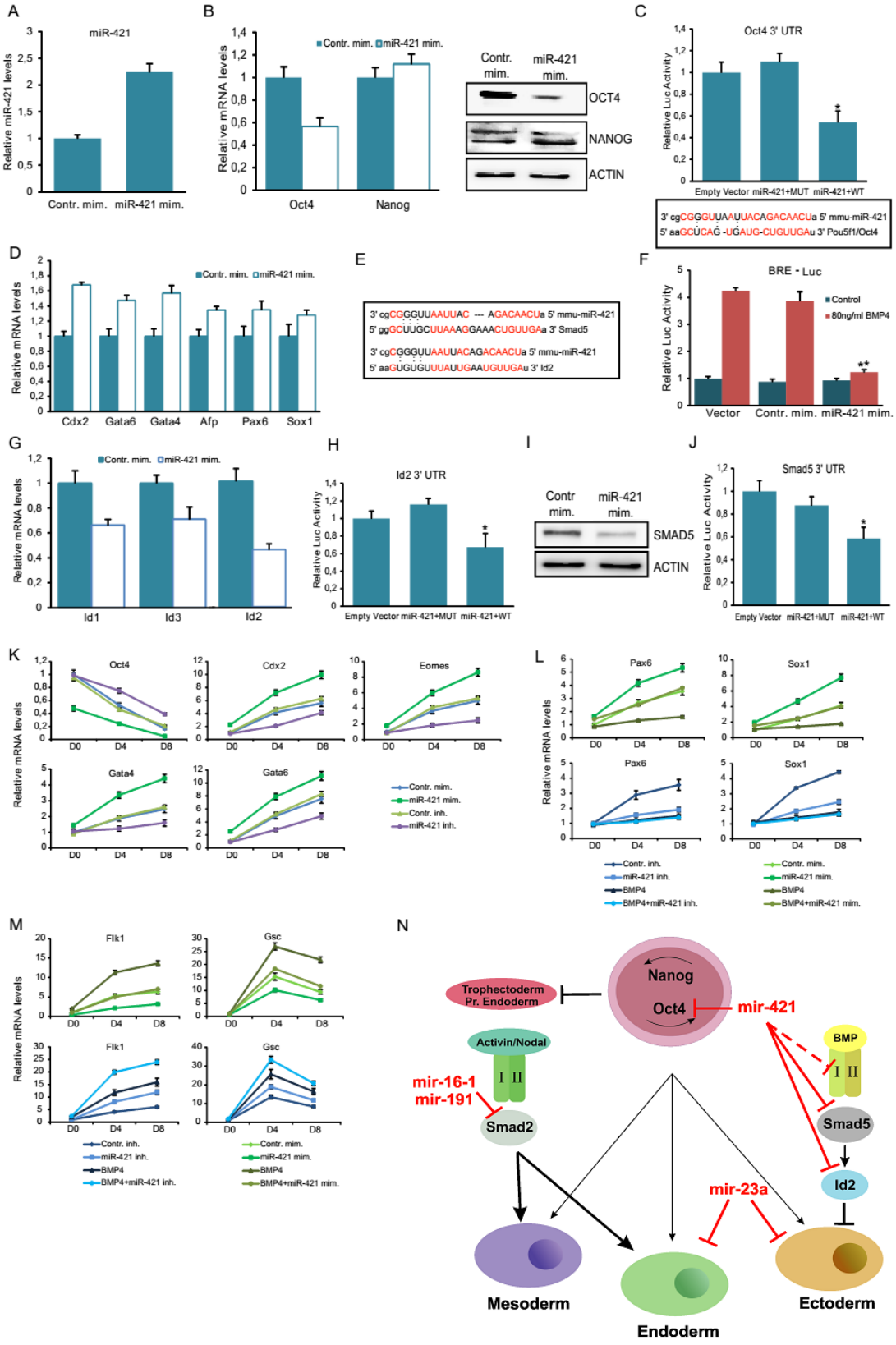


Figure 2.12: miR-421 induces differentiation through suppressing Oct4 and regulating BMP-signaling pathway. **A.** Measurement of miRNA levels by RT-PCR after transient transfection with miR-421

mimic. Error bars indicate +SD (n=3). **B**. mRNA and protein levels of stemness factors (Oct4 and Nanog) after miR-421 overexpression **C**. mir-421 target sites in the 3' UTR of Oct4. Luciferase activity of Oct4 3'UTR upon miR-421 mimic supplementation. Data are shown as mean +SD (n = 4), *p < 0.05. **D.** Relative mRNA levels of differentiation markers in miR-421-induced mESCs. Error bars indicate +SD (n=3). **E.** miR-421 binding sites in the 3' UTR of Smad5 and Id2. **F.** miR-421 overexpression inhibits BRE-Luc activity. Data are shown as mean +SD (n = 4), **p < 0.001. **G**. RT-PCR analysis of BMP4 target genes (Ids) expression levels in miR-421 overexpressed mESCs. Error bars indicate +SD (n=3). **H**. Overexpression of miR-421 decreased the luciferase activity of Id2. Data are shown as mean +SD (n = 4), *p < 0.05. **I.** SMAD5 protein levels and **J.** the Smad5 3'UTR luciferase activity were reduced by miR-421 mimic. Data are shown as mean +SD (n = 4), *p < 0.05. **K.** Relative mRNA levels of differentiation genes at EBs D0, D4, D8 upon miR-421 overexpression or inhibition. Error bars indicate +/-SD (n=3). **L, M.** Relative mRNA levels of ectodermal (**L**) and mesodermal (**M**) differentiation genes at EBs D0, D4, D8 upon miR-421 overexpression or inhibition in the presence of BMP4. Error bars indicate +/-SD (n=3). **N.** Proposed mechanism for the regulation of mESC differentiation by the aforementioned miRNAs.

miR-421 inhibitor were differentiated (Appendix A3). The overexpression of miR-421 favored the suppression of *Oct4* and at the same time enhanced the induction of trophectoderm (*Cdx2, Eomes*) and endoderm (*Gata4, Gata6*) differentiation (**Figure 2.12K**). Concerning the induction of ectodermal markers (*Pax6, Sox1*), miR-421 elevation caused a significant increase, whereas the addition of BMP4 did not allow differentiation towards this lineage. miR-421 inhibitor up-regulated *Oct4* expression and down-regulated the expression of trophectoderm, endoderm and ectoderm differentiation markers (**Figures 2.12K, 2.12L**). Moreover, mesodermal markers (*Flk1, Gsc*) were not induced upon miR-421 overexpression, in contrast to miR-421 inhibition or BMP4 treatment where their induction was significantly raised (**Figures 2.12M**, Appendix A3). In agreement with these data, the concurrent addition of miR-421 mimic and BMP4 did not affect the differentiation induction. It is noteworthy, that ectodermal genes appeared to be decreased, while mesodermal markers were significantly increased in BMP4/miR-421 inhibitor treated cells (**Figures 2.12L, 2.12M,** Appendix A3). The above experimental results suggest that miR-421 is a positive regulator of mESCs differentiation by two mechanisms, suppression of *Oct4* and competition with BMP signaling. Contrary to its function in mESCs, miR-421 has been previously characterized as an oncomiR in several cancers. In neuroblastoma,

miR-421 suppresses ataxia–telangiectasia mutated (ATM) uncoupling DNA damage from cell cycle check points (Rezende, Silva Martins et al. 2015). In pancreatic tumor cells, miR-421 represses *Smad4* that is critical for BMP signal transduction and represses its target gene *Id3*, promoting cell proliferation and colony formation (Otchy, Wolff et al. 2015). Therefore miR-421 regulates Smad4-mediated signaling pathways in cancer cells. Additionally, miR-421 is regulated by TGF-β and BMP4 pathway in pulmonary artery smooth muscle cells, via a conserved Smad binding element (SBE) (Marchand, Proust et al. 2012).

To conclude, this study unveils a miRNA-mediated mechanism for miRNAs that regulate ESC fate decisions . Regarding miR-16-1, miR-191 and miR-421 this effect is due to competition with TGF-β family signaling. Inhibition of Activin/Nodal pathway by miR-16-1 and miR-191 promotes mESC maintenance, whereas competition of miR-421 with the BMP pathway results in mESCs exit from pluripotency and their commitment to ectodermal fate. Conversely, miR-23a is itself regulated by TGF-β/BMP. Taken together, our work reveals a reciprocal antagonism between the investigated miRNAs and TGF-β signaling pathways in regulating ESC differentiation (**Figure 2.12N**). Our findings link these miRNAs with the TGF-β/BMP signaling and may have implications in cancer biology as TGF-β pathway is a critical regulator of tumor growth, invasion and metastasis (Drabsch and ten Dijke 2012). miRNAs that have parallel function in cancer and stem cells may be useful candidate molecules to advance the basic knowledge and design combinatorial strategies for cancer and cell replacement therapies.

Chapter 3: PML, a crucial regulator of mouse embryonic and induced pluripotent stem cells

3.1 Introduction

3.1.1 PML protein structure and PML Nuclear Bodies (PML-NBs)

The promyelotic leukemia (*Pml*) gene was first described in the early 1990s, at the chromosomal translocation breakpoint t(15;17) in acute promyelotic leukemia (APL) (de The, Lavau et al. 1991; Kakizuka, Miller et al. 1991). It was originally identified as a fusion partner of retinoic acid receptor-α (RARα), giving rise to the oncogenic protein (PML-RARα) (**Figure 3.1**). The fusion protein impairs the functions of both parental proteins and blocks the hematopoietic differentiation at the promyelotic stage, promoting the inappropriate survival and proliferation of early myeloid progenitors (de The, Lavau et al. 1991; Kakizuka, Miller et al. 1991).

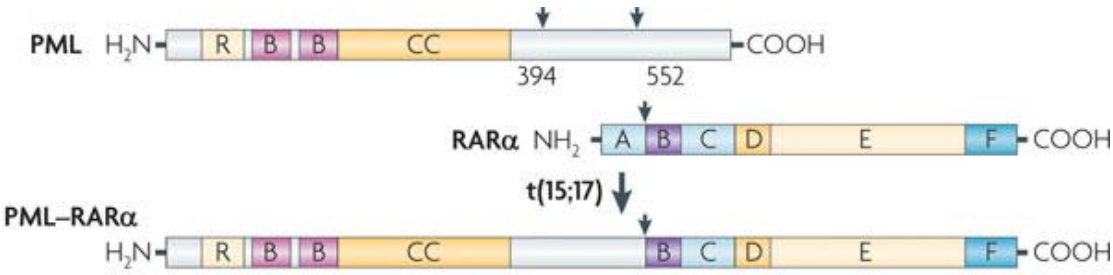


Figure 3.1: Chromosomal translocation between promyelotic leukemia *PML* gene on chromosome 15 and retinoic acid receptor-α (RARα) gene on chromosome 17 in acute promyelotic leukemia (APL) (Hatakeyama 2011).

The *Pml* gene consists of nine exons and is approximately 53kb in length. Alternative splicing of C-terminal exons, results in seven PML isoforms, six nuclear and one cytoplasmic (de The, Lavau et al. 1991) (**Figure 3.2A**). Interestingly, the first three exons, contains three cysteine-rich zinc-binding domains, a RING finger (R), two B-box (B) and a α-helica coiled-coil domain (CC), forming the RBCC/TRIM motif at the N-terminal (418aa) (Borden, Campbell Dwyer et al. 1998). Every isoform share RBCC/TRIM motif while they differ either in the central or in the C-terminal region. The distinct C-terminus of each PML isoform determines its functional identity, since the partner binding specificity relies on RBCC/TRIM domain. Moreover, several motifs were recognized in PML isoforms c-terminal end. The nuclear export signal (NES) was identified only in PMLI, whereas sumo interacting motif (SIM) was found in PMLI-V (Scaglioni, Yung et al. 2006; Shen, Lin et al. 2006). A nuclear localization

signal (NLS) is borne by the most PML isoforms, thus they are predominantly localized in the nucleus. Additionally, three lysine residues were identified in PMLI-VI as small ubiquitin-related modifier (SUMO)-1 modification sites: K65, K160 and K490 (**Figure 3.2B**).

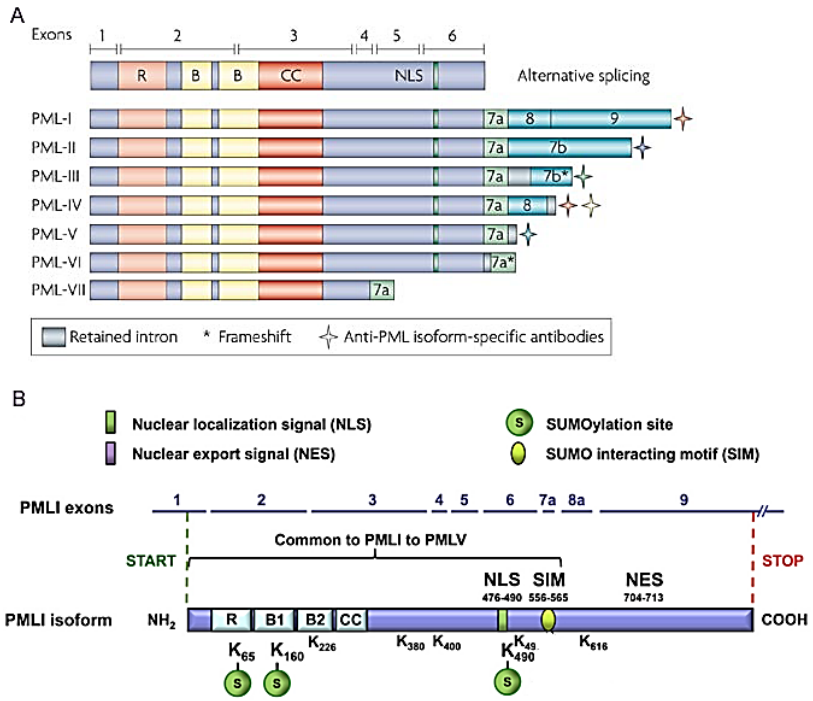


Figure 3.2: PML isoforms and their organization domain. A. C-terminal region alternative splicing results in seven main PML isoforms (Bernardi et al., 2007). **B.** The RBCC/TRIM, the NLS, the NES, and the SIM motifs are represented as well as the three major (K65, K160, and K490) SUMOylation sites (Nisole, Maroui et al. 2013).

The PML protein is the key organizer of spherical subnuclear structures, named PML nuclear bodies (PML-NBs). PML NBs size ranges between 0.1 to 1 µm while their number is typically 1-30 per nucleus, depending on the cell type, cell-cycle phase and differentiation stage. PML deletion leads to PML-NBs loss, pointing out that it is essential for their integrity (Lallemand-Breitenbach and de The 2010). The biogenesis of PML-NBs is based on the dimerization of PML through the RBCC domains, followed by its multimerization for NBs nucleation. Afterwards, sumoylation of PML results in the formation of a spherical body outer shell, while SIM-containing or sumoylated partners are recruited into the body (Bernardi and Pandolfi 2007). PML-NBs are proteinaceous structures tightly bound to the nuclear matrix. An ever growing number and diversity of proteins reside in PML NBs, suggesting PML

implication in virtually all major cellular signalling pathways (Lallemand-Breitenbach and de The 2010).

3.1.2 Transcriptional regulation of PML

Several reports demonstrated that PML expression is tightly regulated at the transcriptional level. The steady-state expression levels of PML depend on the tissue, the cell type, as well as the differentiation stage. However, this pattern changes in response to soluble factors and cellular stress. PML up-regulation is induced by interferons (IFNs) via the signal transducers and activators of transcription (STATs) and INF regulatory factor 3 (IFN3) (Lavau, Marchio et al. 1995). Tumor necrosis factor α (TNF-α) also up-regulates PML expression by promoting its transactivation dependent on STAT1 (Cheng, Liu et al. 2012). Furthermore, interleukin 6 (IL-6) is another cytokine that induces PML transcription through NF-κB and JAK/STAT signaling pathways (Hubackova, Krejcikova et al. 2012). P53 also up-regulates PML expression and several p53 responsive elements are located in the PML promoter region. The p53 homolog, p73, induces also PML transcription, but Akt/PKB can attenuate this activation (Lapi, Di Agostino et al. 2008). Moreover, β-catenin activates PML promoter in a LEF/TCF-independent manner in p53-negative specific cells (Shtutman, Zhurinsky et al. 2002).

3.1.3 Post-translational modifications of PML

Besides transcriptional regulation, PML protein can be post-translationally modified, resulting in the modulation of its stability, PML-NBs biogenesis and protein interaction. All known PML post-translational modifications influence regions of the protein that are common to most of the isoforms.

3.1.3.1 Sumoylation

As mentioned above, PML can be conjugated with SUMO at lysines 65, 160 and 490 (Duprez, Saurin et al. 1999). Lysine 490 resides in exon 6 and is thus absent in cy(Lallemand-Breitenbach and de The 2010)toplasmic PML VII (Jensen, Shiels et al. 2001). PML symoylation is crucial for PML NBs assembly (Bernardi and Pandolfi 2007). For instance, it has been shown that ectopic expression of SUMO proteases

leads to the loss of PML NBs (Nichol, Petruccelli et al. 2009). PML protein, is sumoylated by each of SUMO 1-3. However the functional significance of each form of modification is incompletely understood (Bernardi and Pandolfi 2007). PML is mono-sumoylated by SUMO 1, whereas SUMO 2 and 3 poly-sumoylate PML. Its symoylation is catalyzed by Ubc9, an E2 SUMO conjugating enzyme, through the interaction with the RING domain (Duprez, Saurin et al. 1999).

Sumoylation is a dynamic process that has a central function in regulating the biogenesis and role of both PML proteins and PML NBs. It has been revealed that PML symoylation induces or inhibits its degradation regarding the sumoylation pattern. For example, RNF4/SNURF, an E3 ubiquitin ligase, identifies sumoylated PML and promotes its ubiquitination and subsequent degradation. (Tatham, Geoffroy et al. 2008; Percherancier, Germain-Desprez et al. 2009). On the contrary, PML degradation is prevented through prolyl-isomerase Pin1 activity. These contradictory findings propose that the exact sumoylation pattern on PML may play a critical role in controlling PML stability.

