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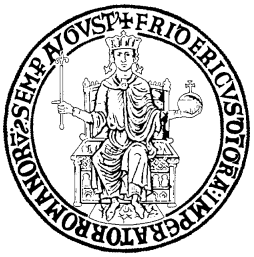

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PhD Program in Neuroscience

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The suppression of miR-23a, miR-24 and miR-27a induces apoptosis during ESC neural differentiation by modulating BMP4 signaling

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TABLE OF CONTENTS

ABSTRACT 3

INTRODUCTION 4

Embryonic Stem cells and neural differentiation 4

Signaling transduction pathways 8

Transcriptional factors regulating ESC pluripotency 11

miRNAs regulating ESC pluripotency and neural differentiation 13

RESULTS 17

miRNAs expression profile in ESCs upon BMP4 treatment 17

miR-23a, miR-24-2 and miR-27a are regulated by BMP4 19

BMP4 regulates miR-23a cluster at transcriptional level 21

miR-23a and miR-23b clusters are expressed in ESCs and during neural differentiation 22

Suppression of miR-23a and miR-23b clusters impairs ESC neural differentiation 24

Impaired differentiation of miRNA KD ESCs is not due to single miRNA or to altered proliferation rate of these cells 26

The suppression of miR-23a and miR-23b clusters increases apoptosis during ESC differentiation 27

Apoptosis starts after ESC to EpiSC transition during ESC differentiation 29

miR-23a and miR-23b clusters protect ESCs from apoptosis 31

Suppression of miR-23a and miR-23b clusters mimics the treatment with low doses of BMP4 32

Smad 5 is a direct target of miR-23a and miR-23b clusters 36

BMP4, miR-23a cluster and Smad5 generate a feedback loop regulating apoptosis during ESC neural differentiation 37

DISCUSSION 39

MATERIALS AND METHODS 42

REFERENCES 48

FIGURES INDEX

1. Pre-implantation mouse embryo development 4
2. Main neural differentiation protocols 6
3. ESC self-renewal and differentiation 7
4. BMP4 and LIF signalin pathways in ESC self-renewal and differentiation 10
5. Biogenesis and regulatory features of the microRNAs 16
6. BMP4 treatment activates BMP4 signaling pathway and miR-125a transcription 17
7. Primary transcripts of up-regulated miRNAs 19
8. BMP4 regulates miR-23a, miR-24-2 and miR-27a 20
9. Phospho-Smads bind genomic upstream region of miR-23a cluster 21
10. Expression levels of miR-23a and miR-23b clusters in ESC neural differentiation 23
11. miRNA suppression reduce Oct3/4 and Nanog levels during SFEB differentiation 25
12. Single miRNAs do not impair stemness and differentiation of ESCs and miRNA clusters do not alter ESC proliferation rate 26
13. Suppression of miR-23a and miR-23b clusters results in apoptosis during SFEB differentiation 28
14. miR-23a and miR-23b cluster suppression do not impair ESC-EpiSC transition 29
15. Apoptotic phenotype occurs after 2 days of SFEB differentiation 30
16. miR-23a and miR-23 clusters have anti-apoptotic function in ESCs 31
17. Different doses of BMP4 have different effects on ESC differentiation 32
18. BMP4 inhibition rescues apoptotic phenotype given by suppression of miR-23a and miR-23b clusters 34
19. Both block of BMP4 receptor and BMP4 effectors rescues apoptosis induced by miRNA suppression 35
20. miR-23a and miR-23b clusters target Smad5 36
21. High doses of BMP4 induce miR-23a transcription and Smad5 post-transcriptional regulation 37
22. BMP4 and miR-23a cluster generate a feedback loop regulating apoptosis in ESC differentiation 38
Table 1. miRNAs up- and down-regulated by BMP4 18
Table 2. Primers used for Real-Time PCR 46

ABSTRACT

Bone morphogenetic protein 4 (BMP4) plays an important role in mouse embryonic stem cells (ESCs) by sustaining pluripotency and blocking differentiation towards neural fate. In this study, we have analyzed the miRNAs regulated by BMP4 in ESCs. We have found that BMP4 signaling directly regulates miR-23a, miR-24-2 and miR-27a through the binding of phospo-Smads on the promoter of the gene encoding all these miRNAs. The suppression of miR-23a, miR-24-2 and miR-27a, together with the suppression of miRNAs of the same families, miR-23b, miR-24-1 and miR-27b, does not impair ESC stemness maintenance and epiblast stem cells (EpiSCs) derivation from ESCs. However, this suppression affects ESC differentiation, thus resulting in the increase of the number of cells undergoing apoptosis soon after the transition from ESCs to EpiSCs. We have demonstrated that the block of BMP4 signaling completely rescues the apoptosis induced by the suppression of miRNAs. Considering that it was already known that BMP4 induces apoptosis during ESC differentiation, our observation suggests that the apoptotic phenotype provoked by miRNA suppression is due to an enhancement of BMP4 signaling. We also demonstrated that miR-23a and miR-23b clusters target Smad5, a downstream effector of BMP4 pathway; this phenomenon explains how the suppression of miRNA clusters enhances BMP4 signaling. In conclusion, the results unveil the existence of a feedback loop, involving Smad5 and miR-23a clusters, that regulates the apoptosis induced by BMP4 during ESC neural differentiation.

INTRODUCTION

Embryonic Stem cells and neural differentiation

Mouse Embryonic Stem cells (ESCs) were firstly established in 1981 from the Inner Cell Mass (ICM) of the pre-implantation embryo at stage of early blastocyst. Early blastocyst is composed of a cluster of cells, the inner cell mass, placed to one side of the vesicle and of an epithelial outer layer, the trophectoderm, enclosing a fluid filled space (the blastocyst cavity). The ICM give rise to all cells of the embryo, while trophectoderm cells give rise to extraembryonic tissues, such as placenta, critical for supporting embryonic development.
During the implantation of the blastocyst, the ICM gives rise to a new epithelial layer, the Primitive Endoderm. Later this epithelium also lines the luminal surface of the mural trophectoderm away from the ICM and contributes to the visceral and parietal endoderm. In addition to the Primitive endoderm, the ICM gives rise to the epiblast in the post-implantation embryo, from which Primitive ectoderm derives (Rossant et al., 2003). Primitive ectoderm in turn give rise to all three embryonic germ layers (ectoderm, mesoderm and definitive endoderm) and primordial germ cells (Pfister et al., 2007)

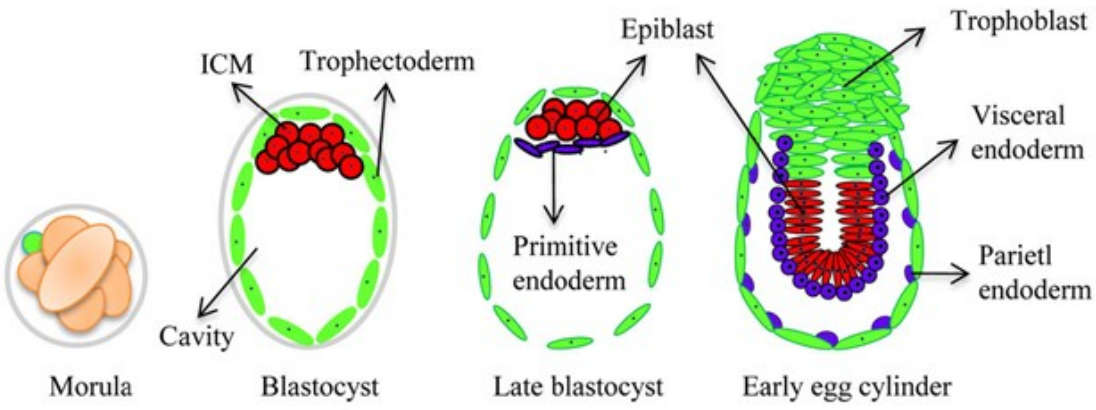

Figure 1. Pre-implantation mouse embryo development.
Morula is formed at 2.5 days from egg fertilization. After cleavage division, compaction and cavitation of morula, blastocyst is formed at 3.5 days. It contains two type of cells: ICM cells that give rise to all cells of the embryo, and trophectoderm cells which give rise to trophoblast. At late blastocyst stage, the ICM is surrounded by a cell type known as primitive endoderm, that gives rise to the yolk sac to support embryonic development. In this stage, ICM cells are at Epiblast state. At early egg cylinder state, primitive endoderm differentiate toward visceral and parietal endoderm while Epiblast cells give rise to primitive ectoderm that can differentiate into all three embryonic germ layers. Adapted from: Huang et al., 2015.

All these steps of embryogenesis can be recapitulated by ESCs. In vitro ESCs proliferate indefinitely without differentiation, a property defined ‘‘self-renewal’’, and at the same time they retain the potential of generating cells of the three germ layers, termed ‘‘pluripotency’’. Traditionally, ESC pluripotency has often been defined as the ability to generate all cell types of an embryo apart from the trophectoderm. This is because an earlier analysis of chimeric mouse embryos, produced by injection ESCs into blastocysts, had shown that these cells are excluded from the trophectoderm lineage (Beddington et al., 1989). However, it has subsequently been found that the ICM and ESCs do still possess the ability to differentiate into the trophectoderm lineage (Pierce et al., 1988; Niwa et al., 2005). Therefore, ESC pluripotency is now defined as the ability to generate all cell types, including the trophectoderm, without the self-organizing ability to generate a whole organism (Niwa et al., 2007).

These remarkable characteristics, self-renewal and pluripotency, made ESCs a powerful source of differentiated cells that could be used in cell replacement therapy. However, the use of ESC in cell replacement therapy remains a main goal in the field. So advances in our understanding of ESC differentiation are necessary to provide new insights for the generation of clinically relevant cell population for cell therapy.

In order to obtain pure cell population to be used in cell therapy, in recent years many protocols have been developed to induce the differentiation of ESCs into cells of the three germ layers, in particular great efforts were made to obtain neural precursors cells (NPCs) from ESCs, with the idea to use these cells in the treatment of neurodegenerative diseases.

The removal of LIF from the conventional serum-free culture medium, with N2 and B27 supplements, is the simplest strategy to obtain neural differentiation of ESCs. Moreover, the addition of fibroblast growth factor (FGF) in this differentiation protocol allows the emergence of cells in the rosette conformations typical of neuroepithelial cells (Ying et al., 2003b). Another method is based on the formation of Embryoid bodies (EB): a defined numbers of ESCs are cultured in nonadhesive condition in absence of LIF and Retinoic acid is added in the last 4 days of differentiation (Bibel et al., 2004). The presence of retinoic acid increases the percentage of neural cells in the EB, but evokes caudalization, so inhibits the production of the cells of the rostral central nervous system (Wichterle et al., 2002; Mizuseki et al., 2003). To avoid the use of retinoic acid and growth factors, Watanabe and collaborators have developed a new differentiation method based on the formation of Serum Free Embryoid bodies (SFEBs). They cultured ESCs in suspension culture conditions without LIF and serum, but in presence of a medium containing Knock-

out Serum Replacement (KSR) that supports the growth of ESC aggregate. In these conditions they obtained a large number of neural precursor and, unlike embryoid bodies cultured in a serum-containing medium, they generated neural precursors without the concomitant induction of mesodermal and endodermal precursors (Watanabe et al., 2005).

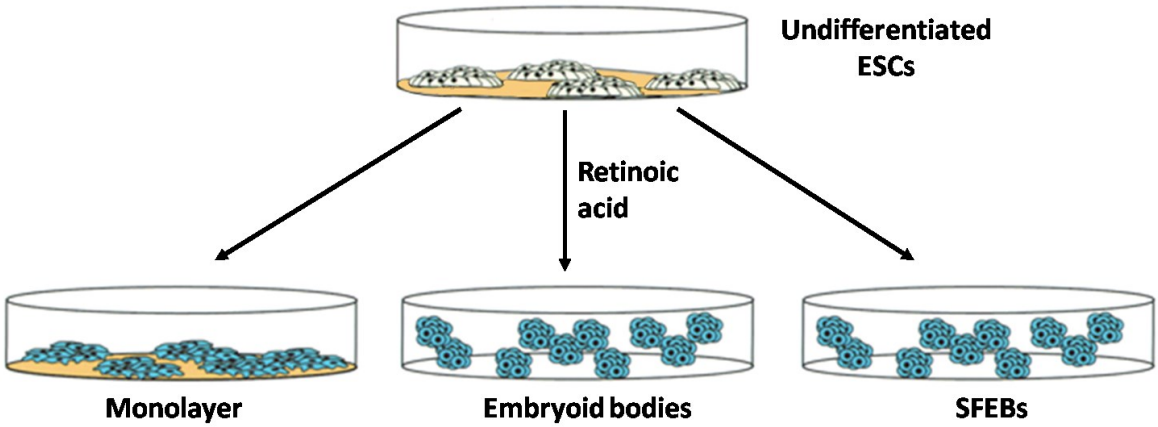

Figure 2. Main neural differentiation protocols

ESCs can be induced to differentiate into neural precursors by using different protocols. The first method is based on the culture of ESCs on gelatin coated dishes in absence of LIF and in the presence of serum-free culture medium with N2 and B27 supplements. The second method consists in plating ESCs at low density in nonadhesive condition and in absence of LIF. In this way, Embryoid bodies are formed and, after 4 days, the addition of Retinoic acid improves neural differentiation. The third method is based on the formation of Serum Free Embryoid bodies (SFEBs). ESCs are plated at low density in nonadhesive condition in serum-free medium without LIF and in presence of KSR.

Using SFEB differentiation method, Zhang and co-workers have demonstrated that neural differentiation occurs in two steps: by the transition from ESCs to Epiblast Stem cells (EpiSCs) and then from EpiSCs to neuroectoderm precursors. They have demonstrated that SFEBs at 2 days of differentiation are comparable to cells of the egg cylinder stage epiblast, therefore SFEB differentiation recapitulates the differentiation steps taking place in vivo from ICM to neuroectoderm formation (Zhang et al., 2010).

EpiSCs can be isolated from the egg cylinder epiblast, moreover, in particular culture conditions, EpiSCs can be also derived from ESCs and maintained in culture by adding activin and Fgf2. Cells obtained in this way have the same characteristics of EpiSCs derived from mouse embryos and express the canonical EpiSC gene Fgf5 (Brons et al.,

2007; Tesar et al., 2007). EpiSCs as well as ESCs can differentiate into precursors of the three germ layers depending on the activity of specific signaling pathways.

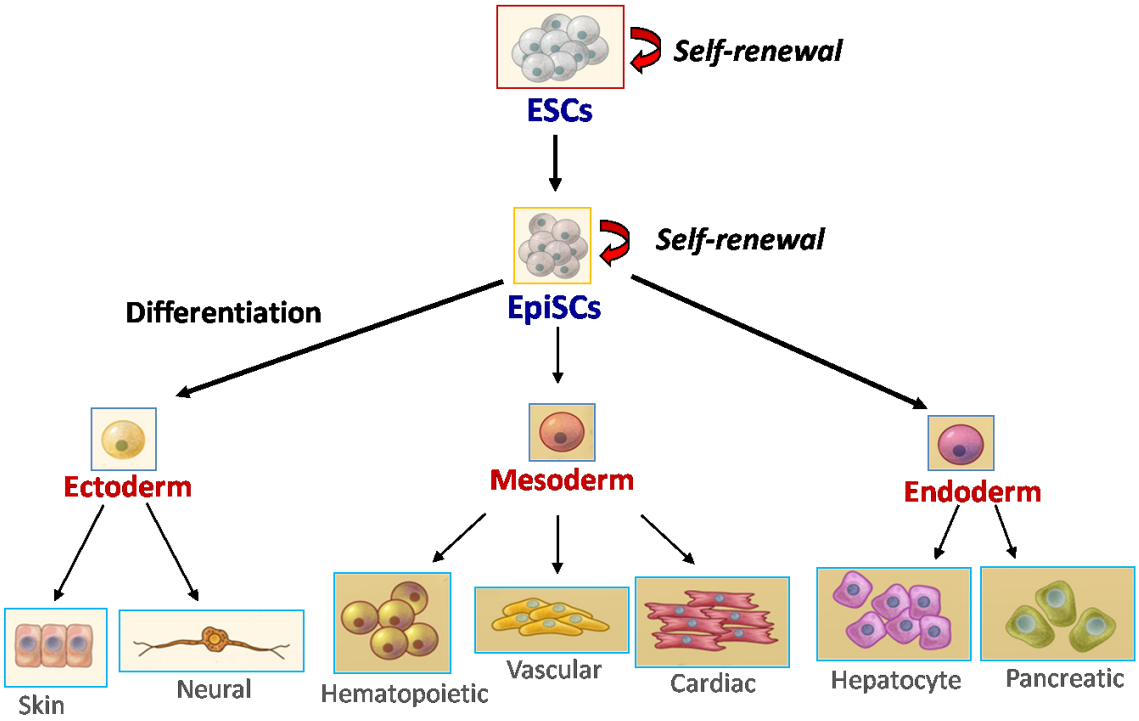

Figure 3. ESC self-renewal and differentiation

Thanks to the ability to self-renew, ESCs can be maintained in vitro indefinitely, whereas thanks to pluripotency these cells can differentiate into cells of all the three germ layers (ectoderm, mesoderm and endoderm), passing through the Epiblast state. Upon proper stimuli, ESCs can differentiate into skin and neural precursors from ectoderm germ layer, or into mesoderm cells, like hematopoietic, vascular and cardiac cells, or into hepatocyte and pancreatic cells from endoderm germ layer.

Adapted from: Murry et al., 2008

Signaling transduction pathways

ESC self-renewal and differentiation are regulated by specific extracellular signals. A key regulator of ESC self-renewal is the leukaemia inhibitory factor (LIF) (Smith et al., 1988). LIF is a member of the IL-6 cytokine family, it binds the LIF receptor (LIFR) which works as a heterodimer together with gp130 receptor and activates Janus-associated tyrosine kinase (JAK) and signal transducer and activation of transcription-3 (STAT3). The ability of LIF to sustain the ESC self-renewal depends on the activation of STAT3 (Niwa et al., 1998), whose activation is sufficient to prevent ESCs differentiation in the presence of serum (Matsuda et al., 1999). Moreover, LIF stimulates the activation of the mitogen-activated protein kinase (MAPK), which induce pro-differentiative signals (Burdon et al., 1999). Therefore LIF sustains self-renewal and prevents ESC differentiation balancing STAT3 and MAPK activation.