3.1.3.2 Phosphorylation

PML phosphorylation is another pivotal modification affecting PML stability. In the presence of growth factors (e.g. IGF1, ERK2), PML is phosphorylated and induces Pin1-mediated degradation, whereas high doses of H_2O_2 disturb the interaction between PML and Pin1, stabilizing PML protein (Reineke, Liu et al. 2010). Everett and coworkers demonstrated that PML phosphorylation also occurs during the cell cycle (specifically during mitosis), but its functional role remains unclear (Everett, Lomonte et al. 1999). Casein kinase 2 (CK2), highly expressed in many tumours, phosphorylates PML and promotes PIAS1-mediated PML degradation (Rabellino, Carter et al. 2012) (Scaglioni, Yung et al. 2006). In similar way, Big MAP Kinase 1 (BMK1) phosphorylates PML at S403 and T409 and induces its degradation, disrupting PML and Mdm2 interaction and inhibiting p53 activity (Yang, Liao et al. 2013).

3.1.3.3 Acetylation

PML is acetylated at lysines 487 and 515 by the histone acetyltransferase (HAT) p300 (Hayakawa et al., 2008). Its acetylation occurs in vivo after the addition of trichostatin A (TSA). Moreover, p300 expression resulted in an increase of PML sumoylation, proposing that acetylation may enhance sumoylation (Hayakawa, Abe et al. 2008).

3.1.4 PML-mediated signaling pathways in cellular activities

PML is involved in central cellular processes including transcription, apoptosis, senescence and response to DNA-damage (**Figure 3.3**). The involvement of PML in such diverse biological functions stems from the numerous proteins that were found to reside or transit through PML-NBs. Non-mutually exclusive models were suggested to describe how PML-NBs exert their biological functions. Firstly, PML-NBs may serve as a storage depot for the accumulation of proteins both in pathological conditions (to sequester foreign or misfolded proteins) and in normal conditions (to accumulate proteins and release them when necessary). Secondly, PML-NBs function as "catalytic surfaces" where proteins accumulate to be post-translationally modified. Alternatively, PML-NBs could serve as active sites for specific nuclear functions like transcriptional and chromatin regulation(Lallemand-Breitenbach and de The 2010).

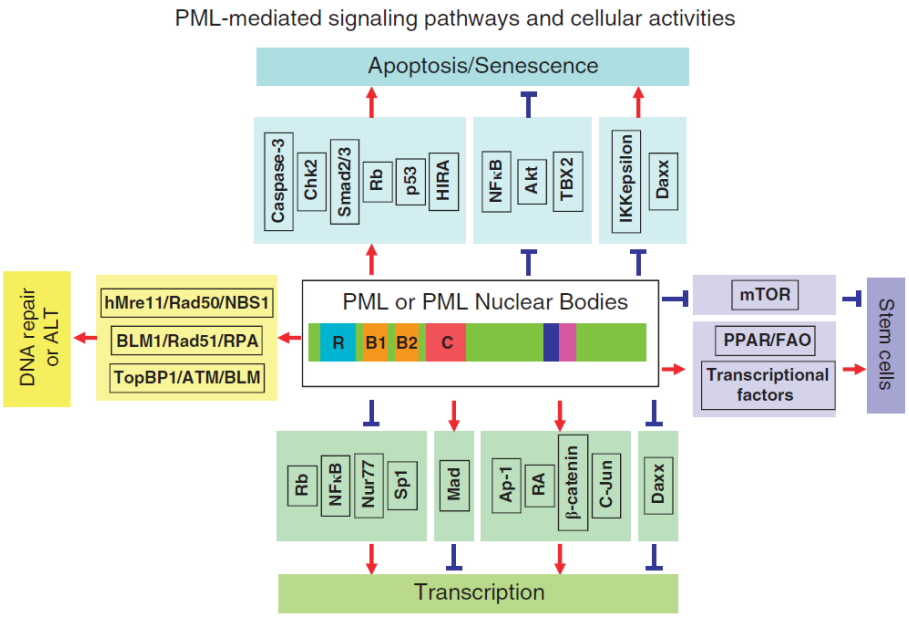


Figure 3.3: PML NB-mediated signaling pathways. PML NBs regulate many diverse cellular function and signaling pathways (Zhou and Bao 2014).

PML can regulate both negatively and positively the transcription process according to its interacting partners (Zhou and Bao 2014). PML-NBs might control transcription either directly by modulating the availability or activity of transcription factors, or indirectly by participating in chromatin-remodeling processes and establishing chromatin architecture that facilitates transcription. For instance, PML-NBs regulate the Major Histocompatibility Complex (MHC) locus transcription, by organizing its genes into distinct, high-order chromatin-loop structure that favors more or less transcriptional activation (Kumar, Bischof et al. 2007). Previous work of our lab showed that IFN gamma (IFNγ)-mediated *MHCII* gene induction generates a spatial epigenetic memory that involves the locus relocalization in proximity to PML-NBs. This leads to accelerated induction upon restimulation that is maintained through several cell generations (Gialitakis, Arampatzi et al. 2010).

PML-NBs were proposed to regulate DNA-damage responses since they were colocalized with DNA repair or single-stranded DNA sites. Numerous DNA repair and checkpoint proteins (e.g. bloom syndrome protein (BLM) and cds1 homolog kinase2 (CHK2)) are dynamically localized in PML-NBs. In general, they are involved in DNA repair processes either by acting as storage that release repair and checkpoints proteins after DNA damage or by actively participating in later phases of DNA repair or in specific repair pathways (Salsman, Jagannathan et al. 2012).

PML apoptotic function is well established by the observation that *Pml*-knockout mice present severe defects in executing apoptosis after different stimuli. A series of interactions of PML with different signaling pathways and other proteins have been shown to promote apoptosis (Salomoni, Dvorkina et al. 2012). For instance, PML controls the expression and activity of tumour suppressor p53. Indeed, p53 is expressed at significantly lower levels in $Pml^{-/-}$ cells compared to WT cells. PML interacts and activates p53 through different mechanisms, including induction of its post-transcriptional modifications, inhibition of its negative regulator (MDM2) (Fogal, Gostissa et al. 2000) (Louria-Hayon, Grossman et al. 2003) and induction of its positive regulator (Casein kinase 1) (Alsheich-Bartok, Haupt et al.

2008). PML is also involved in another cell death mechanism, cell senescence. It is believed that PML-IV induces senescence probably by interacting with the p53/p21 and p16/Rb pathways (Ivanschitz, De The et al. 2013).

A number of human tumors and immortalized cell lines maintain their telomeres in a telomerase-independent way termed Alternative Lengthening of Telomeres (ATL). ALT cells contain a novel kind of PML NBs named ALT-associated PML body (APB). Besides PML, the protein cargo of APBs consists of telomeric DNA, the telomeric repeat binding factor 1/2 (TRF1/2) and proteins involved in DNA synthesis and recombination. A possible scenario for PML-NBs function in ALT mechanism is the promotion of homologous recombination, either by facilitating the association or the stability of recombination complexes, or by creating an appropriate chromatin domain for recombination (Chung, Osterwald et al. 2012).

3.1.5 PML and stem cell regulation

PML is commonly characterized as a bona fide tumour suppressor, because of its involvement in cell apoptosis and senescence. Although, the fact that PML-RARa fusion protein blocks differentiation and favors the survival of early myeloid progenitors and that LICs are eliminated after PML-RARa degradation indicate a positive correlation between PML-RARa and maintenance of cancer stem cells (CSCs) in leukemia (Grignani, Ferrucci et al. 1993). The study of chronic myeloid leukemia (CML) provided insight into the role of PML and CSCs. CML patients have elevated PML levels that are correlated with poor clinical outcome (Ito, Bernardi et al. 2008). PML levels are also increased in HSCs compared to committed cells while *Pml* deletion results in loss of quiescence in HSCs, which leads to their transient amplification and subsequent depletion. Similarly, *Pml*-deficient leukemic initiating cells (LICs) showed reduced survival and were unable to generate CML in transplantation assays, indicating the positive role of PML in LIC maintenance. It is possible that PML exerts its function in LICs by repressing mTOR activity, since $Pml^{-/-}$ HSCs show increased mTOR (mammalian target of rapamycin) activity (Ito, Bernardi et al. 2008). It is known that mTOR positively regulates self-renewal in various systems (Matsubara, Ding et al. 2013). Following the observations that PML have a pro-survival role in CML, Ito and collegueas, revealed that PML regulates the

activation of fatty acid oxidation (FAO) and this metabolic reprogramming is essential for HSC maintenance (Ito, Carracedo et al. 2012).

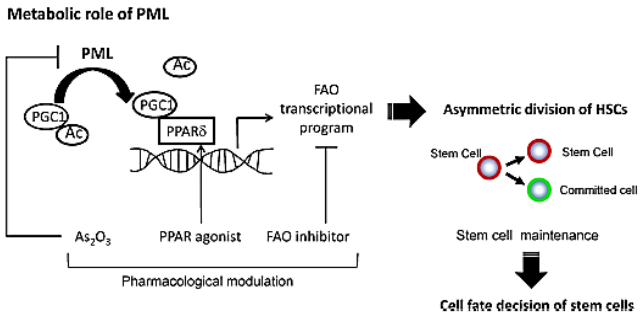


Figure 3.4: A model for regulation of asymmetric division by PML-PPARδ-fatty acid oxidation in HSCs (Ito, Carracedo et al. 2012)

Under normal conditions, peroxisome-proliferator activated receptor delta (PPARδ), a nuclear receptor with a key role in stem cell maintenance, is activated by PML and is reduced during the differentiation. PPARδ is involved in sensing fatty acids and in the activation of the FAO transcriptional program. In vivo and in vitro experiments revealed that conditional loss of *Pparδ* severely affects quiescent, maintenance and HSCs function, whereas treatment with PPAR-δ agonists rescues at some level these phenotypes. Moreover, *Pml* $^{-/-}$ HSCs defects are partially rescued by PPARδ agonists. Taken together, the PML-PPARδ-FAO pathway is crucial for the self-renewal of HSCs. *Pparδ* or *Pml* deletion and FAO inhibition leads to symmetric division of HSCs. Therefore, the PML-PPARδ-FAO is involved in the regulation of HSC asymmetric division (Nakahara, Weiss et al. 2014). Overall, the PML-PPARδ-FAO pathway controls the cell fate decision between self-renewal and HSCs differentiation (**Figure 3.4**). Furthermore, PML was shown to enhance FAO through PPAR signaling by inducing the deacetylation of PPAR-γ-coactivator 1a (PGC1a) by SIRT1 in breast cancer (Carracedo, Weiss et al. 2012).

3.2 Study Objectives

PML is a critical player for multiple cellular processes comprising DNA repair, apoptosis, senescence, oncogenesis and cancer progression. Currently, researchers unveiled intriguing findings regarding PML involvement in the maintenance of CSCs, proposing an essential role for PML in CSC regulation of solid tumors and hematopoietic cancers (Zhou and Bao 2014). Taken that CSCs have common characteristics with stem cells and that PML regulates *Oct4* transcriptional activity in ECCs and mESCs (Chuang, Huang et al. 2011), the aim of this project is to investigate the requirement of PML in ESC properties. The detailed knowledge gained for the mechanism by which PML functions (PML's molecular mechanism of action) in ESCs, may be also applied in CSCs and assist for developing novel cancer therapies. The exact goals of the following part of this thesis are:

- Validating PML expression levels upon ESC differentiation (Previous study in the lab identified that PML is down-regulated upon EBs differentiation by performing cDNA microarrays analysis)
- Generating stable PML KD ESC cell line and examining the effect of its depletion in ESCs prior and upon differentiation
- Performing mRNA expression profiling of ESCs WT and ESCs PML KD in the undifferentiated and differentiated EBs D4
- Generating stable PML OE ESC cell line and analyzing the effect of its induction in ESCs identity
- Examining the function of PML in the generation of iPSCs

3.3 Materials and Methods

3.3.1 Generation of PML KD or OE ESC stable cell lines

The generation of stably expressing PML-shRNA ESC X cell lines was achieved using PML shRNA-pLKO.1 provided by a collaborator of the University of Israel. Briefly, CGR8 cells were grown to 80% confluence and transfected with either empty shRNA or PML shRNA vector. Selection of shRNA transfected cells was achieved with puromycin (2μg/ml). A total of 60 isolated resistant colonies evident after 2 to 4 weeks, were picked and further grown for screening. Puromycin resistant clones were examined using western blot and qPCR. Respectively, PML OE ESC cell lines were generated using PML-IRES-GFP construct or IRES-GFP as a negative control. The isolation of the clones was performed using G418 (200μg/ml).

3.3.2 Isolation of PML KO ESCs

PML KO ESCs were isolated from B57BL/6 *Pml* $^{-/-}$ mice. Females mice were killed at E3.5 and their uteri were immediately separated into 10 ml of DMEM in 100-mm plate. Then, uteri were transferred into 2ml of DMEM in 35mm dish and the blastocysts were flushed out using a 1-ml syringe. Blastocysts were collected under an inverted microscope using a 20-ml pipette and transferred to a 48-well plate containing a feeder layer of MEFs mitotically inactivated 1 day earlier. After 2-3 days, blastocysts attached to the MEF feeder layer and hatched. ES medium was changed every second day while the expanded blastocysts adopted a morphology comparable to ESCs (usually on day 5 or 6).

3.3.3 cDNA microarray analysis

Total RNA was extracted from PML KD and WT ESC cell lines at D0 and D4 of EBs differentiation using RNeasy Microarray Tissue Mini Kit (Qiagen). Then, the RNA was analyzed using Affymetrix Mouse Genome MG430 ST 1.0 array, according the manufacture's instruction. Microarray data was processed to extract the representative intensities from each probe set using Affymetrix Transcriptome Analysis Console (TAC) Software. Fold change >1.5 and $p<0.05$ were used to identify differential expression between the sample groups. Prior to hierarchical clustering, log2 transformation was performed. Functional analysis was performed using the Regulatory Network Enrichment Analysis (RNEA) (Chouvardas et al., 2016). The p

value<0.05 was employed for both significant enriched GO terms and KEGG pathways.

3.3.4 RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was subsequently reverse-transcribed from mRNA by M-MLV Reverse Transcriptase (Takara). Target genes expression was normalized against Actin. The following primers were used in this project:

Actin FOR: 5' GTGTGACGTTGACATCCGTA 3', REV: 5' GTAACAGTCCGCCTAGAAGC 3',
Afp FOR: 5' AAGCTGCGCTCTCTACCACCAGA 3', REV: 5' ACCACAGCCGGACCATT 3',
Bmp4 FOR: 5' TTCCTGGTAACCGAATGCT 3', REV: 5' AAGTGTCGCCTCGAAGTC 3',
Eomes FOR: 5' GCTTCCGGGACAACTACGA 3', REV: 5' GAGAGGAGGCCGTTGGTCT 3',
Fgf5 FOR: 5' GCAGAAGTAGCGCGACGTTT 3', REV: 5' TTGACTTTGCCATCCGGGTAG 3' ,
Fos FOR: 5' GGTTTCAACGCCGACTACGAG 3', REV: 5' AGGTCTGGGCTGGTGGAGAT 3'
Fzd2 FOR: 5'AGTTCCACGGGGAGAAGGG 3', REV: 5' AGCGGGTAGAACTGATGCAC 3'
Gata4 FOR: 5' GCCAACTGCCAACTACCAC 3', REV: 5' GACCTGCTGGCGTCTTAGA 3',
Gata6 FOR: 5' GCCACTGTGGAGACGAGA 3', REV: 5' CATATAGAGCCCCGAAGCA 3',
Hand1 FOR: 5' ACCAGTTACATCGCCTACTTGA 3', REV: 5'CGCGACCACCATCCGTCTT 3'
Id1 FOR: 5' GACTACATCAGGGACCTGCAGC 3', REV: 5' GGCCGCCAAGGCACTGATCTCG 3', Id2 FOR: 5' ATCCCCCAGAACAACAAGGT 3', REV: 5' ACCTTCTTGTTCTGGGGGAT 3',
Id3 FOR: 5' CCAGGTGGAAATCCTGCACC 3', REV: 5' CTCTTGTCCTTGGAGATCACAA 3',
Jmjd3 FOR: 5' GTACAGACCCCCGGAACC 3', REV: 5' TGGTGGAGAAAAGGCCTAAG 3'
Junb FOR: 5' ACCCCTACCGGAGTCTCAA 3', REV: 5' GGAGTAGCTGCTGCTGAGGTTG 3'
Lefty2 FOR: 5' AGCACGCGACCGCTCCC 3', REV:5' CGATGCTCCATTCCGAACAC 3' ,
Nanog FOR: 5' CGCTGCTCCGCTCCATAACT 3', REV: 5' GCGCATGGCTTTCCCTAGTG 3',
Nr0b1 FOR: 5' CTGGTGTGCAGCGTCTGA 3', REV: 5' GTGTTGGTCTCCGGATCTC 3',
Pax6 FOR: 5' GGTGCTGGACAATGAAAACA 3', REV: 5' GGTACAGACCCCCTCGGATAA 3',
Pim-1 FOR: 5' ATGCTCTTGTCCAAAATCAACTCGCTTGCC-3', REV: 5' TGATGAAGTCGAAGAGATCTTGCACCGGCT-3'
Pim-3 FOR: 5' GAGAGGGTCTCCCCAGAGT 3', REV: 5' TGGTGGCACGCTTAGGTTG 3'
Rb1 FOR: 5'-GTGTAAATTCTGCTGCAAAT-3', REV: 5'-GGTCCAAATGTCGGTCTCTC-3'

Oct4 FOR: 5’ CCCTGGGCGTTCTCTTTGGA 3’, REV: 5’ ACCAGGGTCTCCGATTTGCAT 3’, Otx2 FOR: 5’ CCGGAAACAGCGAAGGGA 3’, REV: 5’ GCTGTTGGCGGCACTTAG 3’, Sox17 FOR: 5’ CTCTGCCCTGCCGGGATGG 3’, REV: 5’ AATGTCGGGGTAGTTGCAATA 3’, Stra8 FOR: 5’- GAG GCC CAG CAT ATG TCT AAC-3’, REV: 5’-GCT CTG GTT CCT GGT TTA ATG-3’ T FOR: 5’ GTTCCCGGTGCTGAAGGTAAAT 3’, REV: 5’ GCGAGTCTGGGTGGATGTAGA 3’
Wnt8a FOR: 5’ GGGAACGGTGGAATTGTCCTG 3’ REV: 5’GCAGAGCGGATGGCATGAA 3’

3.3.5 Antibodies

In this study proteins were detected by primary antibodies against Oct3/4 (sc-5279, Santa Cruz), Afp (2137S, Cell Signaling), Nanog (8600S, Cell Signaling), NrOb1 (sc-13034X, Santa Cruz), Actin (sc-47778, Santa Cruz), PML (sc-18423, Santa Cruz), T (sc-17745, Santa Cruz), Snai1 (3879, Cell Signaling), c-myc (Sigma), Cdh1 (3195, Cell signaling), Sox2 (14962, Cell Signalling) and Vim (5741, Cell Signaling).

3.3.6 Co-Immunoprecipitation

IP of in vivo interacting protein complexes was performed using PML OE and PML KO stable ESC cell lines. Cells were lysed in EBC buffer (50mM Tris PH 8, 170mM NaCl, 0.5% NP40, 50mM NaF) containing 1 mM PMSF and protease inhibitors (Roche Applied Science). 200µg whole cell extra were incubated with primary antibody overnight. The following day, 20µl of protein G sepharose beads were added to each sample and incubated at 4°C for 3hr. The beads were centrifuged and washed three times in 1 ml of NENT buffer (10Mm Tris PH 8, 250nM NaCl, 5mM EDTA, 0.5% NP40). SDS sample buffer was added and immunoprecipitated proteins were analyzed by SDS-PAGE, followed by Western blotting as described previously (Niture and Jaiswal, 2009).

3.3.7 iPSC formation

Primary MEFs were isolated from C57BL/6 mice at E13.5. For iPSCs generation, MEFs WT or PML KO (passage 1 or 2) were plated at 0.08–0.1 x 10^6 cells/well in a 6-well plate and incubated overnight. The following day, cells were infected with the lenti-viruses produced by TetO-FUW-OSKM (Addgene # 20342) and FUW-M2rtTA vectors

(Addgene # 20342), for 48h. After four days, cells were reseeded on feeders and treated with 2µg/ml Doxocyclin (Dox) in mESC medium (DMEM, 15% FBS, 200µM non-essential amino acids, 200µM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U/mL LIF (Millipore)), for induction of Yamanaka's factors. The medium was changed every other day. iPSC colonies appeared 9-11 days post-infection. Alkaline phosphatase (AP) staining was performed using Alkaline Phosphatase Detection Kit (Millipore) according to manufacturer's instructions.

3.3.8 Luciferase Assay

PML KD, PML OE and CGR8 ESC cell lines were transfected with APRE-Luc, CAGA-Luc or BRE-Luc reporter plasmids using Lipofectamine 2000 following the manufacture's protocol. Cells were stimulated with LIF (100ng/ml), Activin A (25ng/ml) and BMP4 (80ng/ml) respectively, 16h before harvesting for measurement of luciferase activity using dual-luciferase reporter assay system (Promega).

3.3.9 Teratoma Formation

PML KO or WT iPSCs were harvested using 1X Trypsin and centrifuged at 1200 for 5 min. Cells were then re-suspended in 1X PBS to a final concentration $2x10^6$ cells/100µl and injected intramuscular to 6 to 8-week-old male NOD-SCID mice (100µl per mouse). The mice were monitored every day. Teratomas were arisen between 2 and 4 weeks after engraftment.

3.3.10 Statistical analyses

Student's t-test was used for all statistical analyses. Statistical significance was defined as follows: * means $p < 0.05$; ** means $p < 0.01$. Values were presented as the mean ± SD.

3.4 Results and Discussion

3.4.1 Pml is down-regulated during differentiation and interacts with central pluripotency factors

Previous work from our group displayed that *Pml* expression levels deduced by cDNA microarray analysis of differentiated ESCs, were greatly reduced. To evaluate a possible role of PML in ESC properties, we validated its expression levels in mESCs before and after differentiation. ESCs were differentiated into EBs and the loss of their pluripotency was confirmed by the reduction and subsequent induction of pluripotency (*Nanog, Oct4*) and developmental (*Pax6, T*) genes, respectively (Figure 3.5A, B). Concurrently, PML mRNA and protein levels were markedly decreased upon EBs differentiation (**Figure 3.5A, 3.5B**). Furthermore, under diverse differentiation conditions (LIF withdrawal, retinoic acid (RA) or TSA addition) *Pml* and pluripotency genes expression levels showed a similar trend (**Figure 3.5C, 3.5D**).

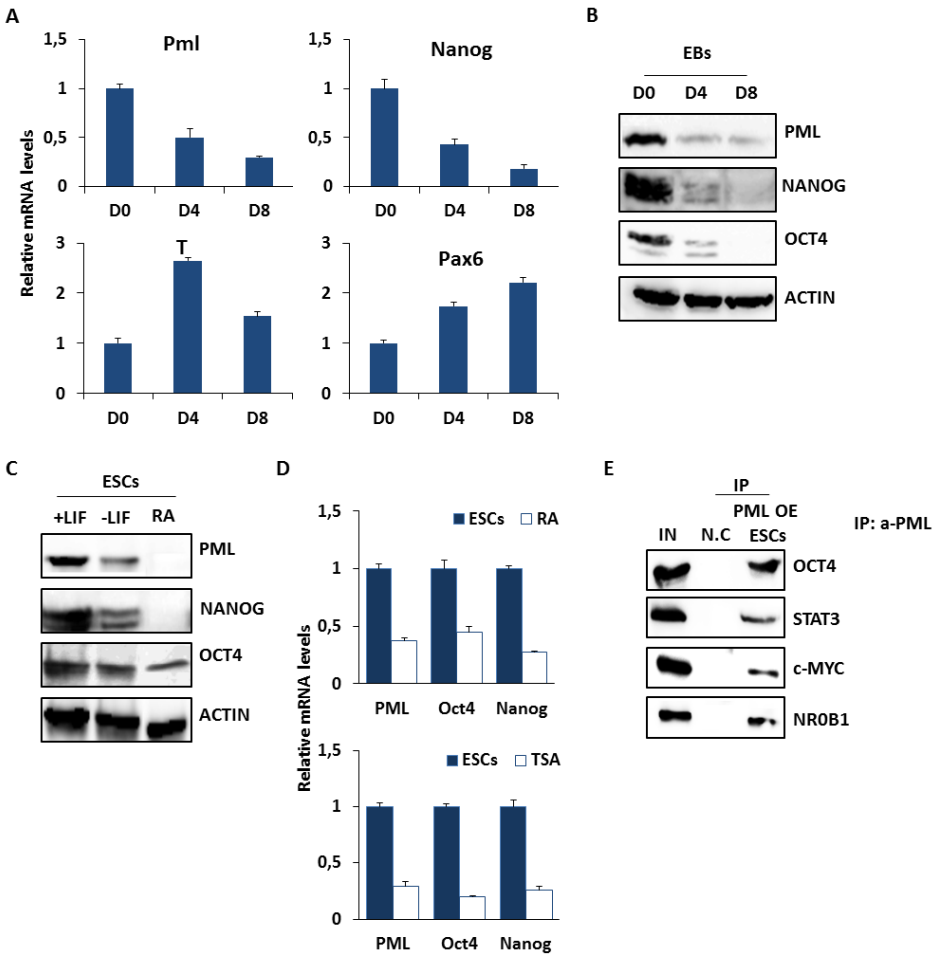


Figure 3.5: PML is reduced upon ESC differentiation and associates with core pluripotency factors. (A, B) *Pml* mRNA (**A**) and protein (**B**) levels were reduced upon EBs diiferentiation. Characteristic pluripotency and differentiation genes were also examined under differentiation conditions. **C.** Differentiation by RA addition or LIF withdrawal decreased PML protein levels. **D.** TSA or RA addition declined *Pml* and pluripotency genes mRNA levels. Error bars indicate +SD of three independent experiments (n=3). **E.** Co-IP of endogenous pluripotency factors (OCT4, c-MYC, STAT3, NR0B1) with endogenous PML from PML OE ESCs compared with PML KD ESCs.

Previous studies demonstrated that PML physically associates with NANOG and OCT4 (Liang, Wan et al. 2008; Chuang, Huang et al. 2011). Our co-immunoprecipitation experiments using PML-overexpressing (OE) ESC cell line, verified OCT4 interaction and further showed that PML associates with STAT3, c-MYC and NR0B1, three essential regulators of mESC identity (**Figure 3.5E**). The decreased expression of PML in the differentiated state and its association with several core pluripotency factors, proposed a potential involvement of PML in ESC pluripotency maintenance.

3.4.2 PML depletion impairs self-renewal and pluripotency of ESCs

To define the functional role of PML in the maintenance of mESC pluripotency, we performed PML depletion via short hairpin RNA (shRNA). The efficiency of PML knockdown (KD) was examined and a remarkable reduction was observed in a stable PML KD ESC line compared to the empty vector control (**Figure 3.6A**). PML KD had no effect on cell morphology (data not shown), but it significantly reduced the expression levels of several pluripotency markers (**Figure 3.6B**). These findings were also observed in PML-knockout (KO) ESCs, that we derived from *Pml*$^{-/-}$ mice (**Figure 3.6B**). Subsequently, we analyzed the cell cycle phase distribution to detect any possible influence by PML KD on it. Surprisingly, the PML KD ESCs showed an obvious loss of S phase, which was correlated with an increase of G1 cells but no significant alteration in G2/M cells (**Figure 3.6C**). It has been previously reported that Stat3, a pivotal mediator of ESC self-renewal, might be responsible for G1-S transition, through activating cell-cycle regulators (e.g. *Junb, c-myc, Pim-1*)(P. Savatier 2004) . At the same time, LIF/Stat3 signaling pathway might enhance self-renewal by elevating the expression of G1-S transition regulators (Burdon, Smith et al. 2002). Hence, to

examine if this assumption is proved in our model, the mRNA levels of *Junb*, *c-myc* and *Pim-1* were measured in PML-KD ESCs and were found to be lower compared to control ESCs (**Figure 3.6B, 3.6D**). Moreover, PML KD interference with LIF/Stat3 signaling pathway was observed using the STAT-dependent reporter (APRE-Luc) in the absence and presence of LIF, where its activation was inhibited (**Figure 3.6E**).

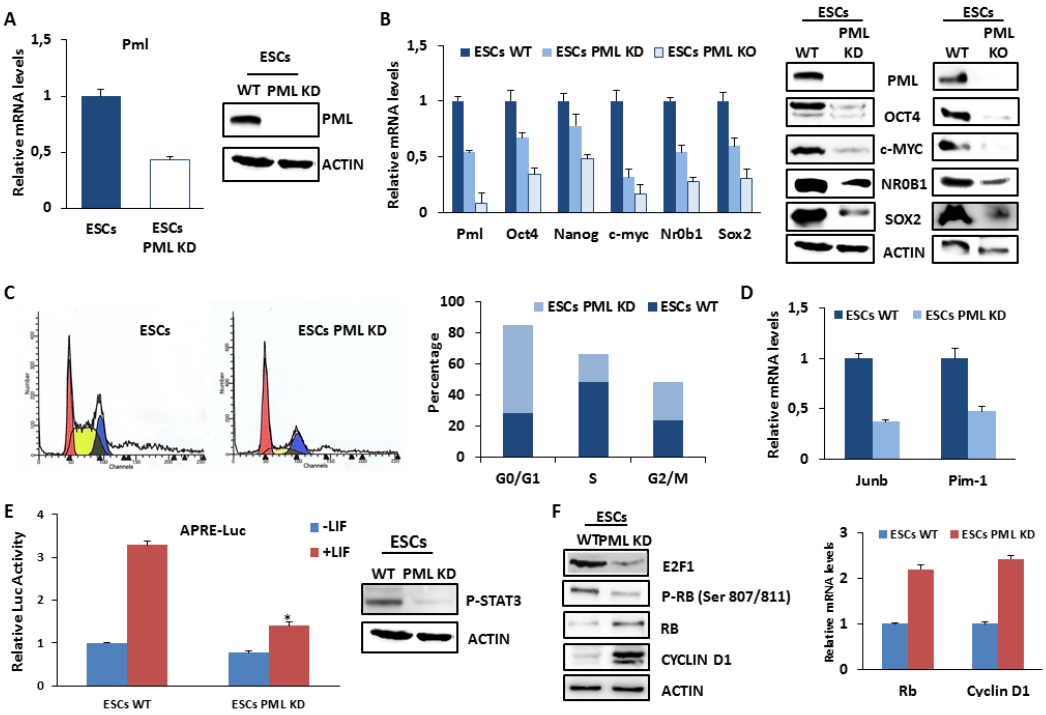


Figure 3.6: PML depletion impairs self-renewal and affects cell-cycle A. *Pml* mRNA and protein levels in PML KD ESC cell line. **B.** Pluripotency genes were significantly decreased in PML KD and PML KO ESCs (left panel). Depletion or deletion of PML led to declined pluripotency factors protein (right panel) **C.** Cell cycle analysis of WT and *PML KD* ESCs stained with PI and analyzed using flow cytometry. **D.** Relative mRNA levels of cell-cycle regulators *Junb* and *Pim-1.* **E.** PML depletion inhibits the activity of APRE-Luc and p-STAT3 proteins levels were also reduced compared to ESCs WT. **F** Protein expression levels of cell cycle regulators (E2F1, phospho-pRB, pRB, CCND1) were detected (left panel), while *Rb* and *Ccnd1* mRNA levels were also measured (right panel). Data are shown as mean + SD of four independent experiments (n=4), *p<0.05

To further confirm this influence, the p-STAT3 protein levels were investigated, exhibiting decreased values in PML KD ESCs (**Figure 3.6E**). Sage et al., proposed a model for the role of retinoblastoma tumor suppressor (pRb) in ESC cell cycle (Sage 2012). In mESCs, pRb is inactivated by hyperphosphorylation owing to high Cyclin/Cdk and E2F activity and permits ESC self-renewal. Upon differentiation, Cdks

and E2Fs are decreased resulting in the inhibition of pRB phosphorylation and induction of its activity (Sage 2012). PML KD ESCs were characterized by reduced protein levels of p-pRB and E2F1 as well as increased levels of total pRB. In accordance with these findings, Ccnd1 expression was also elevated in PML KD ESCs (**Figure 3.6F**). It is known that Ccnd1 is not expressed in the undifferentiated state, whereas is raised upon differentiation (P. Savatier 2004). PML was previously characterized as growth suppressor while its ectopic expression repressed cell growth in different somatic cell lines (HeLa, 3T3 cells), leading to G1 elongation (Mu, Le et al. 1997). Additionally, it was reported that *Pml* deficient MEFs proliferate greatly faster than WT MEFs (Tang, Liang et al. 2013). These findings are contradicted to our results since we identified that upon PML reduction, G1 phase was lengthened. This opposition may be ascribed to the peculiar ESC cell cycle. To summarize, our results revealed that PML depletion resulted in ESC self-renewal and pluripotency impairment and an elongation of G1 phase by controlling the expression of cell-cycle regulators.

3.4.3 PML reduction promotes primed pluripotent state

To further understand how PML KD affects ESC identity, we performed a genome-wide analysis from PML KD and WT ESCs. As shown in Figure 3.7, 3088 genes were deregulated - 1009 were up-regulated (F.C>1.5, $p<0.05$) and 2079 were down-regulated (F.C<-1.5, $p<0.05$) - upon PML depletion. Importantly, the functional analysis using the RNEA software (Chouvardas et al., 2016) revealed that several signaling pathways implicated in ESC pluripotency were affected. For instance, BMP4 and Jak/Stat3 signaling pathways were significantly enriched in WT ESCs, while Activin and FGF signaling pathways were enriched in PML KD ESCs (**Figure 3.7**). Moreover, many genes related to cell proliferation or chromatin organization were also affected by PML depletion, reinforcing our hypothesis that PML may contribute to maintenance of mESC properties. On top of that, several naïve characteristic genes (*Nr0b1, Tbx3, Klf4, Klf5*) were down-regulated in PML KD ESCs, whereas representative primed markers were elevated (*Lefty1, Lefty2, Xist*) (**Figure 3.8A**). These data were confirmed using RT-PCR in both PML KD and KO ESCs (**Figure 3.8B**).