However, LIF is necessary but not sufficient to maintain ESCs in the undifferentiated state in serum-free medium, indicating that there are many factors in serum required to sustain self-renewal and prevent differentiation. One of the most important of these serum factors is Bone Morphogenetic Protein 4 (BMP4). It belongs to the Transforming growth factor (TGF-β) superfamily that includes also TGF-β, Activin, Nodal and other related proteins. These proteins play an important role in the establishment of body plan and tissue differentiation through their effect on cell proliferation, differentiation and migration (Derynck et al., 2003).

TGF-β signaling pathways are induced by heterodimerization of type I and type II receptors upon ligand binding. The phosphorylation of type I receptor by type II receptor allows the recruitment and the phosphorylation of receptor-regulated SMAD proteins (R-SMADs). SMAD 1,5 and 8 are activated by the binding of BMP4 to type I receptors Alk 1,2,3 and 6, whereas SMAD 2,3 are activated by the binding of Nodal/Activin to type I receptors Alk 4,5 and 7. Activated R-SMADs in turn form complexes with the common mediator SMAD4. SMAD complexes accumulate in the nucleus, where they are directly involved in regulating the transcription of target genes (Schmierer et al., 2007). The main BMP4 target genes involved in the maintenance of ESC self renewal are the helix-loop-helix Ids (inhibitors of differentiation). Ids functionally antagonize neurogenic bHLH transcription factors and block the neural differentiation (Gerrard et al., 2005). Exogenous expression of Ids mimic the effect of BMP4 in mouse ESCs (Ying et al., 2003a). The induction of Id genes by BMP4 has been reported also in differentiating ESC cultures (Hollnagel et al., 1999) and is dependent by BMP pathway but not by LIF. An interaction

between BMP4 and LIF has been found by Ying and collaborators (Ying et al., 2003a). Indeed, they have demonstrated that activated STAT3 and Smad1 may colocalize, supporting the hypothesis that BMP4/Smad1 sustains LIF/STAT3 function in mouse ES cells (Ying et al., 2003a). Interestingly, BMP4 sustains self-renewal also through inhibition of ERK by up-regulating Dual-specificity phosphatase 9 (DUSP9) gene that in turn inhibits phosphorlation of ERK (Qi et al., 2004; Li et al., 2012).

As already mentioned, BMP4 signaling has crucial functions in maintaining pluripotency of mouse ESCs but it has also a crucial role in the inhibition of neural differentiation (Kawasaki et al., 2000; Tropepe et al., 2001; Ying et al., 2003a). Very interestingly, Zhang and collaborators suggested that there is a BMP4-sensitive window during ESC neural differentiation (Zhang et al., 2010). They observed that BMP4 suppresses neural differentiation, but once neural commitment is initiated, BMP4 is no longer able to inhibit it. Indeed, in the first stage of ESC differentiation, BMP4 inhibits EpiSC derivation, whereas in the later stages, when the commitment of EpiSCs into precursors of the three germ layers occurs, BMP4 suppresses neural differentiation ad promotes mesoderm and endoderm commitment (Zhang et al., 2010). In addition, the effects of BMP4 on ESC differentiation are dose-dependent; high doses of BMP4 block differentiation and maintain pluripotency, while low doses of BMP4 induce apoptosis of neural precursor cells during epidermal differentiation of ESCs (Gambaro et al., 2006), through the activation of Caspase-3

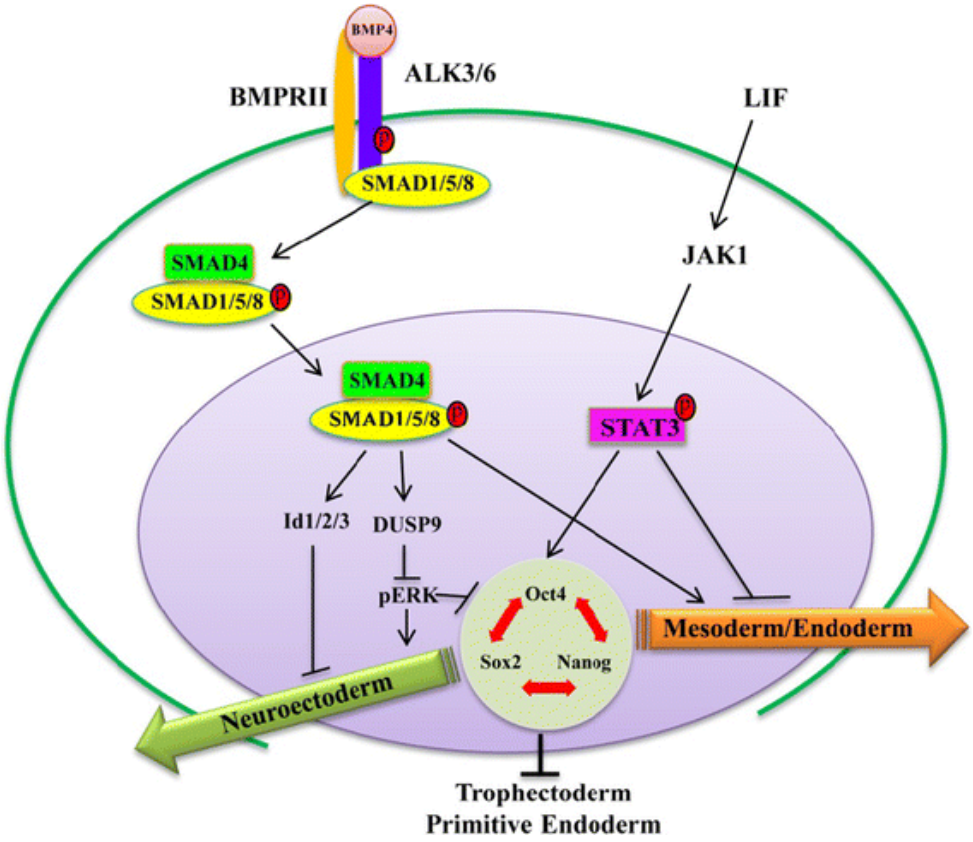

Figure 4. BMP4 and LIF signalin pathways in ESC self-renewal and differentiation

BMP4 ligand binds Alk3/6 receptor that heterodimerizes with type II receptor BMPRII, resulting in the activation of Smad1,5,8 proteins. These proteins form a complex with Smad4 that traslocates into the nucleus. Smad complex induces the transcription of Id1/2/3 and Dusp9 genes which inhibit neuroectoderm differentiation. On the other hand, LIF activates JAK1/STAT3, by sustaining self-renewal and inhibiting mesoderm and endoderm differentaiton of ESCs.

Taken from Huang et al., 2015.

Transcriptional factors regulating ESC pluripotency

ESCs require extrinsic growth factors for the maintenance of pluripotency in culture. These extrinsic stimuli converge in a network of key transcription factors that controls pluripotency. This network includes the homeodomain transcription factor Oct3/4 (Niwa et al., 2000), the high mobility group (HMG)–box transcription factor Sox2 (Avilion et al., 2003) and the variant homeodomain transcription factor Nanog (Chambers et al., 2003).

Oct3/4 is a POU domain-containing transcription factor that is expressed in pluripotent cells during mouse embryogenesis, both ESCs and EpiSCs, even if its expression is weaker in EpiSCs. ESCs during differentiation lose the expression of Oct3/4, indeed this protein is tightly related to pluripotency. The loss of Oct3/4 induces inappropriate trophectoderm differentiation because it interacts with Cdx2 (a trigger for trophectoderm differentiation) to form a repressor complex. This complex interferes with the autoregulation of these two factors, giving rise to a reciprocal inhibition system that establishes their mutually exclusive expression (Niwa et al., 2005). ESC-specific enhancers containing binding sites for Oct3/4 and Sox2 have been identified in genes such as Nanog (Boyer et al., 2005; Kuroda et al., 2005; Rodda et al., 2005), Rex1 (Shi et al., 2006) and in Oct3/4 and Sox2 themselves, suggesting that these two transcription factors are regulated by a positive-feedback loop (Chew et al., 2005).

Another transcription factor having an essential role in maintaining the pluripotent state of the ICM and ESCs is Nanog. It is a homeobox-containing transcription factor expressed in pluripotent cells and absent in differentiated cells. The mechanism through which Nanog regulates stem cell pluripotency is unclear. Based on the differences in gene expression between wild-type and Nanog null cells, it has been proposed that Nanog regulates pluripotency mainly as a transcription repressor for downstream genes that are important for cell differentiation such as Gata4 and Gata6 (Chambers et al., 2003; Mitsui et al., 2003). However, Nanog can also activate the genes necessary for self-renewal such as Rex1 and Oct3/4 (Pan et al., 2006). Nanog disruption in ESCs not only results in the differentiation into primitive endoderm (Liu et al., 2007), but also blocks neuronal differentiation induced by the removal of LIF and BMPs from serum-free culture (Ying et al., 2003b). In addition, Nanog can also block mesoderm specification by repressing brachyury, which encodes the mesoderm-specific T-box transcription factor T. Thus, Nanog can block primitive endodermal differentiation, neuronal differentiation and mesodermal differentiation under different culture conditions.

These results suggest the crucial role of transcriptional factors in ESC pluripotency and differentiation. However in recent years also post-transcriptional gene regulation by microRNAs (miRNAs) is emerging as an essential regulator of ESC development (Cheng et al., 2005).

miRNAs regulating ESC pluripotency and neural differentiation

miRNAs are fine-tuning regulators of gene expression and are involved in a wide spectrum of biological processes including cell proliferation (Delaloy et al., 2010; Niu et al., 2013), cell fate determination and differentiation (Bartel et al., 2004; Chen et al., 2004; Makeyev et al., 2007; Li et al., 2010; Akerblom et al., 2013).

microRNAs are 20–25 nucleotide non-coding RNAs that bind to the 3' untranslated region (UTR) of target mRNAs resulting in mRNA degradation or block of translation (Rana 2007). miRNAs are transcribed by RNA polymerases II and III (Bartel et al., 2004; Rodriguez et al., 2004) as precursor transcripts, called primary microRNAs (pri-miRNAs). Some miRNAs are organized in polycystronic clusters and they are transcribed as a unique pri-miRNA (miRNA cluster). These miRNAs often belong to different miRNA family, so they do not target the same mRNA. Pri-miRNAs are processed in the nucleus into an intermediate form (pre-miRNAs) by Drosha and DiGeorge syndrome critical region gene 8 (DGCR8; also known as Pasha) proteins. Pre-miRNAs can also be generated by a non-canonical mirtron pathway. mirtrons are miRNAs located in the introns of a protein coding gene (Okamura et al., 2007; Westholm et al., 2011). When the host gene is transcribed, spliceosome and debranching enzyme produce a pre-miRNA that is ready to be processed. Pre-miRNAs obtained from canonical and non-canonical pathways are translocated by the exportin5 into the cytoplasm, in which they are further processed by Dicer, an RNase III-like enzyme, into mature miRNAs (Gangaraju and Lin 2009). miRNAs are recognized by RNA-induced silencing complex (RISC), containing an Argonaute family member, that drives repression of mRNA translation and stability.

Thanks to their function in translational attenuation, miRNAs are considered fine regulators of gene expression. Their crucial function in ESCs has been demonstrated by analyzing the phenotypes of DGCR8 and Dicer mutants. In particular, Dicer knock-out ESCs are not able to properly differentiate and exhibit slow proliferation rate (Kanellopoulou et al. 2005), whereas DGCR8 null cells show affected differentiation, proliferation and cell cycle progressing (Wang et al., 2007). ESC specific miRNAs have been identified comparing the expression of miRNAs in ESCs and in differentiated cells. With this approach the miR-290 family has been found to be expressed specifically in undifferentated cells (Houbaviy et al., 2003). This miRNA family is involved in cell cycle regulation and prevents the epigenetic silencing of pluripotent factors Oct3/4, Sox2, Nanog and Myc targeting repressor of *de novo* DNA methyltransferase (DNMT) (Sinkkonen et

al., 2008). Oct3/4, Sox2 and Nanog in turn recognize the predicted promoter region of miR-290 cluster, regulating the expression of these miRNAs in ESCs (Marson et al., 2008). These data indicate that a tight control of miRNA expression is required to maintain stemness.

Interestingly, there are also miRNAs regulating ESC differentiation. Of note, Tarantino and co-workers have found 138 miRNAs up-regulated during ESC differentiation. Among these miRNAs, they found that miR-34a, miR-100, and miR-137, are required for the proper differentiation of ESCs by regulating Sirt1, Smarca5, and Jarid1b genes which are involved in sustaining the undifferentiated state (Tarantino et al., 2010). In addition, several miRNAs have been characterized for their function in the first stages of neurogenesis. In particular, miR-134 is an inducer of ESC differentiation (Gaughwin et al., 2011). It is not expressed in undifferentiated cells but its expression increases in ESCs treated with retinoic acid. It induces differentiation into ectodermal lineages targeting Sox2 and Nanog and regulating indirectly Oct3/4 in combination with miR-296 and miR-470 (Tay et al., 2008; Niu et al., 2013). Moreover, depending on the stage of neural differentiation, miR-134 has also other effects and targets. Indeed it enhances neural precursors proliferation and counteracts their apoptosis and differentiation (Gaughwin et al., 2011). Another crucial miRNA involved in ESC differentiation, self-renewal and pluripotency is let-7 (Melton et al., 2010). Let-7a miRNA is highly expressed in somatic cells, while pri-let-7 immature transcripts are present at high levels in ESCs. In these cells the processing of immature pri-let-7 into mature let-7 is inhibited by binding of Lin28 that blocks Drosha and Dicer action (Newman et al., 2008; Heo et al., 2008). Let-7a in turn induce neural commitment of ESCs by targeting Lin-28 (Rybak et al., 2008). In addition, the inhibition of another miRNA belonging to the let-7 family, let-7b, induces proliferation of neural stem cells (NSCs) and blocks their neuronal differentiation (Zhao et al., 2010). Also some brain-specific miRNAs, such as miR-124a and miR-9, are involved in the neural differentiation of ESCs (Krichevsky et al., 2006). Krichevsky and collaborators found that miR-124a and miR-9 prevent the activation of STAT3, a protein that inhibits neuronal differentiation, thus inducing neural lineage commitment (Krichevsky et al., 2006) . Moreover Saunders and co-worker demonstrated that miR-9, together with miR-181a and b, miR-204, miR-199b and miR-135a, prevent SIRT1 expression during neuronal differentiation (Saunders et al., 2010). We have recently found that another miRNA is involved in a feedback loop regulating ESC differentiation: miR-125a. In particular, we uncovered that BMP4 directly up-regulates miR-125a, that, in turn, targets a co-receptor of

BMP4 receptor, Dies1. Its suppression results in a reduced sensibility of ESCs to BMP4 and in an unbalance of BMP4 and Nodal/Activin pathways in favor of the latter. The resulted up-regulation of Nodal/Activin pathway blocks ESC differentiation maintaining cells in the epiblast state, demonstrating that this feedback loop is required for the proper differentiation of ESCs (Parisi et al., 2012). Thus, our findings suggest that this miR-125-dependent loop sets the sensitivity of ESCs to BMP4. In addition, we have found that miR-125b, another member of miR-125 family, also targets Dies1, down-regulating BMP4 signal transduction. Interestingly, we have found that unlike miR-125a, miR-125b is not directly regulated by TGFβ signals, suggesting that miRNAs having the same function could be regulated in independent manners to fine tuning the response of the cells to extracellular cytokines (Battista et al., 2013). In conclusion, these findings suggest that the study of miRNAs regulated by BMP4 is crucial to better understand the molecular mechanisms involved in ESC differentiation.

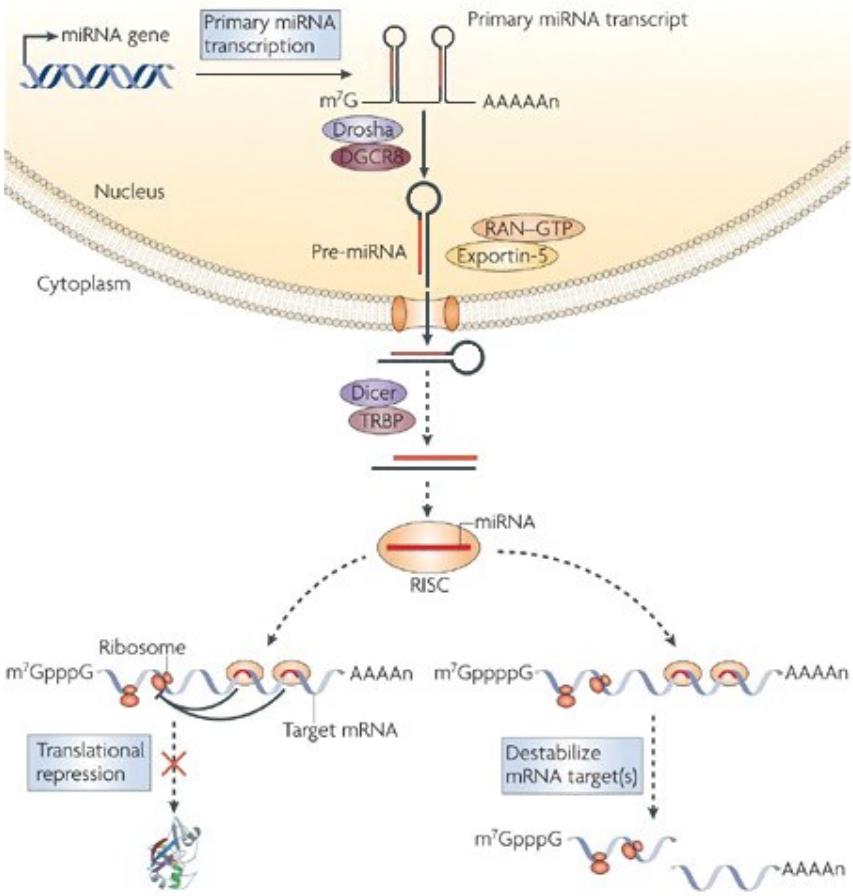

Figure 5. Biogenesis and regulatory features of the microRNAs

microRNA genes are transcribed as primary miRNA transcripts. These are processed into pre-microRNAs in the nucleus by a Microprocessor complex, which contains the RNase III enzyme Drosha and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8). Pre-microRNAs are then transported by exportin 5, which is a karyopherin, and RanGTP into the cytoplasm, where they are further processed by the RNAse III enzyme Dicer and by the double-stranded RNA-binding protein TRBP (TAR (HIV) RNA binding protein 2). This results in double-stranded 20–25 nucleotide (nt) intermediates with 2 nt overhangs on the 3' end. One of the RNA strands is then loaded by Dicer into an RNA-induced silencing complex (RISC), which contains an Argonaute-subfamily member, that then targets the 3' untranslated region of the target mRNAs. An imperfect match between the microRNA and the mRNA results in translational repression, whereas a perfect match between the microRNA and the mRNA results in degradation of mRNA targets. Taken from Lodish et al., 2008.