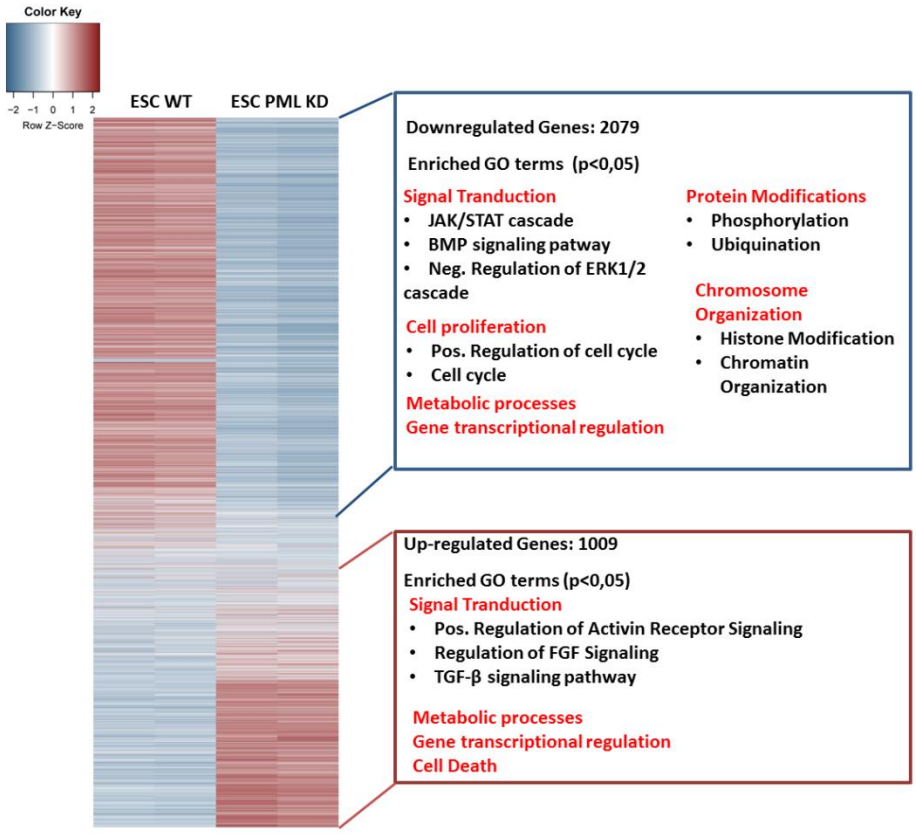


Figure 3.7: Heat map representing differential expression of genes in PML KD ESC compared to ESC WT. GO analysis was performed using RNEA software. Representative enriched terms were also shown.

Additionally, the protein levels of OTX2 and CDH1 were measured in PML KD and WT ESCs (**Figure 3.8C**). Recently, it was shown that Otx2 – an important brain development transcription factor- is critical for ESC conversion into EpiSCs and stabilizes the primed state by inhibiting the mesendoderm to neural shift (Acampora, Di Giovannantonio et al. 2013). On the contrary, Murayama et al., reported that enforced expression of Cdh1, can drive the transition from primed to naïve PCs in the presence of the LIF, elucidating its importance in mESC pluripotency maintenance (Murayama, Masaki et al. 2015). Furthermore, we verified that PML KD negatively affects BMP4 signaling by reducing the activity of BRE-Luc reporter and the expression levels of its transcriptional targets Ids 1-3 (**Figure 3.8D, 3.8E**). As mentioned above, BMP4 along with LIF/Stat3 signaling is required for the maintenance of naïve pluripotent state, while Activin/Nodal and Fgf/Erk signaling are connected with the primed state. Given PML KD effect on BMP4 and LIF/Stat3

signaling pathways, we explored the involvement of PML in Activin/Nodal signaling. As illustrated in **Figures 3.8B** and **3.8F**, PML KD increased the mRNA levels of the Nodal/Activin specific genes (e.g. *Acvr2a*) and enhanced the activity of Smad3-responsive element CAGA-Luc reporter upon stimulation with 25ng/µl Activin A. These findings pointed out that PML may be essential for the maintenance of ground pluripotent state.

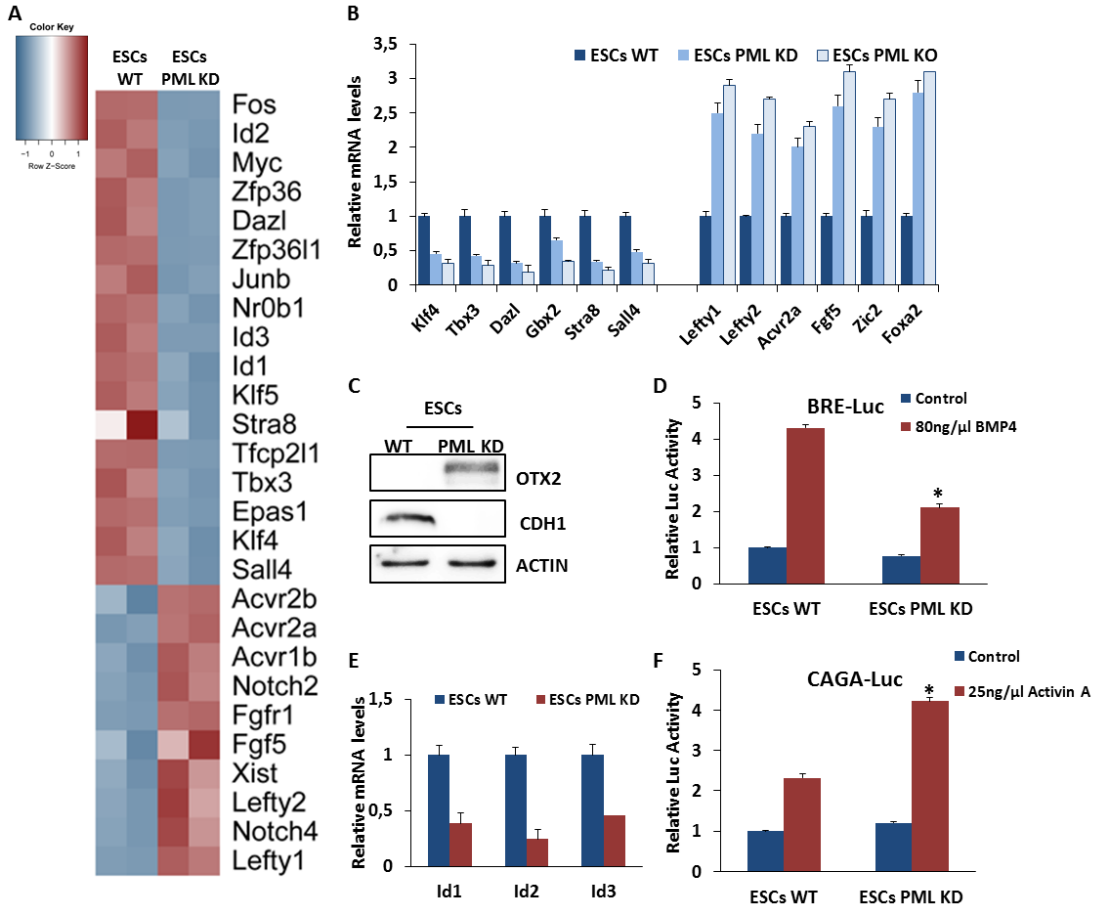


Figure 3.8: PML reduction promotes primed pluripotent state. A. Heat map with the top representative naïve and primed genes. **B.** mRNA levels of characteristic naïve and primed genes in PML KD, PML KO and control ESCs. **C.** OTX2 and CDH1 protein levels prior and after PML inhibition in ESCs. **D.** Luciferase activity of BRE-Luc reporter was suppressed upon PML depletion. **E.** mRNA levels of BMP signaling targets genes (*Id1, Id2, Id3*) in PML KD ESCs and WT ESCs. **F.** PML reduction resulted in the inhibition of CAGA-Luc reporter activity. Error bars indicate +SD of three independent experiments (n=4), *p<0.05.

3.4.4 PML reduction favors mesodermal and represses endodermal lineage specification

To identify the role of PML during mESC differentiation, we induced PML KD and WT ESCs to form EBs and then analyzed the gene expression profile on EBs D4, using cDNA microarray analysis. Upon PML KD ESC differentiation, 836 genes were up-regulated (F.C>1.5, p<0.05) and 400 genes were down-regulated (F.C<-1.5, p<0.05) (**Figure 3.9A**). Importantly, according to Gene Ontology (GO), PML KD preferentially induces mesoderm and represses endoderm formation (**Figure 3.9A**). The representative differentiation markers levels were also corroborated through RT-PCR and Western Blot (**Figure 3.9B, 3.9C**). Concerning the ectoderm differentiation, Nestin (*Nes*) was significantly up-regulated, while the other ectodermal genes remained constant (**Figure 3.9D**). At the same time, we found that the reduction of *Nr0b1* and *Nanog* during differentiation was greatly decreased in PML KD ESCs (**Figure 3.9E**). Our results are in line with previous studies, proposing that Nr0B1 and Nanog inhibit endoderm differentiation (Zhang, Liu et al. 2014). Hence, it seems that PML is essential for ESC proper differentiation towards the three germ layers.

A potential molecular mechanism might be based on Tbx3 regulation by PML. Recently, it was stated that Tbx3 is essential for repressing mesoderm differentiation through targeting T and Wnt pathway genes (*Wnt8a, Fzd2, Wnt3a*) (Waghray, Saiz et al. 2015). Wnt signaling is important for ESC pluripotency maintenance as well as for the generation of primitive streak (mesendoderm) during gastrulation (Tortelote, Hernandez-Hernandez et al. 2013). It is believed that this conflicting role of Wnt pathway in pluripotency against differentiation is partially controlled by the expression of Tbx3. Consistent with the above works, we found that in the absence of PML expression, *Tbx3* was down-regulated (**Figure 3.9F**), while T and Wnt markers (*Wnt3a, Fzd2, Wnt8*) were overexpressed and induced mesoderm differentiation (**Figures 3.9B, 3.9C, 3.9F**). According to GO, Wnt signaling is enriched in PML KD ESCs upon EBs formation, reinforcing our notion that PML KD ESCs promotes mesoderm differentiation. On the other hand, another group unveiled that Tbx3 connects with Jmjd3 (an epigenetic modifier-histone demethylase), activating the enhancer of Eomes. Chromatin is then reorganized and Eomes is permitted to promote itself and endoderm regulators transcriptional activation mediated by Jmjd3 (Kartikasari, Zhou

et al. 2013). Furthermore, it was also reported that *Eomes* $^{-/-}$ ESCs fail to give rise to endoderm lineage (Costello, Pimeisl et al. 2011). In our model, we found that the mRNA of *Eomes* and *Jmjd3* were decreased, in agreement with *Tbx3* reduction and the aforementioned studies (**Figure 3.9F**). Previously, it was also stated that Tbx3 physically interacts with PML in proliferating cells (e.g MEFs), reinforcing our assumption that PML and Tbx3 are directly related (Martin, Benhamed et al. 2012). Taken together, the above results reinforce the assumption that PML controls cell fate decision through Tbx3 regulation.

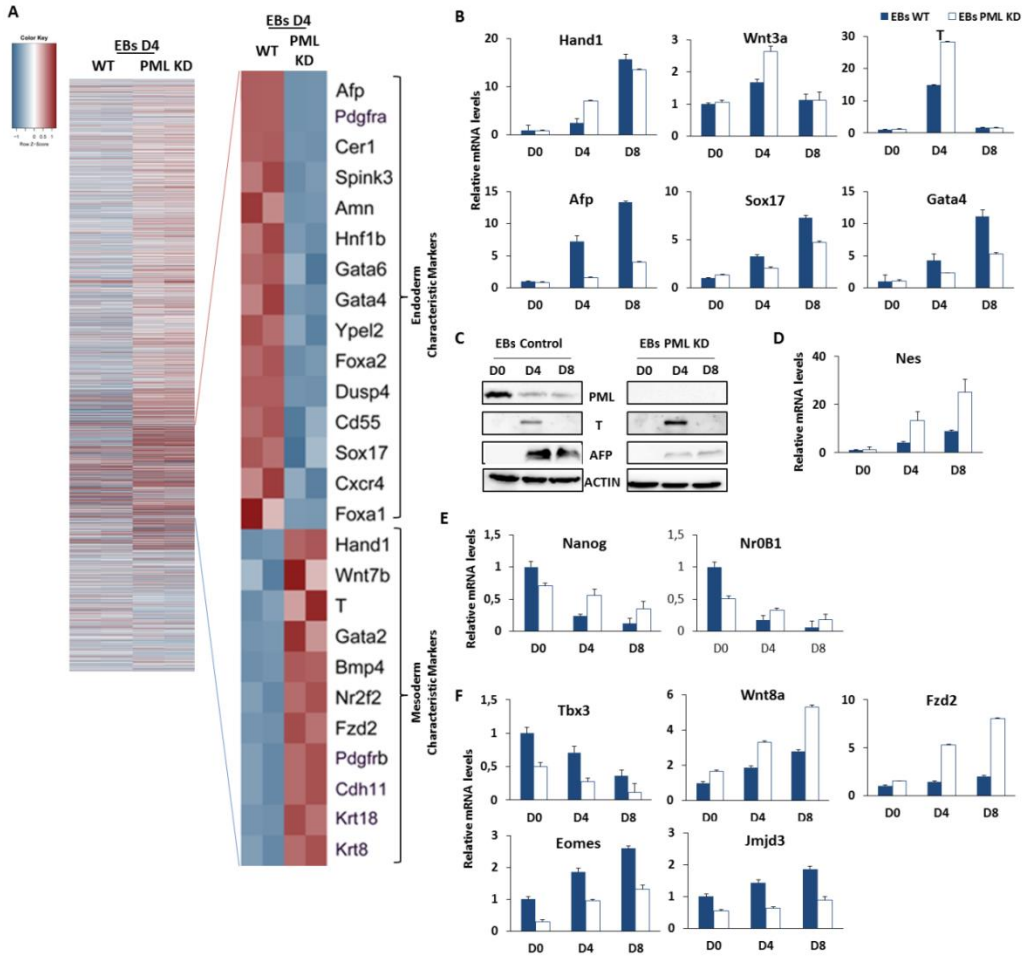


Figure 3.9: PML inhibition promotes mesoderm and represses endoderm differentiation. A. The heat map displaying the probe sets/genes differentially expressed in PML KD and WT EBs D4 (F.C>1.5, p<0.05) The top endodermal genes and mesodermal deregulated genes by PML decrease are listed on the right. **B.** Validation of microarray results using RT-PCR (N = 4) in PML KD and WT EBs. **C.** Western blot analysis of PML, T and AFP expression in PML KD EBs compared to WT EBs D. *Nes* mRNA levels upon differentiation of PML KD or WT ESCs. **E.** *Nanog* and *Nr0b1* mRNA levels during EBs differentiation in PML KD and WT ESCs. **F.** Tbx3, Wnt pathway genes, Eomes and Jmjd3 mRNA levels

upon differentiation of PML KD or WT ESCs. Data are shown as mean + SD of four independent experiments (n=4).

3.4.5 PML OE maintains ground pluripotent state and promotes endoderm differentiation

To further delineate the functional role of PML, we generated stable PML OE ESC lines. Protein expression levels of PML and pluripotency markers were measured and showed elevated levels compared to control ESCs (**Figure 3.10A**). In addition, characteristic naïve markers were significantly increased, in contrast to primed state specific genes that were reduced (**Figure 3.10B**).

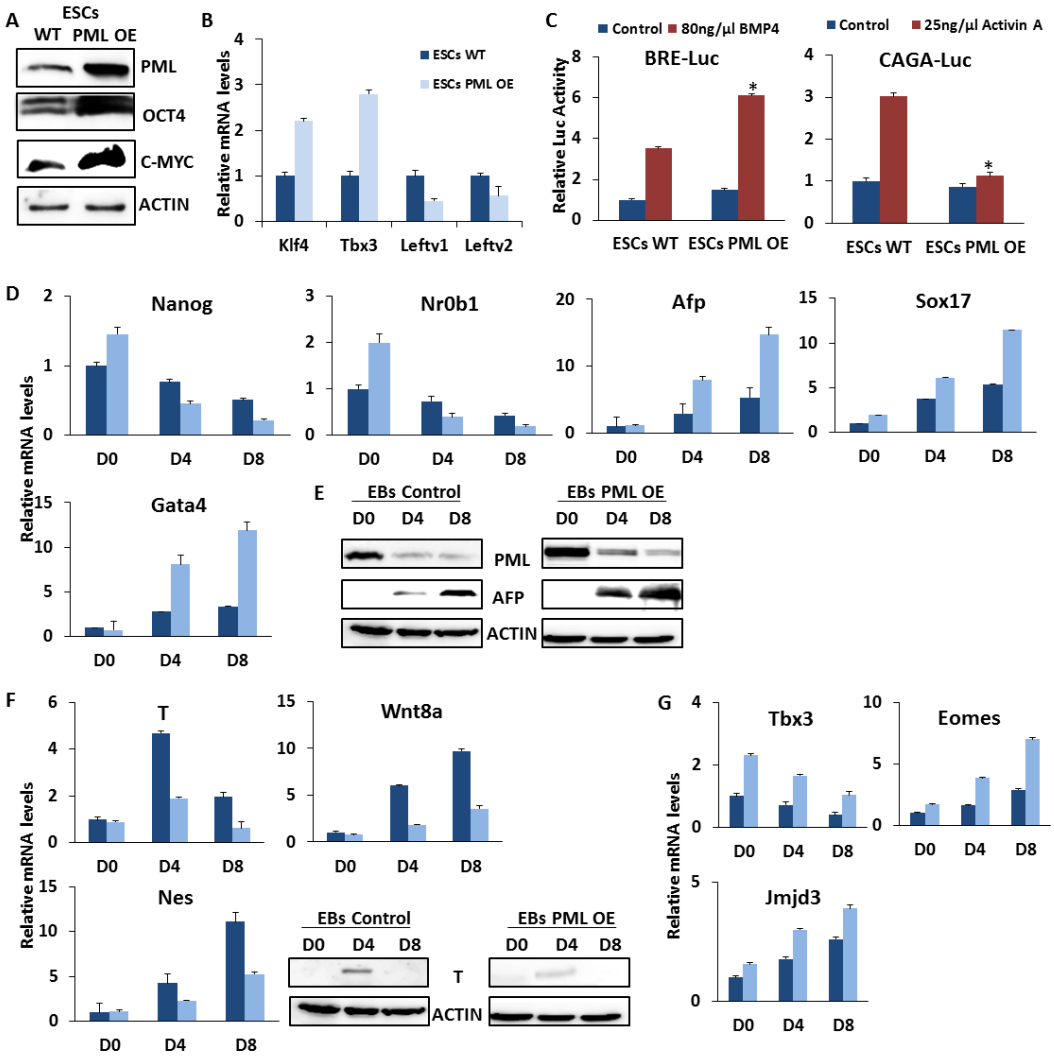


Figure 3.10: Enforced expression of PML maintains naïve pluripotent state and induces endoderm specification. A. PML and pluripotency factors protein levels in PML OE ESC cell line. **B.** mRNA levels

of representative naïve and primed genes in PML OE ESCs. **C.** PML enforced expression promotes BRE-Luc reporter activity, while CAGA-Luc activity is significantly inhibited. Data are shown as mean + SD of four independent experiments (n=4), p<0.05. **D.** *Nanog*, *Nr0b1* and characteristic endodermal genes (*Afp, Sox17, Gata4*) mRNA levels. **E.** Protein levels of PML and AFP upon differentiation of PML OE and WT ESCs. **F.** Mesoderm (*T, Wnt3a, Wnt8a*) and ectoderm (*Nes*) specific genes mRNA levels and T protein levels during EBs differentiation of PML OE and WT ESCs. **E.** mRNA levels of *Tbx3*, *Eomes* and *Jmjd3* in the course of EBs differentiation. Data are shown as mean + SD of three independent experiments (n=3).