RESULTS

miRNAs expression profile in ESCs upon BMP4 treatment

In order to identify the miRNAs regulated by BMP4 and involved in neural differentiation of ESCs, we performed a miRNA expression profile in ESCs treated with BMP4 in a chemical defined medium (KSR) without serum and in the presence of LIF. Of note, the absence of serum avoids the influence of growth factors and switchs off TGFβ signaling. To verify the activation of BMP4 signaling upon BMP4 treatment, we measured the levels of Smad phosphorylation and Smad target genes, Id1 and Id3, after 1h of BMP4 treatment. As shown in figure 6, BMP4 treatment induces a strong phosphorylation of Smad1/5/8 and significantly enhances the transcription of Id1 and Id3 genes (Figure 6a). Moreover, we measured the accumulation of miR-125a, a miRNA directly regulated by BMP4 (Parisi et al., 2012), after 24h of BMP4 treatment, and we found increased levels of mature miR-125a, as expected (Figure 6b).

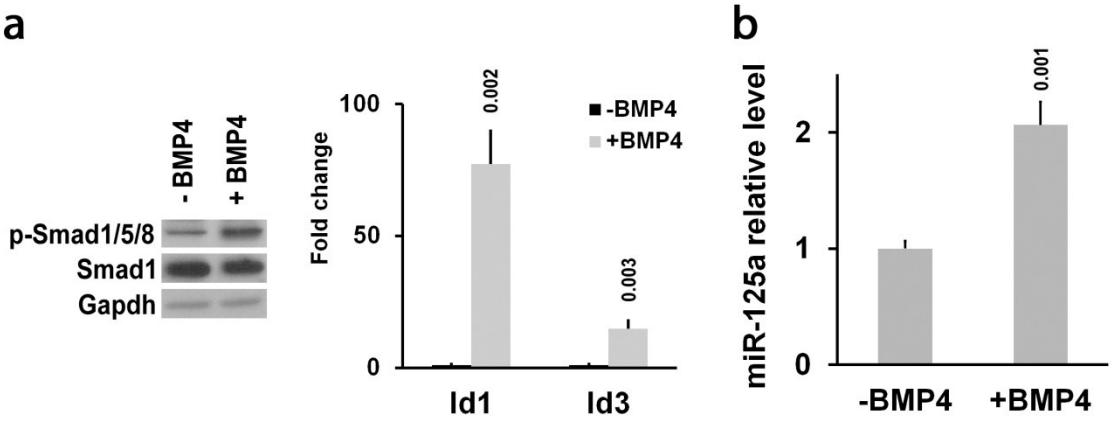

Figure 6. BMP4 treatment activates BMP4 signaling pathway and miR-125a transcription.
a) ESCs were cultured overnight in serum free medium with KSR and LIF and treated with 20ng/ml of BMP4 for 1 hour. Smads protein levels were analyzed by western blotting (left panel), Ids expression levels were analyzed by qPCR (right panel). **b)** ESCs were cultured overnight with KSR and LIF and treated with 20ng/ml of BMP4 for 24 hours. Accumulation of mature miRNA was analyzed by qPCR. Data are reported as fold change relative to untreated (-BMP4) ESCs (fold change = 1). Numbers over the bars indicate the P-Value.

To perform miRNA expression profile we used two miRNA qPCR panels containing 384 pairs of dried down LNA primer sets for the detection of each miRNA annotated in the Sanger miRBase version 17 database. This procedure provided exceptional sensitivity enabling accurate quantification of very low microRNA levels and a single-base discrimination among very similar microRNAs. We measured the levels of miRNAs in ESCs treated or not with BMP4 for 24h, when processing of all pri-miRNAs is completed

and we selected miRNAs on the basis of Standard Error (S.E.) and fold change. The results of miRNA expression profile allowed us to cluster microRNAs in two groups: miRNAs up-regulated and down-regulated by BMP4 (Table 1). As expected, we found miR-125a among up-regulated miRNAs, in agreement with our previous observation (Parisi et al., 2012).

Up-regulated miRNAs	Down-regulated miRNAs
mmu-miR-125a	mmu-miR-10a
mmu-miR-142	mmu-miR-135a
mmu-miR-187	mmu-miR-139
mmu-miR-193b	mmu-miR-141
mmu-miR-196a	mmu-miR-153
mmu-miR-199a	mmu-miR-181b
mmu-miR-211	mmu-miR-1937b
mmu-miR-23a	mmu-miR-20b
mmu-miR-24	mmu-miR-217
mmu-miR-27a	mmu-miR-219
mmu-miR-27b	mmu-miR-30a
mmu-miR-29b	mmu-miR-363
mmu-miR-302d	mmu-miR-369
mmu-miR-324	mmu-miR-375
mmu-miR-339	mmu-miR-431
mmu-miR-362	mmu-miR-449a
mmu-miR-379	mmu-miR-449c
mmu-miR-503	mmu-miR-466d
mmu-miR-582	mmu-miR-496
mmu-miR-9	mmu-miR-673
	mmu-miR-674
	mmu-miR-677
	mmu-miR-770
	mmu-miR-7a
	mmu-miR-7b
	mmu-miR-99a

Table 1. miRNAs up- and down-regulated by BMP4
ESCs were treated with BMP4 for 24h and then all mature miRNAs annotated in the Sanger miRBase version 17 database were measured using miRNA qPCR panels. Data are presented as fold change over miRNA levels in control untreated samples. miRNAs were classified as up-regulated and down-regulated upon BMP4 treatment using as cut-off >1.25- and <0.75- fold change, respectively.

miR-23a, miR-24-2 and miR-27a are regulated by BMP4

In order to understand whether up- and down-regulated miRNAs are directly regulated by BMP4, we measured the levels of their primary transcripts (pri-miRNAs) upon 1h of BMP4 treatment. Indeed, after 1h of BMP4 treatment, direct targets of BMP4 are already transcribed. Using real time PCR procedure, we found increased levels of miR-23a, miR-24-2, miR-27a as well as miR-125a transcripts (Figure 7).

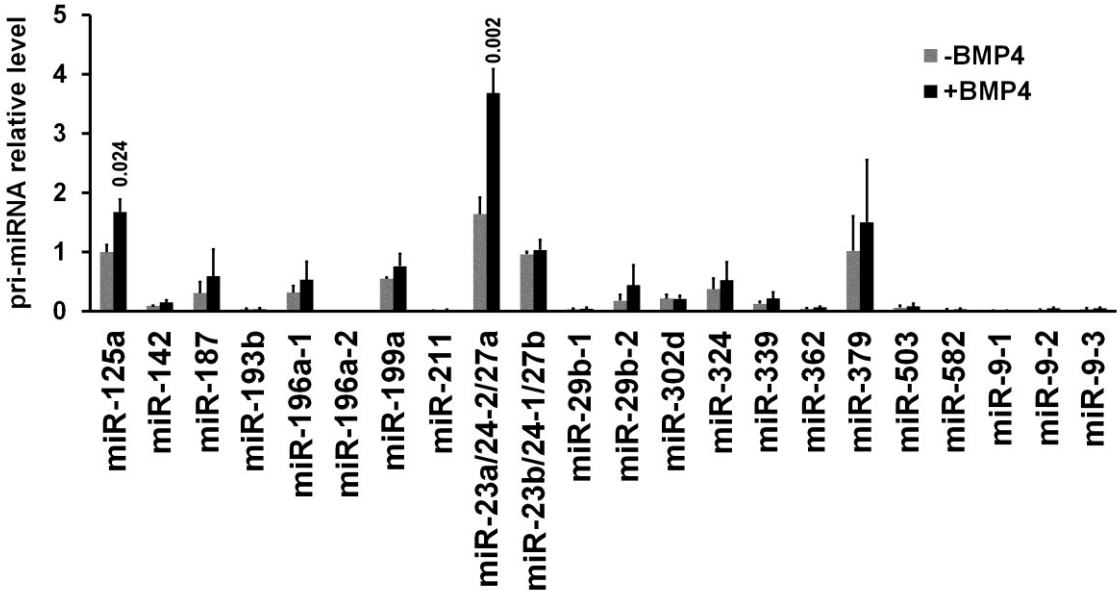

Figure 7. Primary transcripts of up-regulated miRNAs.
ESCs were treated with BMP4 for 1h and pri-miRNA transcript levels were measured by qPCR. Data are shown as fold change over the level of miR-125a in untreated (-BMP4) ESCs (fold change = 1). Numbers over the bars indicate the P-Value.