Moreover, the impact of PML OE on reporter signaling pathways activity, verified the importance of PML in the maintenance of ground pluripotency (**Figure 3.10C**). Upon EBs differentiation, PML OE favored the suppression of *Nr0B1* and *Nanog* and at the same time enhanced the induction of endoderm (*Afp, Sox17, Gata4*) differentiation markers (**Figure 3.10D, 3.10E**). Concerning the induction of mesodermal, Wnt pathway (*T, Wnt8*) and ectodermal (*Nes*) markers, PML OE caused a significant increase (**Figure 3.10F**). Additionally, we found that the mRNA levels of *Tbx3, Eomes* and *Jmjd3* were elevated in PML OE ESCs (**Figure 3.10G**). These experimental results verified our hypothesis that PML is a central regulator of mESC identity.

3.4.6 PML is required for reprogramming efficiency of MEFs into iPSCs

Since PML is required for mESC self-renewal and pluripotency, we investigated its role in the efficiency of reprogramming. To assess PML functional importance, we isolated MEFs from PML deficient mice for iPSC generation. *PML*$^{-/-}$ MEFs and WT MEFs were reprogrammed with Yamanaka factors (OSKM) and formed iPSC colonies. OSKM transduced-MEFs WT formed colonies by days 9-10, while OSKM transduced-MEFs *PML*$^{-/-}$ formed by days 14-15. Based on alkaline phosphatase (AP)-positive colony numbers, PML deletion greatly decreased the efficiency of reprogramming (approximately 10 fold) compared to control (**Figure 3.11A**).

As stated in chapter 1, reprogramming entails several molecular steps including MET in the first stage (Samavarchi-Tehrani, Golipour et al. 2010). The fact that transduced-MEFs *PML*$^{-/-}$ colonies appeared later, may suggest an early defect of reprograming. It is known that Cdh1 and Occludin (Ocln) are essential for colonies formation and promote epithelial-mesenchymal transition (EMT), while Snai1, Zeb1/2 and Vimentin (Vim) maintain mesenchymal phenotype by suppressing

epithelial markers (Thiery, Acloque et al. 2009). Although *Pml*$^{-/-}$ MEFs express significantly lower levels of mesenchymal markers owing to the TGF-β inactivation (**Figure 3.11B**) (Lin, Bergmann et al. 2004), their epithelial morphological switch was not obvious at day 12 of reprogramming process (Figure 3.11C). On the contrary, MEFs WT showed a clear MET after OSKM transduction (**Figure 3.11C**). Then, we analyzed the expression levels of Oct4 and Nanog at day 12 and we found that OSKM transduced-MEFs WT showed higher expression levels of pluripotency markers, in contrast to *Pml*$^{-/-}$ MEFs (**Figure 3.11D**).

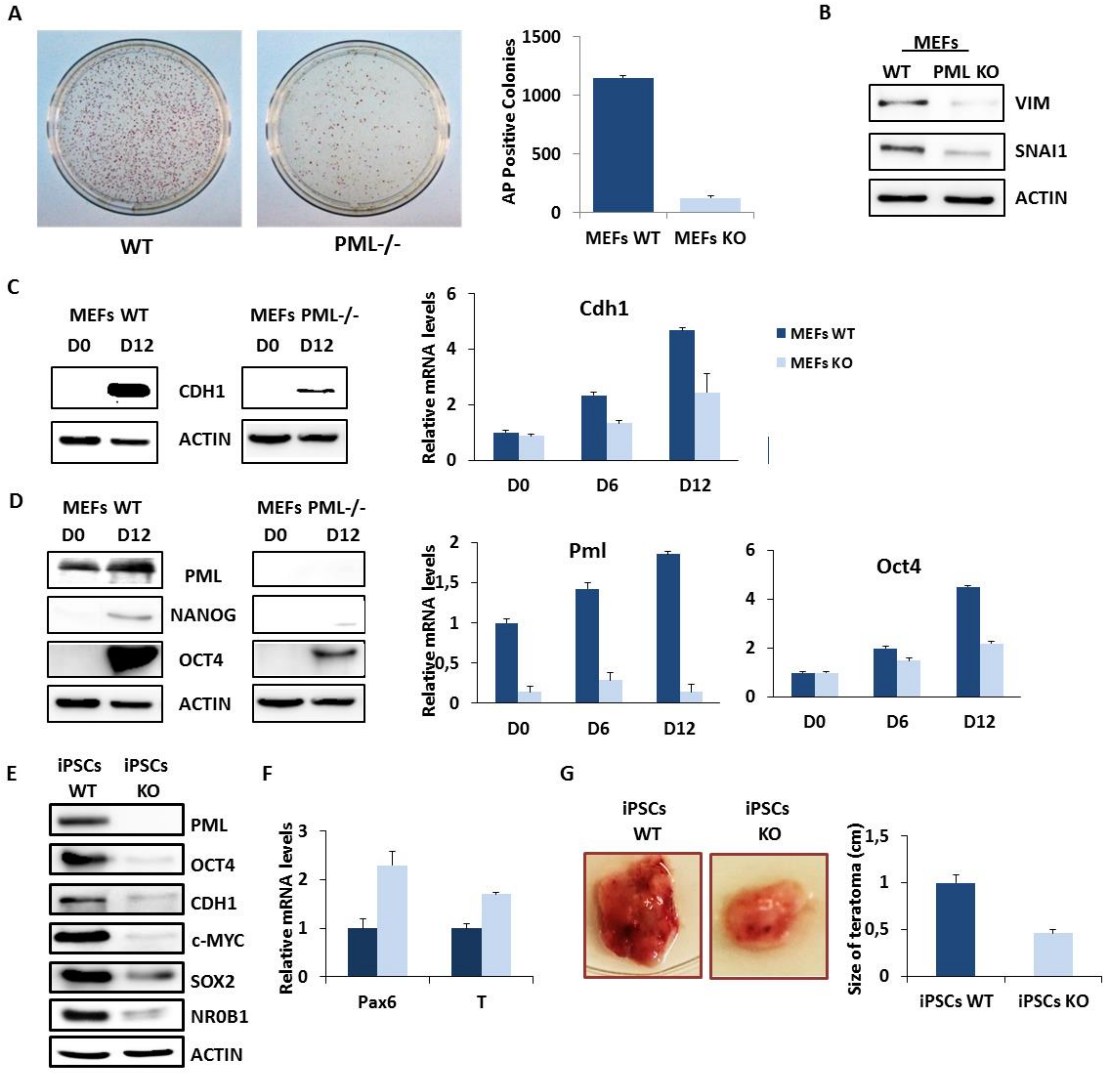


Figure 3.11: PML is essential for efficiency of MEFs reprogramming to iPSCs. A. AP staining of iPSCs colonies 28 days after OSKM lentiviral transduction. AP-positive colony numbers are shown on the right. **B.** Protein levels of mesenchymal characteristic markers (VIM, SNAI1). **C, D**. Expression levels of epithelial (**C**) and pluripotency markers (**D**) at D12 of reprogramming process. **E.** Protein levels of pluripotency markers in iPSCs WT and PML KO. **F.** mRNA levels of two representative developmental

genes (*Pax6, T*) in iPSCs WT and PML KO. **G**. Size of teratomas of iPSCs WT and iPSCs PML KO. Data are shown as mean + SD of four independent experiments (n=4).

Afterwards, pluripotency and differentiation properties were examined in the derived iPSC colonies. Interestingly, the representative undifferentiated stem cell marker, OCT4, SOX2, c-MYC, CDH1, NR0B1, were higher in WT-iPSC than *Pml*$^{-/-}$ iPSC (**Figure 3.11E**). Unlike WT-iPSC, *Pml*$^{-/-}$ iPSC expressed higher levels of differentiated characteristic genes (*Pax6, T*) (**Figure 3.11F**). Finally, we tested the pluripotency of these colonies by teratoma induction in vivo and we found that PML deletion inhibited the efficiency of iPSC teratoma formation (**Figure 3.11G**).

Our data are supported by two recent studies (Liu, Sun et al. 2013; Unternaehrer, Zhao et al. 2014). Liu et al., proposed a sequential EMT-MET mechanism at the beginning of reprogramming. Particularly, they suggested that during the first days of reprogramming (days 1-3) an EMT is required for the final generation of iPSCs. Treatment with several TGF-β molecules at day 1.5 greatly enhanced, while TGF-β treatment at day 12 significantly decreased the efficiency of reprogramming, suggesting a bi-functional role of TGF-b, an early stimulatory and a late inhibitory (Liu, Sun et al. 2013). Consistent with this observation, a year later, Unternaehrer and colleagues revealed that the depletion of Snai1 diminished, whereas its overexpression elevated reprogramming efficiency in human and mouse cells (Unternaehrer, Zhao et al. 2014). On the other hand, Pandolfi group showed that PML is a crucial regulator of TGF-β signaling. *Pml*$^{-/-}$ MEFs showed a significant reduction of Smad2/3 phosphorylation and impaired TGf-β target genes expression (Lin, Bergmann et al. 2004). Moreover, mesenchymal characteristic markers (Snai1) were expressed at lower levels, since TGF-β signaling is a critical regulator of EMT. Collectively, we concluded that PML positively contributes to the reprogramming of somatic cells by most likely enhancing EMT at the very early stages (d1-d3).

To date, several factors were identified to control the abilities of ESC either negatively or positively. Nevertheless, it is essential to detect new critical factors involved in the regulation of ESC molecular mechanism. Our results demonstrated for the first time that PML plays crucial role in the maintenance of ESC identity. We displayed that PML reduction led to pluripotency factors decrease and to the

induction of primed pluripotent state. Our microarray analysis further revealed that PML depletion negatively affects both cell cycle progression through mediating several factors related to it as well as signaling pathways essential for the naïve pluripotent state maintenance. Interestingly, we also unveiled that PML determined ESC cell fate commitment, specifically by promoting endoderm and suppressing mesoderm lineage. A possible model proposed is related to Tbx3 regulation (**Figure 3.12A**). Tbx3 negatively controls mesodermal differentiation through targeting T and Wnt-pathways genes, whereas it favors endodermal specification by positively regulating Eomes and Jmjd3 (Tortelote, Hernandez-Hernandez et al. 2013; Waghray, Saiz et al. 2015). Herein, PML knockdown causes a significant reduction of Tbx3, resulting in the alteration of cell fate decisions. Finally, PML facilitates the reprogramming process through a possible regulation of TGF-β and EMT (**Figure 3.12B**), deducing its necessity in the molecular network of ESCs. There is no doubt that PML is crucial for establishing ESC properties and it would be interesting to understand in depth the underlying mechanism and correlate it with its role in cancer cells.

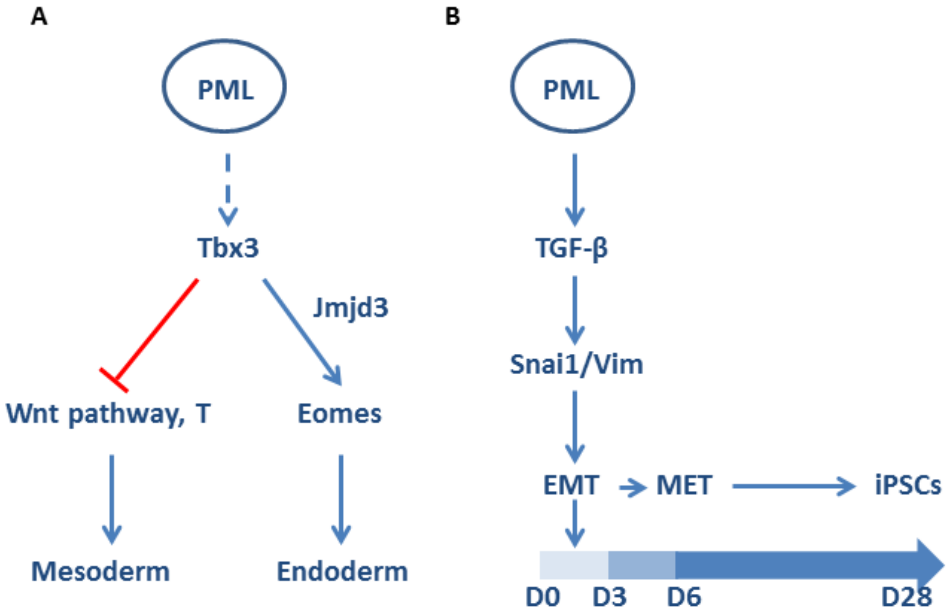


Figure 3.12: A proposed model for PML involvement in (A) mESC cell fate decision and (B) the reprogramming of somatic cells to iPSCs

Chapter 4: Conclusion

Molecular regulation of ESCs pluripotency is an ongoing question of strong interest in stem cell research. ESCs are characterized by a complicated regulatory network—including transcription factors, signaling pathways, and epigenetic factors (chromatin modifiers and miRNAs), which is crucial for the maintenance of ESC identity. Over the past years remarkable progress was made to elucidate the overall picture of this network and new factors are day-to-day identified as essential regulators for deciphering new molecular mechanisms of ESC biology.

This study unveiled two independent mechanisms that govern ESC pluripotency. miRNAs and PML were demonstrated to be critical for ESC properties and contribute to the pluripotency network. Firstly, four new miRNAs (miR-16-1, miR-191, miR-23a, miR-421), which were differentially expressed upon differentiation, were studied. Among them, miR-191 and miR-16-1 are highly expressed in ESCs and repress Activin-Nodal signaling, resulting in the inhibition of mesendoderm formation. miR-23a, which is also down-regulated in the differentiated state, suppresses differentiation towards the endoderm and ectoderm lineages. On the other hand, miR-421 was identified to act as a differentiation-associated regulator that promotes trophectodermal, ectodermal and endodermal lineages through the direct repression of the core pluripotency transcription factor *Oct4* and the BMP-signaling components, *Smad5* and *Id2*. These findings uncover a regulatory network between the studied miRNAs and both branches of TGF-β/BMP signaling pathways revealing their importance for ESC lineage decisions. Secondly, in this study we also provided strong evidence for the pivotal role of PML in ESC self-renewal and pluripotency. PML is declined during differentiation while experimental manipulation of its expression intensely influences ESC identity by regulating several transcription factors and signaling pathways, establishing in this way its necessity in this network. Furthermore, our results illustrate that PML expression is required for the reprogramming of somatic cells to iPSCs as its deletion acts as a barrier to this process, through TGF-β signaling inactivation. Together, our data decode a new mechanism for PML involvement in the regulation of ESC pluripotency circuit.

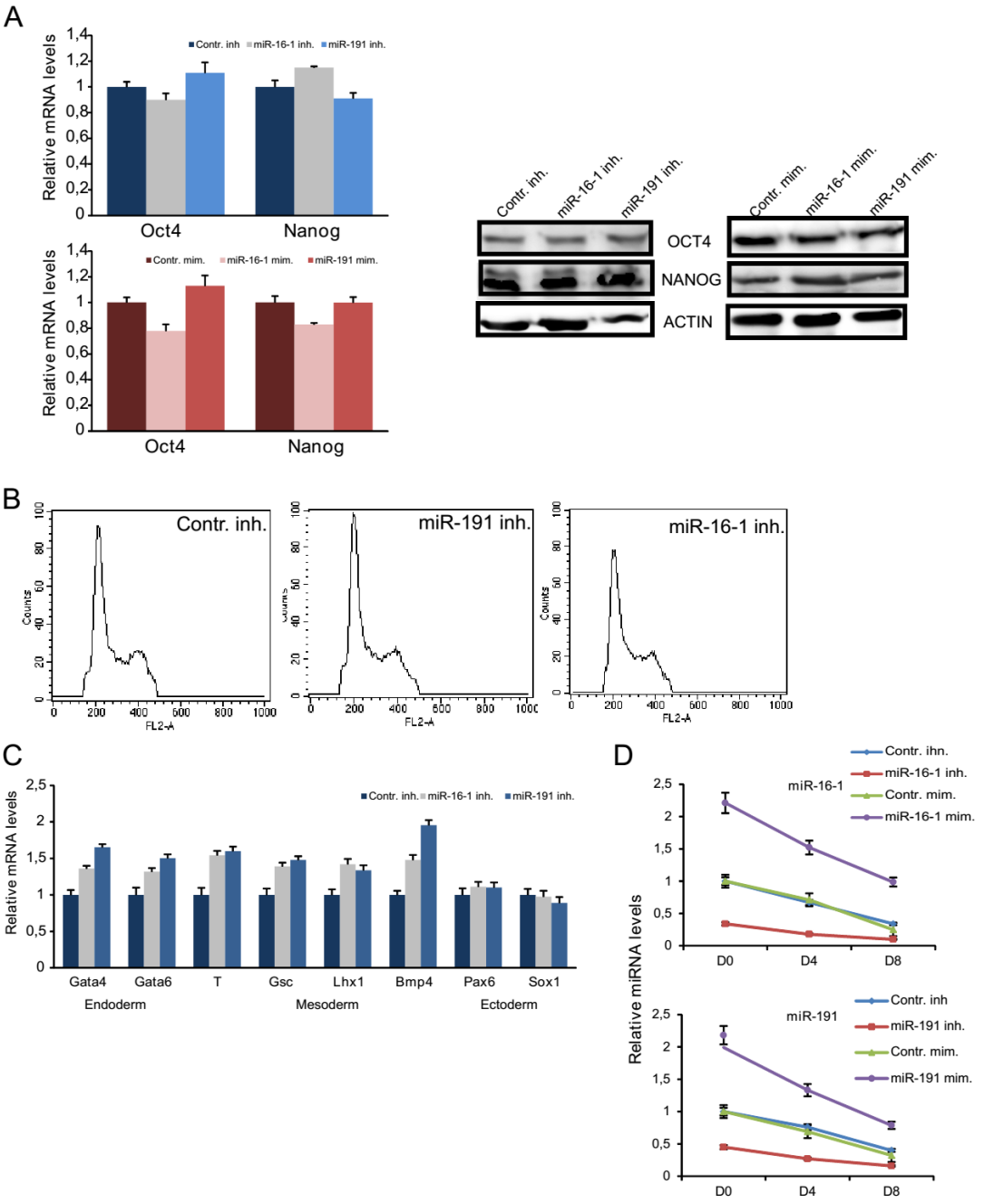
Previous studies have demonstrated the functional role of both PML and the aforementioned miRNAs in cancer biology (Guan and Kao 2015), being involved in several cancer types acting either as tumor suppressors or oncogenes (Guan and Kao

2015). Meantime, ESCs and tumor cells share many common properties exemplified by self-renewal, rapid proliferation and inhibition of differentiation. ESCs have inherent tumorigenic potential and they generate benign tumors and teratomas when injected in immunodeficient mice. Reprogramming of somatic cells into pluripotency by oncogenes -*Myc* and *Klf4*- suggests a strong link between pluripotency and tumorigenicity. Currently, growing experimental evidence has revealed that tumors contain a variable number of cells that have ESC abilities, termed cancer stem cells (CSCs). Studies in the fields of ESCs, iPSCs and CSCs provide strong evidence of cross-complementing benefits. Insight of the tumorigenic properties of stem cells and their differentiated descendants is required before their use in cell replacement therapies. Understanding the biology and gene circuits shared by both normal stem cells and CSCs, is important for efficient cancer treatment (Hadjimichael, Chanoumidou et al. 2015). Due to the fact that PML and the above miRNAs act in both stem and cancer cells, the discovery of their underlying molecular mechanisms in ESCs is a promising information, which may provide depth knowledge in cancer biology and help in developing new drugs to target CSCs.