Of note, miR-23a, miR-24-2 and miR-27a derive from a unique miRNA cluster gene, miR-23a cluster, located on chromosome 8. These miRNAs belong to different families, therefore they do not share the seed sequence and, as a consequence, do not target the same mRNAs. To confirm that the induction of these three miRNAs by BMP4 treatment results in the accumulation of the mature form, we measured levels of miRNAs by Q-PCR analysis with specific LNA probes. As shown in Figure 8a, we found a significant increase of mature miR-23a, miR-24-2 and miR-27a upon 24h of BMP4 treatment. Moreover, to better characterize the expression regulation of miR-23a cluster we inhibited BMP4 signaling. Indeed, if BMP4 directly regulates miR-23a cluster, the block of BMP4 signaling should reduce the expression of these miRNAs. Thus, we treated ESCs with the BMP4 antagonist Dorsomorphin to block BMP4 signaling. Alternatively, we silenced BMP4 effectors Smad1 and Smad5 by RNA interference. In both cases we found a marked decrease of the primary transcript of miR-23a cluster (Figure 8b-d).

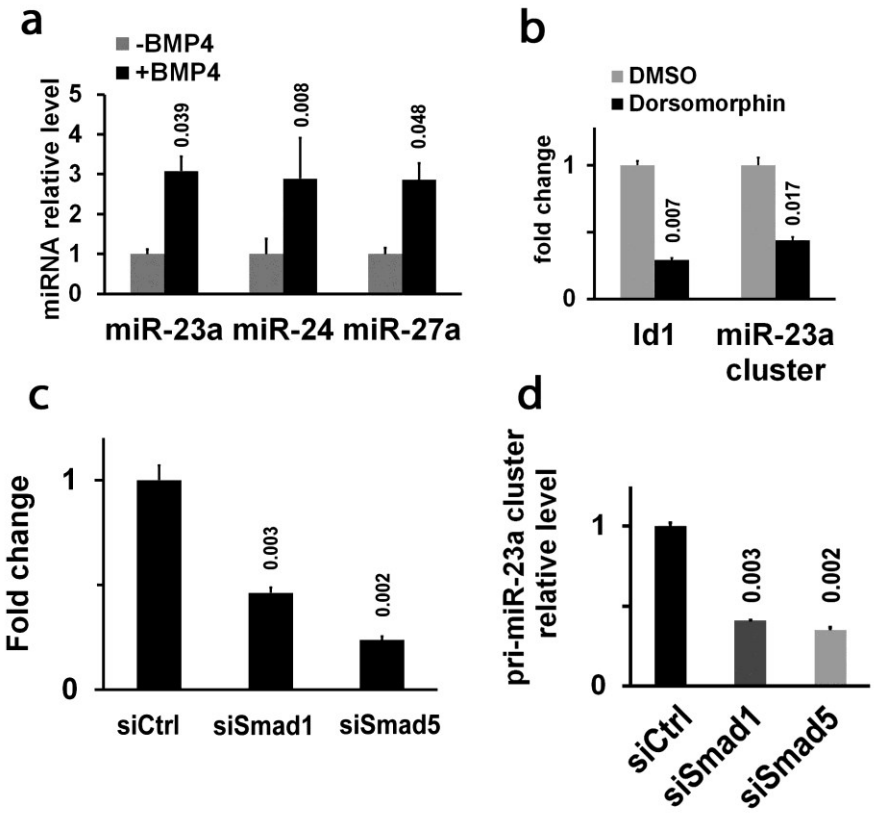

Figure 8. BMP4 regulates miR-23a, miR-24-2 and miR-27a.
a) Undifferentiated cells were treated with BMP4 and after 24h miRNA levels were measured by qPCR. **b)** ESCs were treated with Dorsomorphin for 1h and Id1 mRNA levels and pri-miRNA of miR-23a cluster were measured by qPCR . **c)** Silencing of Smad1 and Smad5 was evaluated after 24h of siRNA transfection by qPCR. **d)** ESCs were transfected with the indicated siRNAs and after 24h pri-miR-23a cluster levels were evaluated by qPCR. Data are presented as fold change relative to control (-BMP4, DMSO, siCtrl) ESCs (fold change = 1). Numbers over the bars indicate the P-Value.

BMP4 regulates miR-23a cluster at transcriptional level

To understand whether miR-23a cluster is regulated by BMP4 at transcriptional level, we searched for Smad binding sites on the promoter of miR-23a cluster gene by using Chromatin Immunoprecipitation (ChIP). In particular, we treated ESCs with BMP4 for 1h and then we immunoprecipitated chromatin with phospho-Smad1/5/8 antibody and we found activated Smads bound to Id1 promoter, as expected (Ying et al., 2003a) (Figure 9a). At the same time, we searched for Smad binding sites in the region of 10 kb upstream of the transcriptional start site of miR-23a cluster gene. We found significant enrichments in the chromatin immunoprecipitated with anti-Smads antibody in five regions upstream of miR-23a cluster gene, demonstrating that miR-23a cluster is regulated at transcriptional level by BMP4 (Figure 9b). In agreement with these results, sequence analysis of upstream region of miR-23a cluster revealed many consensus binding sites for Smads (Fei et al., 2010).

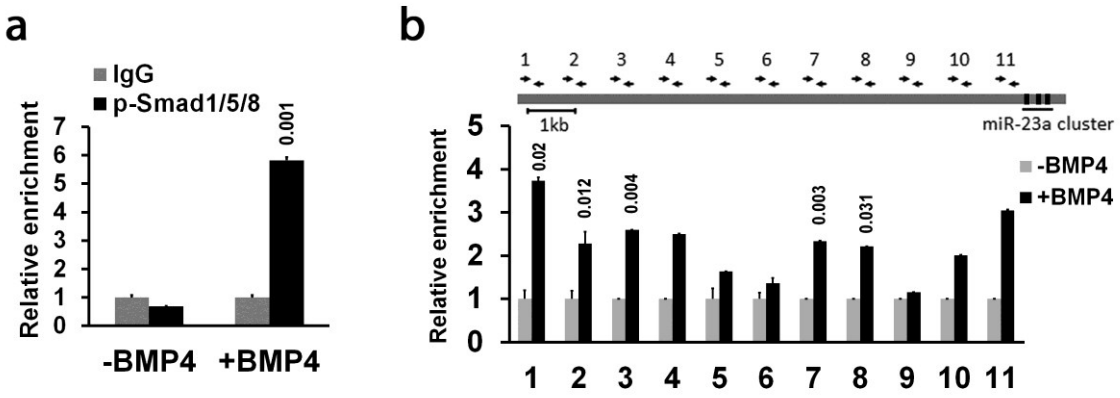

Figure 9. Phospho-Smads bind genomic upstream region of miR-23a cluster.
a) ESCs were treated with BMP4 for 1h and ChIP assay was performed using anti-phospho-Smad1/5/8 antibody. Id1 immunoprecipitation was evaluated by ChIP-qPCR. Data are expressed as fold enrichment relative to the IgG controls (fold change = 1). **b)** Undifferentiated cells were treated with BMP4 for 1h and the binding of phospho-Smad1/5/8 was evaluated by ChIP assay. DNA was immunoprecipitated with anti-phospho-Smad1/5/8 antibody and specific primer (indicated by arrows on the top of the panel) were used to amplify regions upstream to the transcriptional start site of miR-23a cluster. Data are shown as fold change over untreated (-BMP4) ESCs (fold change = 1). Numbers over the bars indicate the P-Value.

miR-23a and miR-23b clusters are expressed in ESCs and during neural differentiation

In the study of miR-23a cluster functions, we first wondered whether miR-23a, miR-24-2 and miR-27a are expressed in ESCs and during neural differentiation. We induced neural differentiation through the formation of serum-free embryoid bodies (SFEBs), that results in the generation of epiblast stem cells (EpiSCs) before day 2, and then differentiate into neuroectodermal precursors. We measured the levels of miR-23a, miR-24-2 and miR-27a in undifferentiated cells and during SFEB differentiation, and we found that they are expressed in ESCs and their expression decreases during differentiation, even if it is still significant at 4 days of SFEB differentiation (Figure 10a). Another miRNA cluster, named miR-23b cluster, is located on chromosome 13 and it encodes for miR-23b, miR-24-1 and miR-27b. miR-24-1 is identical to miR-24-2, whereas miR-23a and miR-27a belong to the same families of miR-23b and miR-27b, respectively. Since miRNAs of the same families share the same targets, we also investigated the expression of miR-23b cluster, and we found that this miRNA transcript shares the same expression profile of miR-23a cluster (Figure 10b). Accordingly, also mature miR-23b and miR-27b are expressed in undifferentiated cells and decrease until 4 days of SFEB differentiation (Figure 10c). Interestingly, BMP4 treatment induces the expression of mature forms of miRNAs belonging to miR-23b cluster (Figure 10d). However, 1h of BMP4 treatment does not induce the transcription of this cluster, suggesting that BMP4 does not regulate directly miR-23b cluster (Figure 10b).