Appendices

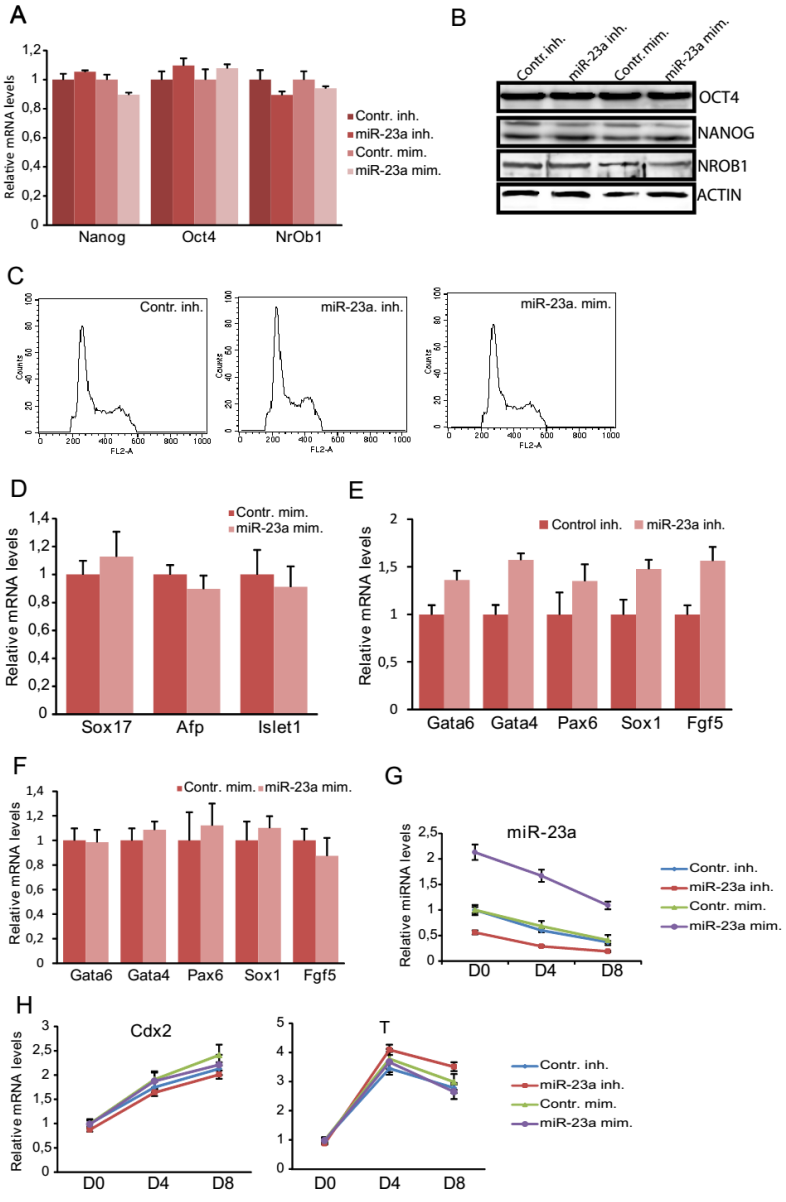
Appendix A1

Related to Figure 2.10. A. mRNA and protein levels of stemness markers upon the inhibition or overexpression of miR-16-1/miR-191 in mESCs. **B.** Analysis of cell cycle distribution of mESCs transfected with miR-16-1 and miR-191 inhibitors compared to control. **C.** Relative mRNA expression levels of differentiation markers in miR-16-1 and miR-191-inhibited mESCs. Error bars indicated +SD of three independent experiments (n=3). **D.** Relative miRNA expression levels during EB differentiation of mESCs transfected with miR-16-1/miR-191 inhibitors or mimics compared to controls. Error bars indicated +/-SD of three independent experiments (n=3).



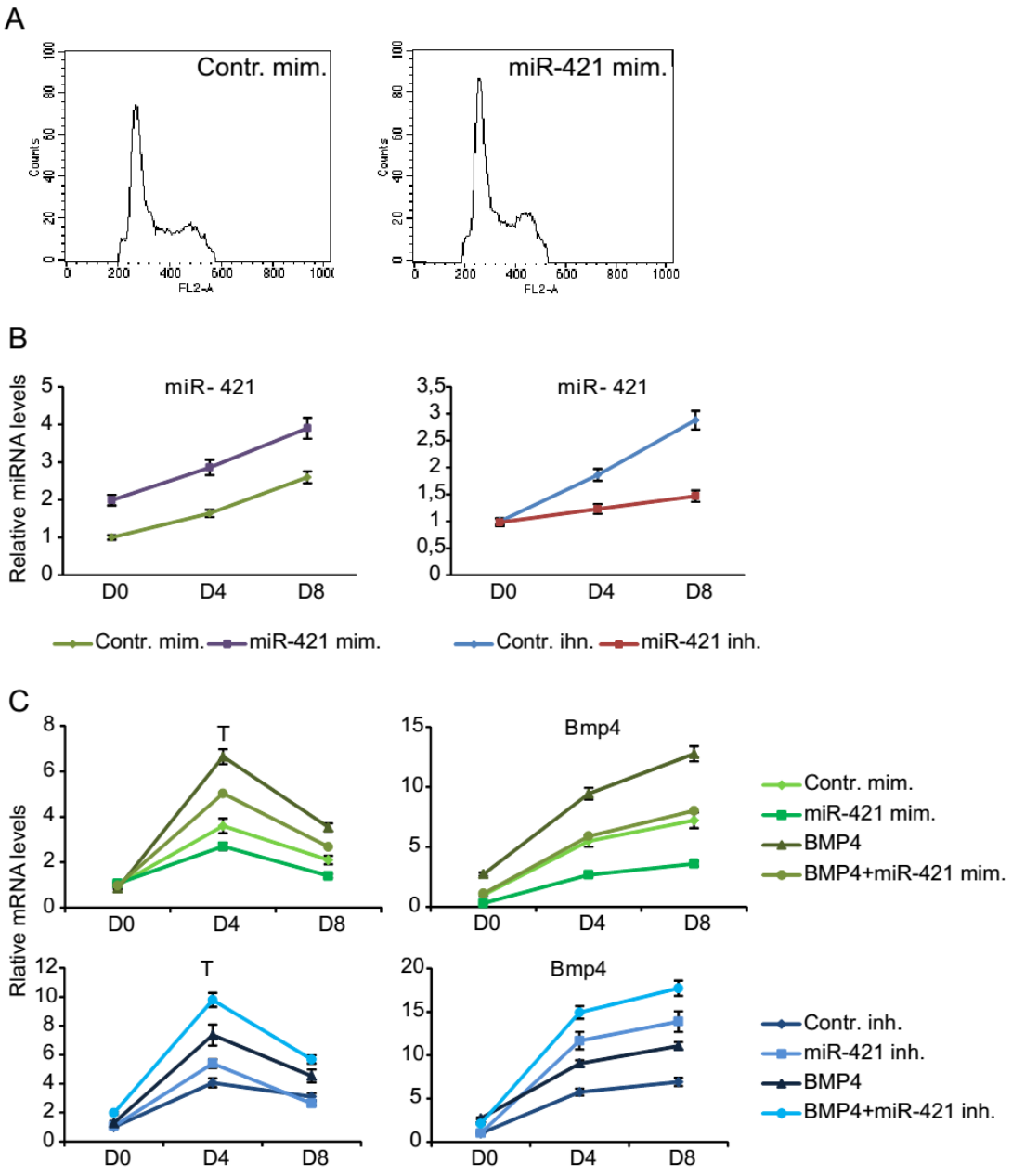
Appendix A2

Related to Figure 2.11. A, B. Relative mRNA (**A**) and protein (**B**) levels of pluripotency factors (Oct4, Nanog, NrOb1) in response to miR-23a inhibitor or mimic. Error bars indicated +SD of three independent experiments (n=3). **C.** Cell cycle distribution of miR-23a inhibited/overexpressed mESCs compared to control. **D.** Relative mRNA expression levels of the indicated differentiation genes upon miR-23a overexpression. Error bars indicated +SD of three independent experiments (n=3). **E, F.** Relative mRNA levels of genes associated with the three germ layers in miR-23a inhibited or overexpressed mESCs. Error bars indicated +SD of three independent experiments (n=3). **G.** Relative miRNA expression levels of miR-23a inhibited or overexpressed mESCs upon differentiation compared to controls. Data shown as mean +/- SD of three independent experiments (n=3). **H.** RT-PCR indicating the levels of characteristic trophectoderm and mesoderm genes (*Cdx2*, *T*) at EBs D0, D4 and D8, upon miR-23a overexpression or inhibition. Error bars indicate +/-SD of three independent experiments (n=3).



Appendix A3

Related to Figure 3.12. A. Cell cycle analysis of mESCs before and after miR-421 overexpression. **B.** Relative miRNA expression level of miR-421 overexpressed and inhibited mESCs during EB differentiation compared to control. Error bars indicated +/- SD of three independent experiments (n=3). **C.** RT-PCR indicating the levels of characteristic mesoderm genes (*T, Bmp4*) at EBs D0, D4 and D8, upon miR-421 overexpression or inhibition. Error bars indicate +/-SD of three independent experiments (n=3).



References

Acampora, D., L. G. Di Giovannantonio, et al. (2013). "Otx2 is an intrinsic determinant of the embryonic stem cell state and is required for transition to a stable epiblast stem cell condition." Development **140**(1): 43-55.

Aksoy, I., V. Giudice, et al. (2014). "Klf4 and Klf5 differentially inhibit mesoderm and endoderm differentiation in embryonic stem cells." Nat Commun **5**: 3719.

Aksoy, I., R. Jauch, et al. (2013). "Oct4 switches partnering from Sox2 to Sox17 to reinterpret the enhancer code and specify endoderm." EMBO J **32**(7): 938-953.

Aksoy, I., C. Sakabedoyan, et al. (2007). "Self-renewal of murine embryonic stem cells is supported by the serine/threonine kinases Pim-1 and Pim-3." Stem Cells **25**(12): 2996-3004.

Alsheich-Bartok, O., S. Haupt, et al. (2008). "PML enhances the regulation of p53 by CK1 in response to DNA damage." Oncogene **27**(26): 3653-3661.

Apostolou, E. and K. Hochedlinger (2013). "Chromatin dynamics during cellular reprogramming." Nature **502**(7472): 462-471.

Aqeilan, R. I., G. A. Calin, et al. (2010). "miR-15a and miR-16-1 in cancer: discovery, function and future perspectives." Cell Death Differ **17**(2): 215-220.

Avilion, A. A., S. K. Nicolis, et al. (2003). "Multipotent cell lineages in early mouse development depend on SOX2 function." Genes Dev **17**(1): 126-140.

Babiarz, J. E., J. G. Ruby, et al. (2008). "Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs." Genes Dev **22**(20): 2773-2785.

Bandi, N., S. Zbinden, et al. (2009). "miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer." Cancer Res **69**(13): 5553-5559.

Bao, S., F. Tang, et al. (2009). "Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells." Nature **461**(7268): 1292-1295.

Baudino, T. A., C. McKay, et al. (2002). "c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression." Genes Dev **16**(19): 2530-2543.

Beck, F., T. Erler, et al. (1995). "Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes." Dev Dyn **204**(3): 219-227.

Benetti, R., S. Gonzalo, et al. (2008). "A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases." Nat Struct Mol Biol **15**(3): 268-279.

Berezikov, E., W. J. Chung, et al. (2007). "Mammalian mirtron genes." Mol Cell **28**(2): 328-336.

Bernardi, R. and P. P. Pandolfi (2007). "Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies." Nat Rev Mol Cell Biol **8**(12): 1006-1016.

Bernstein, E., S. Y. Kim, et al. (2003). "Dicer is essential for mouse development." Nat Genet **35**(3): 215-217.

Bhaskaran, M. and M. Mohan (2014). "MicroRNAs: history, biogenesis, and their evolving role in animal development and disease." Vet Pathol **51**(4): 759-774.

Boeuf, H., C. Hauss, et al. (1997). "Leukemia inhibitory factor-dependent transcriptional activation in embryonic stem cells." J Cell Biol **138**(6): 1207-1217.

Bohm, J., F. J. Kaiser, et al. (2007). "Synergistic cooperation of Sall4 and Cyclin D1 in transcriptional repression." Biochem Biophys Res Commun **356**(3): 773-779.

Bohnsack, M. T., K. Czaplinski, et al. (2004). "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs." RNA **10**(2): 185-191.

Borden, K. L., E. J. Campbell Dwyer, et al. (1998). "An arenavirus RING (zinc-binding) protein binds the oncoprotein promyelocyte leukemia protein (PML) and relocates PML nuclear bodies to the cytoplasm." J Virol **72**(1): 758-766.

Boulton, T. G., N. Stahl, et al. (1994). "Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors." J Biol Chem **269**(15): 11648-11655.

Bourillot, P. Y., I. Aksoy, et al. (2009). "Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog." Stem Cells **27**(8): 1760-1771.

Boyer, L. A., T. I. Lee, et al. (2005). "Core transcriptional regulatory circuitry in human embryonic stem cells." Cell **122**(6): 947-956.

Brons, I. G., L. E. Smithers, et al. (2007). "Derivation of pluripotent epiblast stem cells from mammalian embryos." Nature **448**(7150): 191-195.

Burdon, T., A. Smith, et al. (2002). "Signalling, cell cycle and pluripotency in embryonic stem cells." Trends Cell Biol **12**(9): 432-438.

Cai, X., C. H. Hagedorn, et al. (2004). "Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs." RNA **10**(12): 1957-1966.

Calin, G. A., A. Cimmino, et al. (2008). "MiR-15a and miR-16-1 cluster functions in human leukemia." Proc Natl Acad Sci U S A **105**(13): 5166-5171.

Calin, G. A., C. D. Dumitru, et al. (2002). "Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **99**(24): 15524-15529.

Camus, A., A. Perea-Gomez, et al. (2006). "Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo." Dev Biol **295**(2): 743-755.

Caporali, A. and C. Emanueli (2011). "MicroRNA-503 and the extended microRNA-16 family in angiogenesis." Trends Cardiovasc Med **21**(6): 162-166.

Card, D. A., P. B. Hebbar, et al. (2008). "Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells." Mol Cell Biol **28**(20): 6426-6438.

Carracedo, A., D. Weiss, et al. (2012). "A metabolic prosurvival role for PML in breast cancer." J Clin Invest **122**(9): 3088-3100.

Cartwright, P., C. McLean, et al. (2005). "LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism." Development **132**(5): 885-896.

Casanova, E. A., O. Shakhova, et al. (2011). "Pramel7 mediates LIF/STAT3-dependent self-renewal in embryonic stem cells." Stem Cells **29**(3): 474-485.

Cauffman, G., H. Van de Velde, et al. (2005). "Oct-4 mRNA and protein expression during human preimplantation development." Mol Hum Reprod **11**(3): 173-181.

Chak, L. L. and K. Okamura (2014). "Argonaute-dependent small RNAs derived from single-stranded, non-structured precursors." Front Genet **5**: 172.

Chambers, I., D. Colby, et al. (2003). "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells." Cell **113**(5): 643-655.

Chambers, I., J. Silva, et al. (2007). "Nanog safeguards pluripotency and mediates germline development." Nature **450**(7173): 1230-1234.

Chandran, P. A., A. Keller, et al. (2014). "The TGF-beta-inducible miR-23a cluster attenuates IFN-gamma levels and antigen-specific cytotoxicity in human CD8(+) T cells." J Leukoc Biol **96**(4): 633-645.