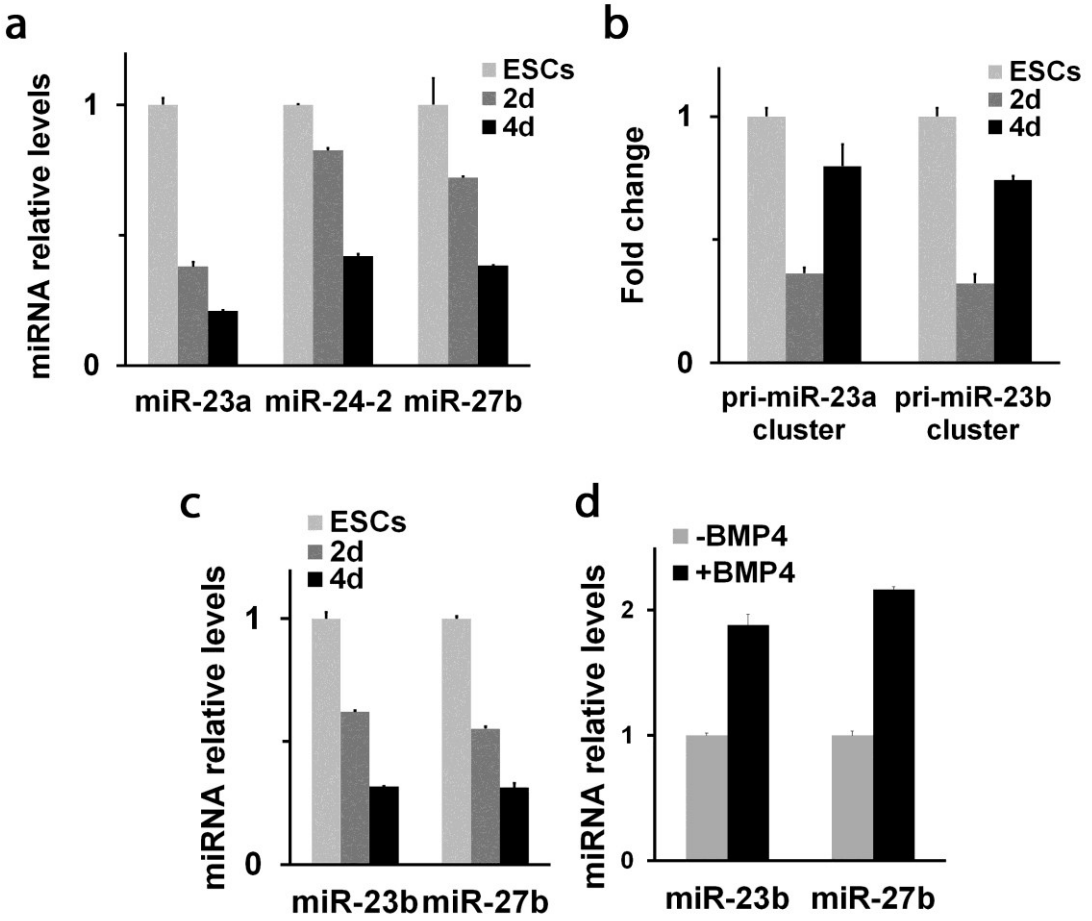

Figure 10. Expression levels of miR-23a and miR-23b clusters in ESC neural differentiation
a) ESCs were differentiated in neuroectodermal precursors using SFEB differentiation method. miRNA levels were evaluated in undifferentiated cells, in 2 days SFEBs and 4 days SFEBs by qPCR. **b)** Primary transcripts of miR-23a and miR-23b clusters were measured in ESCs and during SFEB differentiation using qPCR. **c)** miRNAs belonging to miR-23b cluster were measured in undifferentiated cells and during SFEB differentiation by qPCR. **d)** ESCs were stimulated with BMP4 for 24h and mature miR-23b and miR-27b were evaluated by qPCR. Data are presented as fold change of miRNA levels in undifferentiated (ESCs) and untreated (-BMP4) ESCs (fold change = 1).

Suppression of miR-23a and miR-23b clusters impairs ESC neural differentiation

Considering that miRNAs of miR-23a and miR-23b clusters are expressed at high levels in undifferentiated cells and they share the targets, so they could compensate each other, we decided to study their functions inhibiting them with specific anti-miRs (Figure 11a). We first analyzed the effect of their suppression on ESC self-renewal. The analysis of stemness markers Oct3/4, Nanog, Rex1 and Dax1 by Q-PCR and Oct3/4 by western blot shows that the suppression of miR-23a and miR-23b clusters does not affect ESC self-renewal (Figure 11b). Then we investigated the effect of inhibition of these two clusters during differentiation. To this aim we suppressed miRNAs with specific anti-miRNAs in ESCs and then we induced neuroectodermal differentiation through SFEB formation. At 4 days of differentiation, SFEBs are composed mainly of cells positive for neuroectodermal markers Pax6 and Sox1, but they still retain a few cells positive for stemness markers. Upon miRNA suppression we did not find any changes in the expression of neuroectodermal markers, but we found a significant decrease in Oct3/4 and Nanog expression (Figure 11c).

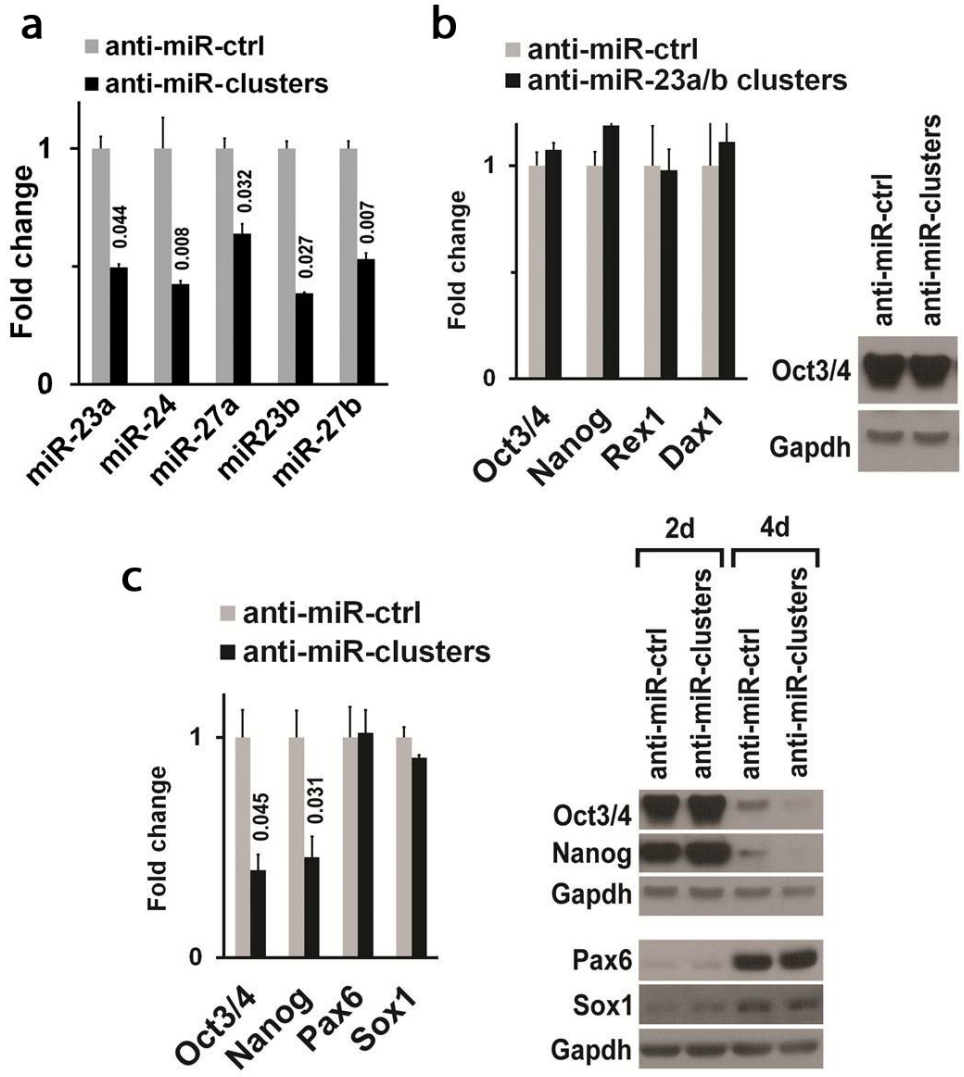

Figure 11. miRNA suppression reduce Oct3/4 and Nanog levels during SFEB differentiation. **a)** ESCs were transfected with specific anti-miRNAs and, after 24h, miRNA supression was evaluated by qPCR. **b)** In ESCs, miR-23a and miR-23b clusters were suppressed by transfection of specific anti-miRNAs. Stemness markers were evaluated after 24h from anti-miRNA transfection by qPCR (left panel) and by western blot (right panel). **c)** ESCs were transfected with anti-miRNAs of miR-23a and miR-23b clusters and then induced to differentiate as SFEBs. At 4 days of SFEB differentiation stemness markers (Oct3/4 and Nanog) and neuroectoderm markers (Pax6 and Sox1) were measured by qPCR (left panel). These markers were also analyzed by western blot (right panel) at 2 days and 4 days of SFEB differentiation. Data are presented as fold change over control (anti-miR-ctrl) ESCs (fold change = 1). Numbers over the bars indicate the P-Value.

Impaired differentiation of miRNA KD ESCs is not due to single miRNA or to altered proliferation rate of these cells

Considering that in our experiments we transfected all miRNAs of miR-23a and miR-23b clusters, we wondered whether impaired differentiation of miRNA KD cells could be due to the effect of single miRNA KD. Therefore, we transfected single miRNAs in ESCs and then we induced SFEB differentiation. In these condition we did not find any changes neither in the expression of stemness markers nor in the expression of neuroectodermal markers in ESCs and at 4 days of SFEB differentiation (Figure 12a).

Moreover, to examine whether rapid decrease of stemness markers upon suppression of the two clusters could be due to an altered proliferation rate of pluripotent cells, we performed a BrdU assay. As shown in Figure 12b, the inhibition of these two clusters does not impair ESC proliferation.