Chappell, J., Y. Sun, et al. (2013). "MYC/MAX control ERK signaling and pluripotency by regulation of dual-specificity phosphatases 2 and 7." Genes Dev **27**(7): 725-733.

Charron, J., B. A. Malynn, et al. (1990). "High-frequency disruption of the N-myc gene in embryonic stem and pre-B cell lines by homologous recombination." Mol Cell Biol **10**(4): 1799-1804.

Chen, W. M., Z. X. Chen, et al. (2008). "[Biological characteristics of human fetal osteoblastic 1.19 cell line]." Zhongguo Shi Yan Xue Ye Xue Za Zhi **16**(2): 339-344.

Chen, X., H. Xu, et al. (2008). "Integration of external signaling pathways with the core transcriptional network in embryonic stem cells." Cell **133**(6): 1106-1117.

Cheng, X., Y. Liu, et al. (2012). "Promyelocytic leukemia protein (PML) regulates endothelial cell network formation and migration in response to tumor necrosis factor alpha (TNFalpha) and interferon alpha (IFNalpha)." J Biol Chem **287**(28): 23356-23367.

Chenoweth, J. G., R. D. McKay, et al. (2010). "Epiblast stem cells contribute new insight into pluripotency and gastrulation." Dev Growth Differ **52**(3): 293-301.

Chhabra, R., R. Dubey, et al. (2010). "Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases." Mol Cancer **9**: 232.

Chong, M. M., G. Zhang, et al. (2010). "Canonical and alternate functions of the microRNA biogenesis machinery." Genes Dev **24**(17): 1951-1960.

Chouvardas, P., Kollias G. and Nikolaou C. (2016) "Inferring active regulatory networks from gene expression data using a combination of prior knowledge and enrichment analysis", BMC Bioinformatics (in press)

Chuang, Y. S., W. H. Huang, et al. (2011). "Promyelocytic leukemia protein in retinoic acid-induced chromatin remodeling of Oct4 gene promoter." Stem Cells **29**(4): 660-669.

Chung, I., S. Osterwald, et al. (2012). "PML body meets telomere: the beginning of an ALTernate ending?" Nucleus **3**(3): 263-275.

Clipsham, R., K. Niakan, et al. (2004). "Nr0b1 and its network partners are expressed early in murine embryos prior to steroidogenic axis organogenesis." Gene Expr Patterns **4**(1): 3-14.

Costello, I., I. M. Pimeisl, et al. (2011). "The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation." Nat Cell Biol **13**(9): 1084-1091.

Darnell, J. E., Jr. (1997). "STATs and gene regulation." Science **277**(5332): 1630-1635.

Davies, J., D. Newnham, et al. (2015). "A recommended read." Nurs Stand **30**(16): 28-29.

de The, H., C. Lavau, et al. (1991). "The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR." Cell **66**(4): 675-684.

Derynck, R. (1994). "TGF-beta-receptor-mediated signaling." Trends Biochem Sci **19**(12): 548-553.

Dhillon, A. K., N. Fairlie, et al. (2015). "Pelvic Actinomyces israelii abscess: a differential diagnosis of a pelvic mass." BMJ Case Rep **2015**.

Drabsch, Y. and P. ten Dijke (2012). "TGF-beta signalling and its role in cancer progression and metastasis." Cancer Metastasis Rev **31**(3-4): 553-568.

Duprez, E., A. J. Saurin, et al. (1999). "SUMO-1 modification of the acute promyelocytic leukaemia protein PML: implications for nuclear localisation." J Cell Sci **112 (Pt 3)**: 381-393.

Ema, M., D. Mori, et al. (2008). "Kruppel-like factor 5 is essential for blastocyst development and the normal self-renewal of mouse ESCs." Cell Stem Cell **3**(5): 555-567.

Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." Nature **292**(5819): 154-156.

Everett, R. D., P. Lomonte, et al. (1999). "Cell cycle regulation of PML modification and ND10 composition." J Cell Sci **112 (Pt 24)**: 4581-4588.

Fei, T., S. Zhu, et al. (2010). "Smad2 mediates Activin/Nodal signaling in mesendoderm differentiation of mouse embryonic stem cells." Cell Res **20**(12): 1306-1318.

Feng, B., J. Jiang, et al. (2009). "Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb." Nat Cell Biol **11**(2): 197-203.

Festuccia, N., R. Osorno, et al. (2012). "Esrrb is a direct Nanog target gene that can substitute for Nanog function in pluripotent cells." Cell Stem Cell **11**(4): 477-490.

Finch, B. W. and B. Ephrussi (1967). "RETENTION OF MULTIPLE DEVELOPMENTAL POTENTIALITIES BY CELLS OF A MOUSE TESTICULAR TERATOCARCINOMA DURING PROLONGED CULTURE in vitro AND THEIR EXTINCTION UPON HYBRIDIZATION WITH CELLS OF PERMANENT LINES." Proc Natl Acad Sci U S A **57**(3): 615-621.

Flynt, A. S., E. J. Thatcher, et al. (2009). "miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos." J Cell Biol **185**(1): 115-127.

Fogal, V., M. Gostissa, et al. (2000). "Regulation of p53 activity in nuclear bodies by a specific PML isoform." EMBO J **19**(22): 6185-6195.

Gangaraju, V. K. and H. Lin (2009). "MicroRNAs: key regulators of stem cells." Nat Rev Mol Cell Biol **10**(2): 116-125.

Gialitakis, M., P. Arampatzi, et al. (2010). "Gamma interferon-dependent transcriptional memory via relocalization of a gene locus to PML nuclear bodies." Mol Cell Biol **30**(8): 2046-2056.

Greber, B., G. Wu, et al. (2010). "Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells." Cell Stem Cell **6**(3): 215-226.

Griffiths-Jones, S. (2006). "miRBase: the microRNA sequence database." Methods Mol Biol **342**: 129-138.

Grignani, F., P. F. Ferrucci, et al. (1993). "The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells." Cell **74**(3): 423-431.

Guan, D. and H. Y. Kao (2015). "The function, regulation and therapeutic implications of the tumor suppressor protein, PML." Cell Biosci **5**: 60.

Gunning, G. A., M. Murray, et al. (2016). "Inter-comparison of radon detectors for one to four week measurement periods." J Radiol Prot **36**(1): 104-116.

Guo, G., J. Yang, et al. (2009). "Klf4 reverts developmentally programmed restriction of ground state pluripotency." Development **136**(7): 1063-1069.

Hadjimichael, C., K. Chanoumidou, et al. (2015). "Common stemness regulators of embryonic and cancer stem cells." World J Stem Cells **7**(9): 1150-1184.

Han, J., P. Yuan, et al. (2010). "Tbx3 improves the germ-line competency of induced pluripotent stem cells." Nature **463**(7284): 1096-1100.

Han, J., F. Zhang, et al. (2012). "RNA interference-mediated silencing of NANOG reduces cell proliferation and induces G0/G1 cell cycle arrest in breast cancer cells." Cancer Lett **321**(1): 80-88.

Hassan, M. Q., J. A. Gordon, et al. (2010). "A network connecting Runx2, SATB2, and the miR-23a~27a~24-2 cluster regulates the osteoblast differentiation program." Proc Natl Acad Sci U S A **107**(46): 19879-19884.

Hatakeyama, S. (2011). "TRIM proteins and cancer." Nat Rev Cancer **11**(11): 792-804.

Hay, D. C., L. Sutherland, et al. (2004). "Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells." Stem Cells **22**(2): 225-235.

Hayakawa, F., A. Abe, et al. (2008). "Acetylation of PML is involved in histone deacetylase inhibitor-mediated apoptosis." J Biol Chem **283**(36): 24420-24425.

Holland, J. D., A. Klaus, et al. (2013). "Wnt signaling in stem and cancer stem cells." Curr Opin Cell Biol **25**(2): 254-264.

Hu, G., J. Kim, et al. (2009). "A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal." Genes Dev **23**(7): 837-848.

Hubackova, S., K. Krejcikova, et al. (2012). "Interleukin 6 signaling regulates promyelocytic leukemia protein gene expression in human normal and cancer cells." J Biol Chem **287**(32): 26702-26714.

Ito, K., R. Bernardi, et al. (2008). "PML targeting eradicates quiescent leukaemia-initiating cells." Nature **453**(7198): 1072-1078.

Ito, K., A. Carracedo, et al. (2012). "A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance." Nat Med **18**(9): 1350-1358.

Ivanova, N., R. Dobrin, et al. (2006). "Dissecting self-renewal in stem cells with RNA interference." Nature **442**(7102): 533-538.

Ivanschitz, L., H. De The, et al. (2013). "PML, SUMOylation, and Senescence." Front Oncol **3**: 171.

Jang, H. J., H. W. Seo, et al. (2013). "Gene expression and DNA methylation status of chicken primordial germ cells." Mol Biotechnol **54**(2): 177-186.

Jensen, K., C. Shiels, et al. (2001). "PML protein isoforms and the RBCC/TRIM motif." Oncogene **20**(49): 7223-7233.

Jiang, J., Y. S. Chan, et al. (2008). "A core Klf circuitry regulates self-renewal of embryonic stem cells." Nat Cell Biol **10**(3): 353-360.

Jouneau, A., C. Ciaudo, et al. (2012). "Naive and primed murine pluripotent stem cells have distinct miRNA expression profiles." RNA **18**(2): 253-264.

Kahan, B. W. and B. Ephrussi (1970). "Developmental potentialities of clonal in vitro cultures of mouse testicular teratoma." J Natl Cancer Inst **44**(5): 1015-1036.

Kakizuka, A., W. H. Miller, Jr., et al. (1991). "Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML." Cell **66**(4): 663-674.

Kamachi, Y., M. Uchikawa, et al. (2000). "Pairing SOX off: with partners in the regulation of embryonic development." Trends Genet **16**(4): 182-187.

Kanellopoulou, C., S. A. Muljo, et al. (2005). "Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing." Genes Dev **19**(4): 489-501.

Kartikasari, A. E., J. X. Zhou, et al. (2013). "The histone demethylase Jmjd3 sequentially associates with the transcription factors Tbx3 and Eomes to drive endoderm differentiation." EMBO J **32**(10): 1393-1408.

Kelly, K. F., D. Y. Ng, et al. (2011). "beta-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism." Cell Stem Cell **8**(2): 214-227.

Kim, J., J. Chu, et al. (2008). "An extended transcriptional network for pluripotency of embryonic stem cells." Cell **132**(6): 1049-1061.

Kumar, P. P., O. Bischof, et al. (2007). "Functional interaction between PML and SATB1 regulates chromatin-loop architecture and transcription of the MHC class I locus." Nat Cell Biol **9**(1): 45-56.

Lallemand-Breitenbach, V. and H. de The (2010). "PML nuclear bodies." Cold Spring Harb Perspect Biol **2**(5): a000661.

Landgraf, P., M. Rusu, et al. (2007). "A mammalian microRNA expression atlas based on small RNA library sequencing." Cell **129**(7): 1401-1414.

Lapi, E., S. Di Agostino, et al. (2008). "PML, YAP, and p73 are components of a proapoptotic autoregulatory feedback loop." Mol Cell **32**(6): 803-814.

Lavau, C., A. Marchio, et al. (1995). "The acute promyelocytic leukaemia-associated PML gene is induced by interferon." Oncogene **11**(5): 871-876.

Lee, K. L., S. K. Lim, et al. (2011). "Graded Nodal/Activin signaling titrates conversion of quantitative phospho-Smad2 levels into qualitative embryonic stem cell fate decisions." PLoS Genet **7**(6): e1002130.

Lee, R. C., R. L. Feinbaum, et al. (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-854.

Lee, Y., M. Kim, et al. (2004). "MicroRNA genes are transcribed by RNA polymerase II." EMBO J **23**(20): 4051-4060.

Lewis, B. P., C. B. Burge, et al. (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." Cell **120**(1): 15-20.

Li, R., J. Liang, et al. (2010). "A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts." Cell Stem Cell **7**(1): 51-63.

Li, T., H. Li, et al. (2016). "microRNA-23a inhibits osteogenic differentiation of human bone marrow-derived mesenchymal stem cells by targeting LRP5." Int J Biochem Cell Biol **72**: 55-62.

Li, Y., Q. Gao, et al. (2012). "WNT/beta-catenin-signaling pathway stimulates the proliferation of cultured adult human Sertoli cells via upregulation of C-myc expression." Reprod Sci **19**(11): 1232-1240.

Li, Y., W. Li, et al. (2014). "Downregulation of tumor suppressor menin by miR-421 promotes proliferation and migration of neuroblastoma." Tumour Biol **35**(10): 10011-10017.

Li, Y., J. McClintick, et al. (2005). "Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4." Blood **105**(2): 635-637.

Liang, J., M. Wan, et al. (2008). "Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells." Nat Cell Biol **10**(6): 731-739.

Lin, H. K., S. Bergmann, et al. (2004). "Cytoplasmic PML function in TGF-beta signalling." Nature **431**(7005): 205-211.

Liu, Q., H. Fu, et al. (2008). "miR-16 family induces cell cycle arrest by regulating multiple cell cycle genes." Nucleic Acids Res **36**(16): 5391-5404.

Liu, X., H. Sun, et al. (2013). "Sequential introduction of reprogramming factors reveals a time-sensitive requirement for individual factors and a sequential

EMT-MET mechanism for optimal reprogramming." Nat Cell Biol **15**(7): 829-838.

Lodato, M. A., C. W. Ng, et al. (2013). "SOX2 co-occupies distal enhancer elements with distinct POU factors in ESCs and NPCs to specify cell state." PLoS Genet **9**(2): e1003288.

Logan, C. Y. and R. Nusse (2004). "The Wnt signaling pathway in development and disease." Annu Rev Cell Dev Biol **20**: 781-810.

Loh, Y. H., Q. Wu, et al. (2006). "The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells." Nat Genet **38**(4): 431-440.

Louria-Hayon, I., T. Grossman, et al. (2003). "The promyelocytic leukemia protein protects p53 from Mdm2-mediated inhibition and degradation." J Biol Chem **278**(35): 33134-33141.

Lutticken, C., U. M. Wegenka, et al. (1994). "Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130." Science **263**(5143): 89-92.

Maherali, N. and K. Hochedlinger (2008). "Induced pluripotency of mouse and human somatic cells." Cold Spring Harb Symp Quant Biol **73**: 157-162.

Maherali, N., R. Sridharan, et al. (2007). "Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution." Cell Stem Cell **1**(1): 55-70.

Mandal, P. K. and D. J. Rossi (2013). "Reprogramming human fibroblasts to pluripotency using modified mRNA." Nat Protoc **8**(3): 568-582.

Mann, K. (2015). "The calcified eggshell matrix proteome of a songbird, the zebra finch (Taeniopygia guttata)." Proteome Sci **13**: 29.

Marchand, A., C. Proust, et al. (2012). "miR-421 and miR-30c inhibit SERPINE 1 gene expression in human endothelial cells." PLoS One **7**(8): e44532.

Marson, A., S. S. Levine, et al. (2008). "Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells." Cell **134**(3): 521-533.

Martello, G., P. Bertone, et al. (2013). "Identification of the missing pluripotency mediator downstream of leukaemia inhibitory factor." EMBO J **32**(19): 2561-2574.

Martello, G., T. Sugimoto, et al. (2012). "Esrrb is a pivotal target of the Gsk3/Tcf3 axis regulating embryonic stem cell self-renewal." Cell Stem Cell **11**(4): 491-504.

Martello, G., L. Zacchigna, et al. (2007). "MicroRNA control of Nodal signalling." Nature **449**(7159): 183-188.

Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." Proc Natl Acad Sci U S A **78**(12): 7634-7638.

Martin, N., M. Benhamed, et al. (2012). "Physical and functional interaction between PML and TBX2 in the establishment of cellular senescence." EMBO J **31**(1): 95-109.

Matheson, A. M. and J. T. Sakata (2015). "Relationship between the Sequencing and Timing of Vocal Motor Elements in Birdsong." PLoS One **10**(12): e0143203.

Mathieu, J. and H. Ruohola-Baker (2013). "Regulation of stem cell populations by microRNAs." Adv Exp Med Biol **786**: 329-351.

Matsubara, S., Q. Ding, et al. (2013). "mTOR plays critical roles in pancreatic cancer stem cells through specific and stemness-related functions." Sci Rep **3**: 3230.

Matsuda, T., T. Nakamura, et al. (1999). "STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells." EMBO J **18**(15): 4261-4269.

Melton, C., R. L. Judson, et al. (2010). "Opposing microRNA families regulate self-renewal in mouse embryonic stem cells." Nature **463**(7281): 621-626.

Mitchell, G. W., C. G. Guglielmo, et al. (2015). "Measurement of Whole-Body CO2 Production in Birds Using Real-Time Laser-Derived Measurements of Hydrogen (delta(2)H) and Oxygen (delta(18)O) Isotope Concentrations in Water Vapor from Breath." Physiol Biochem Zool **88**(6): 599-606.

Mitsui, K., Y. Tokuzawa, et al. (2003). "The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells." Cell **113**(5): 631-642.

Miyoshi, N., H. Ishii, et al. (2011). "Reprogramming of mouse and human cells to pluripotency using mature microRNAs." Cell Stem Cell **8**(6): 633-638.

Monteys, A. M., R. M. Spengler, et al. (2010). "Structure and activity of putative intronic miRNA promoters." RNA **16**(3): 495-505.

Moon, J. H., J. S. Heo, et al. (2011). "Reprogramming fibroblasts into induced pluripotent stem cells with Bmi1." Cell Res **21**(9): 1305-1315.

Morey, L., A. Santanach, et al. (2015). "Pluripotency and Epigenetic Factors in Mouse Embryonic Stem Cell Fate Regulation." Mol Cell Biol **35**(16): 2716-2728.

Moustakas, A. and C. H. Heldin (2009). "The regulation of TGFbeta signal transduction." Development **136**(22): 3699-3714.

Mu, Z. M., X. F. Le, et al. (1997). "Stable overexpression of PML alters regulation of cell cycle progression in HeLa cells." Carcinogenesis **18**(11): 2063-2069.

Muheim, R., S. Sjoberg, et al. (2016). "Polarized light modulates light-dependent magnetic compass orientation in birds." Proc Natl Acad Sci U S A.

Murayama, H., H. Masaki, et al. (2015). "Successful reprogramming of epiblast stem cells by blocking nuclear localization of beta-catenin." Stem Cell Reports **4**(1): 103-113.

Murchison, E. P., J. F. Partridge, et al. (2005). "Characterization of Dicer-deficient murine embryonic stem cells." Proc Natl Acad Sci U S A **102**(34): 12135-12140.

Musto, A., A. Navarra, et al. (2014). "miR-23a, miR-24 and miR-27a protect differentiating ESCs from BMP4-induced apoptosis." Cell Death Differ.

Nagpal, N., H. M. Ahmad, et al. (2013). "MicroRNA-191, an estrogen-responsive microRNA, functions as an oncogenic regulator in human breast cancer." Carcinogenesis **34**(8): 1889-1899.

Nagpal, N. and R. Kulshreshtha (2014). "miR-191: an emerging player in disease biology." Front Genet **5**: 99.

Nakahara, F., C. N. Weiss, et al. (2014). "The role of PML in hematopoietic and leukemic stem cell maintenance." Int J Hematol **100**(1): 18-26.

Nakatake, Y., N. Fukui, et al. (2006). "Klf4 cooperates with Oct3/4 and Sox2 to activate the Lefty1 core promoter in embryonic stem cells." Mol Cell Biol **26**(20): 7772-7782.

Newman, M. A., J. M. Thomson, et al. (2008). "Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing." RNA **14**(8): 1539-1549.

Ng, H. H. and M. A. Surani (2011). "The transcriptional and signalling networks of pluripotency." Nat Cell Biol **13**(5): 490-496.

Niakan, K. K., H. Ji, et al. (2010). "Sox17 promotes differentiation in mouse embryonic stem cells by directly regulating extraembryonic gene expression and indirectly antagonizing self-renewal." Genes Dev **24**(3): 312-326.

Nichol, J. N., L. A. Petruccelli, et al. (2009). "Expanding PML's functional repertoire through post-translational mechanisms." Front Biosci (Landmark Ed) **14**: 2293-2306.

Nichols, J. and A. Smith (2009). "Naive and primed pluripotent states." Cell Stem Cell **4**(6): 487-492.

Nichols, J., B. Zevnik, et al. (1998). "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4." Cell **95**(3): 379-391.

Nisole, S., M. A. Maroui, et al. (2013). "Differential Roles of PML Isoforms." Front Oncol **3**: 125.

Niwa, H. (2011). "Wnt: what's needed to maintain pluripotency?" Nat Cell Biol **13**(9): 1024-1026.

Niwa, H., T. Burdon, et al. (1998). "Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3." Genes Dev **12**(13): 2048-2060.

Niwa, H., J. Miyazaki, et al. (2000). "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells." Nat Genet **24**(4): 372-376.

Niwa, H., K. Ogawa, et al. (2009). "A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells." Nature **460**(7251): 118-122.

O'Loghlen, A., A. M. Munoz-Cabello, et al. (2012). "MicroRNA regulation of Cbx7 mediates a switch of Polycomb orthologs during ESC differentiation." Cell Stem Cell **10**(1): 33-46.

Ohgushi, M. and Y. Sasai (2011). "Lonely death dance of human pluripotent stem cells: ROCKing between metastable cell states." Trends Cell Biol **21**(5): 274-282.

Okita, K., T. Ichisaka, et al. (2007). "Generation of germline-competent induced pluripotent stem cells." Nature **448**(7151): 313-317.

Otchy, T. M., S. B. Wolff, et al. (2015). "Acute off-target effects of neural circuit manipulations." Nature **528**(7582): 358-363.

P. Savatier, A. M. (2004). "Cell-cycle control in Embryonic Stem Cells." Handbook of Stem Cells **1**: 53-62.

Pellegrini, M. J., A. P. Schiff, et al. (2015). "Conversion of Tibiotalar Arthrodesis to Total Ankle Arthroplasty." J Bone Joint Surg Am **97**(24): 2004-2013.

Pera, M. F., J. Andrade, et al. (2004). "Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin." J Cell Sci **117**(Pt 7): 1269-1280.

Percherancier, Y., D. Germain-Desprez, et al. (2009). "Role of SUMO in RNF4-mediated promyelocytic leukemia protein (PML) degradation: sumoylation of PML and phospho-switch control of its SUMO binding domain dissected in living cells." J Biol Chem **284**(24): 16595-16608.

Polo, J. M., E. Anderssen, et al. (2012). "A molecular roadmap of reprogramming somatic cells into iPS cells." Cell **151**(7): 1617-1632.

Qi, J., J. Y. Yu, et al. (2009). "microRNAs regulate human embryonic stem cell division." Cell Cycle **8**(22): 3729-3741.

Qin, S., Y. Zhu, et al. (2014). "MicroRNA-191 correlates with poor prognosis of colorectal carcinoma and plays multiple roles by targeting tissue inhibitor of metalloprotease 3." Neoplasma **61**(1): 27-34.

Rabellino, A., B. Carter, et al. (2012). "The SUMO E3-ligase PIAS1 regulates the tumor suppressor PML and its oncogenic counterpart PML-RARA." Cancer Res **72**(9): 2275-2284.

Rahman, M. S., N. Akhtar, et al. (2015). "TGF-beta/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation." Bone Res **3**: 15005.

Redmer, T., S. Diecke, et al. (2011). "E-cadherin is crucial for embryonic stem cell pluripotency and can replace OCT4 during somatic cell reprogramming." EMBO Rep **12**(7): 720-726.

Reineke, E. L., Y. Liu, et al. (2010). "Promyelocytic leukemia protein controls cell migration in response to hydrogen peroxide and insulin-like growth factor-1." J Biol Chem **285**(13): 9485-9492.

Reinhart, B. J., F. J. Slack, et al. (2000). "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans." Nature **403**(6772): 901-906.

Rezende, L. D., N. R. Silva Martins, et al. (2015). "Epidemiological aspects of lice (Menacanthus species) infections in laying hen flocks from the State of Minas Gerais, Brazil." Br Poult Sci.

Ro, S., C. Park, et al. (2007). "Tissue-dependent paired expression of miRNAs." Nucleic Acids Res **35**(17): 5944-5953.

Rony, I. K., A. Baten, et al. (2015). "Inducing pluripotency in vitro: recent advances and highlights in induced pluripotent stem cells generation and pluripotency reprogramming." Cell Prolif **48**(2): 140-156.

Rosner, M. H., M. A. Vigano, et al. (1990). "A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo." Nature **345**(6277): 686-692.

Ruby, J. G., C. H. Jan, et al. (2007). "Intronic microRNA precursors that bypass Drosha processing." Nature **448**(7149): 83-86.

Sage, J. (2012). "The retinoblastoma tumor suppressor and stem cell biology." Genes Dev **26**(13): 1409-1420.

Salomoni, P., M. Dvorkina, et al. (2012). "Role of the promyelocytic leukaemia protein in cell death regulation." Cell Death Dis **3**: e247.

Salsman, J., M. Jagannathan, et al. (2012). "Proteomic profiling of the human cytomegalovirus UL35 gene products reveals a role for UL35 in the DNA repair response." J Virol **86**(2): 806-820.

Samavarchi-Tehrani, P., A. Golipour, et al. (2010). "Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming." Cell Stem Cell **7**(1): 64-77.

Sampath, D., C. Liu, et al. (2012). "Histone deacetylases mediate the silencing of miR-15a, miR-16, and miR-29b in chronic lymphocytic leukemia." Blood **119**(5): 1162-1172.

Sato, N., L. Meijer, et al. (2004). "Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor." Nat Med **10**(1): 55-63.

Scaglioni, P. P., T. M. Yung, et al. (2006). "A CK2-dependent mechanism for degradation of the PML tumor suppressor." Cell **126**(2): 269-283.

Scholer, H. R., G. R. Dressler, et al. (1990). "Oct-4: a germline-specific transcription factor mapping to the mouse t-complex." EMBO J **9**(7): 2185-2195.

Schwarz, D. S., G. Hutvagner, et al. (2003). "Asymmetry in the assembly of the RNAi enzyme complex." Cell **115**(2): 199-208.

Sengupta, S., J. Nie, et al. (2009). "MicroRNA 92b controls the G1/S checkpoint gene p57 in human embryonic stem cells." Stem Cells **27**(7): 1524-1528.

Shen, T. H., H. K. Lin, et al. (2006). "The mechanisms of PML-nuclear body formation." Mol Cell **24**(3): 331-339.

Shimozaki, K., K. Nakashima, et al. (2003). "Involvement of Oct3/4 in the enhancement of neuronal differentiation of ES cells in neurogenesis-inducing cultures." Development **130**(11): 2505-2512.

Shtutman, M., J. Zhurinsky, et al. (2002). "PML is a target gene of beta-catenin and plakoglobin, and coactivates beta-catenin-mediated transcription." Cancer Res **62**(20): 5947-5954.

Silva, J., J. Nichols, et al. (2009). "Nanog is the gateway to the pluripotent ground state." Cell **138**(4): 722-737.

Singh, S. K., M. N. Kagalwala, et al. (2008). "REST maintains self-renewal and pluripotency of embryonic stem cells." Nature **453**(7192): 223-227.

Sinkkonen, L., T. Hugenschmidt, et al. (2008). "MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells." Nat Struct Mol Biol **15**(3): 259-267.

Smith, K. N., A. M. Singh, et al. (2010). "Myc represses primitive endoderm differentiation in pluripotent stem cells." Cell Stem Cell **7**(3): 343-354.

Sridharan, R., J. Tchieu, et al. (2009). "Role of the murine reprogramming factors in the induction of pluripotency." Cell **136**(2): 364-377.

Stadtfeld, M., N. Maherali, et al. (2008). "Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse." Cell Stem Cell **2**(3): 230-240.

Stahl, N., T. G. Boulton, et al. (1994). "Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components." Science **263**(5143): 92-95.

Strumpf, D., C. A. Mao, et al. (2005). "Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst." Development **132**(9): 2093-2102.

Sun, C., Y. Nakatake, et al. (2009). "Dax1 binds to Oct3/4 and inhibits its transcriptional activity in embryonic stem cells." Mol Cell Biol **29**(16): 4574-4583.

Sun, C., Y. Nakatake, et al. (2008). "Stem cell-specific expression of Dax1 is conferred by STAT3 and Oct3/4 in embryonic stem cells." Biochem Biophys Res Commun **372**(1): 91-96.

Tai, C. I. and Q. L. Ying (2013). "Gbx2, a LIF/Stat3 target, promotes reprogramming to and retention of the pluripotent ground state." J Cell Sci **126**(Pt 5): 1093-1098.

Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell **126**(4): 663-676.

Tam, W. L., C. Y. Lim, et al. (2008). "T-cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways." Stem Cells **26**(8): 2019-2031.

Tang, M. K., Y. J. Liang, et al. (2013). "Promyelocytic leukemia (PML) protein plays important roles in regulating cell adhesion, morphology, proliferation and migration." PLoS One **8**(3): e59477.

Tarantino, C., G. Paolella, et al. (2010). "miRNA 34a, 100, and 137 modulate differentiation of mouse embryonic stem cells." FASEB J **24**(9): 3255-3263.

Tatham, M. H., M. C. Geoffroy, et al. (2008). "RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation." Nat Cell Biol **10**(5): 538-546.

Tay, Y., J. Zhang, et al. (2008). "MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation." Nature **455**(7216): 1124-1128.

Tay, Y. M., W. L. Tam, et al. (2008). "MicroRNA-134 modulates the differentiation of mouse embryonic stem cells, where it causes post-transcriptional attenuation of Nanog and LRH1." Stem Cells **26**(1): 17-29.

ten Berge, D., D. Kurek, et al. (2011). "Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells." Nat Cell Biol **13**(9): 1070-1075.

Tesar, P. J., J. G. Chenoweth, et al. (2007). "New cell lines from mouse epiblast share defining features with human embryonic stem cells." Nature **448**(7150): 196-199.

Thiery, J. P., H. Acloque, et al. (2009). "Epithelial-mesenchymal transitions in development and disease." Cell **139**(5): 871-890.

Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." Science **282**(5391): 1145-1147.

Tortelote, G. G., J. M. Hernandez-Hernandez, et al. (2013). "Wnt3 function in the epiblast is required for the maintenance but not the initiation of gastrulation in mice." Dev Biol **374**(1): 164-173.

Unternaehrer, J. J., R. Zhao, et al. (2014). "The epithelial-mesenchymal transition factor SNAIL paradoxically enhances reprogramming." Stem Cell Reports **3**(5): 691-698.

Uranishi, K., T. Akagi, et al. (2013). "Dax1 associates with Esrrb and regulates its function in embryonic stem cells." Mol Cell Biol **33**(10): 2056-2066.

Vallier, L., S. Mendjan, et al. (2009). "Activin/Nodal signalling maintains pluripotency by controlling Nanog expression." Development **136**(8): 1339-1349.

Vallier, L., T. Touboul, et al. (2009). "Early cell fate decisions of human embryonic stem cells and mouse epiblast stem cells are controlled by the same signalling pathways." PLoS One **4**(6): e6082.

van den Berg, D. L., T. Snoek, et al. (2010). "An Oct4-centered protein interaction network in embryonic stem cells." Cell Stem Cell **6**(4): 369-381.

Velkey, J. M. and K. S. O'Shea (2003). "Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells." Genesis **37**(1): 18-24.

Viswanathan, S. R., G. Q. Daley, et al. (2008). "Selective blockade of microRNA processing by Lin28." Science **320**(5872): 97-100.

Wada, S., Y. Kato, et al. (2011). "Translational suppression of atrophic regulators by microRNA-23a integrates resistance to skeletal muscle atrophy." J Biol Chem **286**(44): 38456-38465.

Waghray, A., N. Saiz, et al. (2015). "Tbx3 Controls Dppa3 Levels and Exit from Pluripotency toward Mesoderm." Stem Cell Reports **5**(1): 97-110.

Wang, Y., S. Baskerville, et al. (2008). "Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation." Nat Genet **40**(12): 1478-1483.

Wang, Y., R. Medvid, et al. (2007). "DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal." Nat Genet **39**(3): 380-385.

Wellner, U., J. Schubert, et al. (2009). "The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs." Nat Cell Biol **11**(12): 1487-1495.

Wilson, S. I., A. Rydstrom, et al. (2001). "The status of Wnt signalling regulates neural and epidermal fates in the chick embryo." Nature **411**(6835): 325-330.

Winter, J., S. Jung, et al. (2009). "Many roads to maturity: microRNA biogenesis pathways and their regulation." Nat Cell Biol **11**(3): 228-234.

Wray, J., T. Kalkan, et al. (2011). "Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation." Nat Cell Biol **13**(7): 838-845.

Xu, N., T. Papagiannakopoulos, et al. (2009). "MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells." Cell **137**(4): 647-658.

Yang, Q., L. Liao, et al. (2013). "BMK1 is involved in the regulation of p53 through disrupting the PML-MDM2 interaction." Oncogene **32**(26): 3156-3164.

Yeo, J. C. and H. H. Ng (2011). "Transcriptomic analysis of pluripotent stem cells: insights into health and disease." Genome Med **3**(10): 68.

Ying, Q. L., J. Nichols, et al. (2003). "BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3." Cell **115**(3): 281-292.

Ying, Q. L. and A. G. Smith (2003). "Defined conditions for neural commitment and differentiation." Methods Enzymol **365**: 327-341.

Yoda, M., D. Cifuentes, et al. (2013). "Poly(A)-specific ribonuclease mediates 3'-end trimming of Argonaute2-cleaved precursor microRNAs." Cell Rep **5**(3): 715-726.

Yu, J., M. A. Vodyanik, et al. (2007). "Induced pluripotent stem cell lines derived from human somatic cells." Science **318**(5858): 1917-1920.

Zhang, J., G. Liu, et al. (2014). "Dax1 and Nanog act in parallel to stabilize mouse embryonic stem cells and induced pluripotency." Nat Commun **5**: 5042.

Zhang, J., W. L. Tam, et al. (2006). "Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1." Nat Cell Biol **8**(10): 1114-1123.

Zhang, K., L. Li, et al. (2010). "Distinct functions of BMP4 during different stages of mouse ES cell neural commitment." Development **137**(13): 2095-2105.

Zhang, S., B. Cui, et al. (2014). "Ovarian cancer stem cells express ROR1, which can be targeted for anti-cancer-stem-cell therapy." Proc Natl Acad Sci U S A **111**(48): 17266-17271.

Zhang, X., L. Cui, et al. (2012). "Gastric juice microRNA-421 is a new biomarker for screening gastric cancer." Tumour Biol **33**(6): 2349-2355.

Zhang, Y., W. Gong, et al. (2012). "Downregulation of human farnesoid X receptor by miR-421 promotes proliferation and migration of hepatocellular carcinoma cells." Mol Cancer Res **10**(4): 516-522.

Zhao, R. and G. Q. Daley (2008). "From fibroblasts to iPS cells: induced pluripotency by defined factors." J Cell Biochem **105**(4): 949-955.

Zhong, X. Y., J. H. Yu, et al. (2012). "MicroRNA-421 functions as an oncogenic miRNA in biliary tract cancer through down-regulating farnesoid X receptor expression." Gene **493**(1): 44-51.

Zhou, L., X. Zhang, et al. (2013). "Association of a genetic variation in a miR-191 binding site in MDM4 with risk of esophageal squamous cell carcinoma." PLoS One **8**(5): e64331.

Zhou, S., B. Wang, et al. (2016). "miR-421 is a diagnostic and prognostic marker in patients with osteosarcoma." Tumour Biol.

Zhou, W. and S. Bao (2014). "PML-mediated signaling and its role in cancer stem cells." Oncogene **33**(12): 1475-1484.