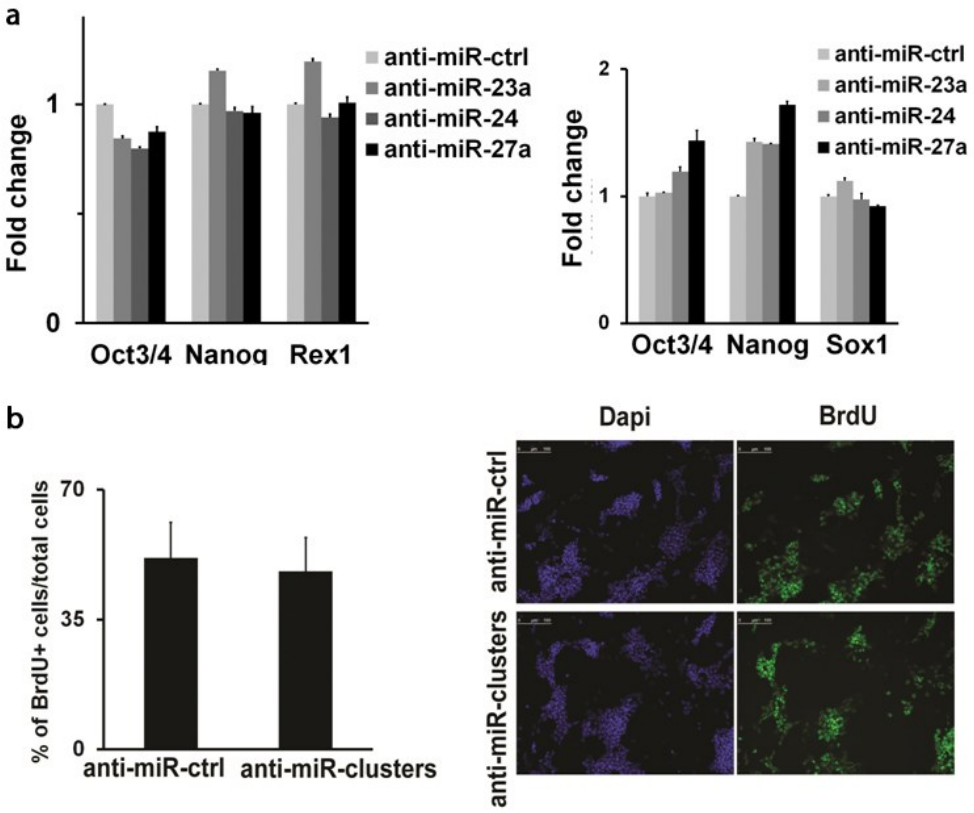

Figure 12. Single miRNAs do not impair stemness and differentiation of ESCs and miRNA clusters do not alter ESC proliferation rate.

a) Single anti-miRNA belonging to miR-23a and miR-23b clusters were transfected in ESCs and then cells were induced to differentiate. Left panel shows expression of stemness markers in undifferentiated cells, right panel shows stemness and neuroectoderm markers at 4 days of SFEB differentiation. Data are expressed as fold change relative to control (anti-miR-ctrl) cells (fold change = 1). **b)** ESCs were transfected with anti-miR-clusters and after 24h BrdU was added to culture medium for 1h. ESC proliferation rate was analyzed by immunofluorescence with anti-BrdU antibody (right panel). Percentage of BrdU positive cells is reported in graph (left panel).

The suppression of miR-23a and miR-23b clusters increases apoptosis during ESC differentiation

To better examine the composition of SFEBs derived from ESCs transfected with specific anti-miRNAs for miR-23a and miR-23b clusters, we performed immunostaining on sectioned SFEBs. We found that in normal conditions 4 days differentiated SFEBs contain mainly Sox1-positive cells surrounding groups of Oct3/4-positive cells located in the centre of the cell aggregate. Interestingly, in SFEBs derived from miRNA KD ESCs we found a great reduction or absence of Oct3/4-positive cells along with extensive cell death in the centre of SFEBs (Figure 13a). These results suggested that the reduced expression of Oct3/4 in miRNA KD ESCs could be due to an induced apoptosis upon the miRNA suppression.

To investigate apoptotic phenotype given by miRNA suppression, we analyzed the activation of Caspase 3, an hallmark of apoptosis, in ESCs differentiated into neuroectoderm precursors. During SFEB differentiation only a small number of cells undergoes apoptosis, whereas upon miRNA suppression, about 10% of SFEBs showed a widespread presence of Cleaved Caspase 3 positive cells mainly in the centre of EBs surrounded by Sox1-positive cells (Figure 13b). The apoptotic effect due to miR-23a and miR-23b cluster suppression was also supported by the increased activation of PARP, that is cleaved in apoptotic cells (Figure 13c). To further characterize apoptotic phenotype given by miRNA suppression, we performed FACS analysis using propidium iodide (PI). This assay is widely used for the evaluation of apoptosis because apoptotic cells are characterized by DNA fragmentation and, as a consequence, hypodiploidy. PI, a fluorochrome capable of binding and labeling DNA, allows the identification of hypodiploid cells. Using FACS, we found an increased percentage of hypodiploid cells in SFEBs derived from miRNA KD ESCs (Figure 13d). Taken together, these results indicate that miRNAs of the 23a and 23b clusters protect ESCs from apoptosis during differentiation.

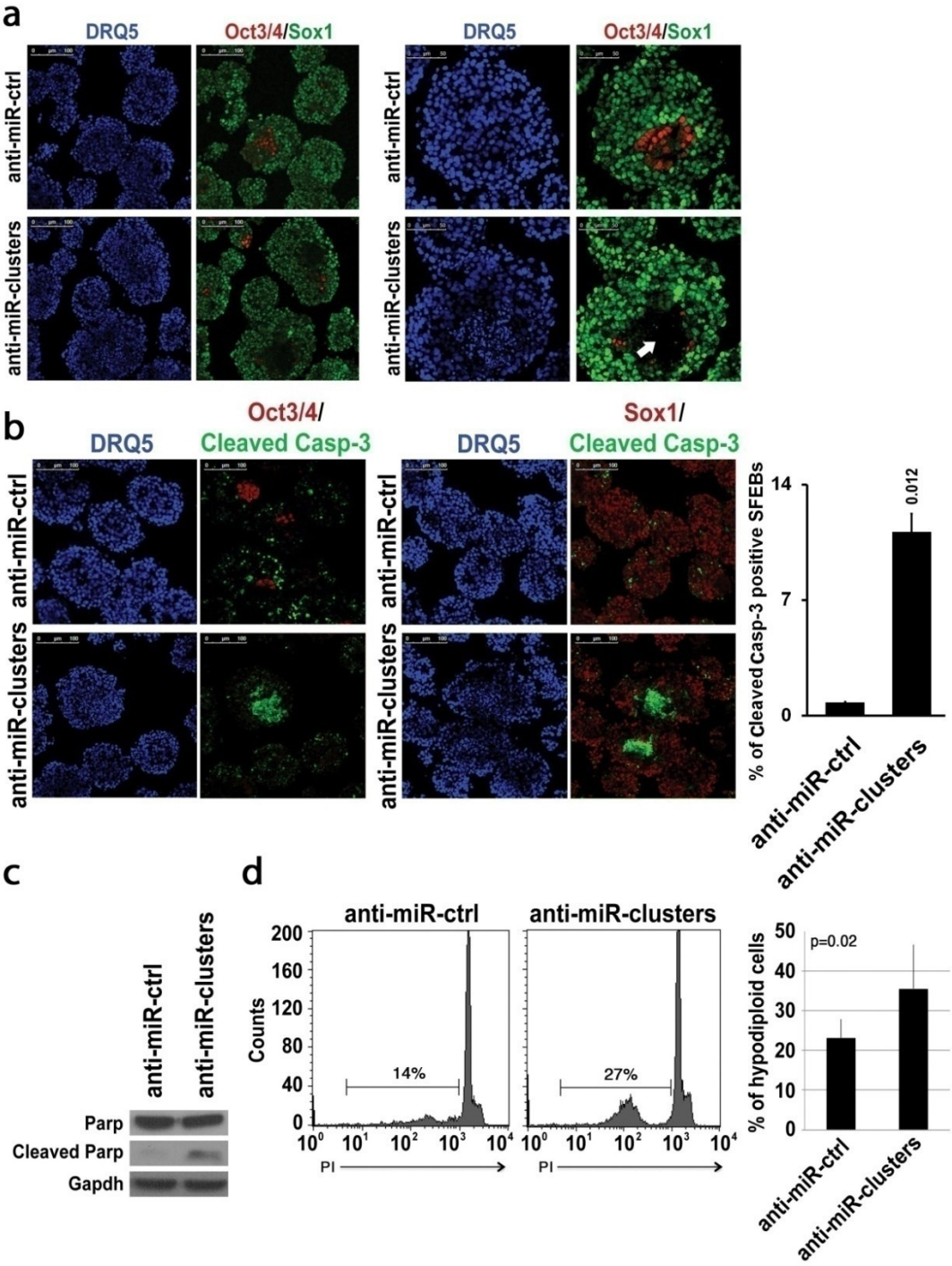

Figure 13. Suppression of miR-23a and miR-23b clusters results in apoptosis during SFEB differentiation

a) ESCs transfected with anti-miR-clusters were differentiated as SFEBs. At 4 days of differentiation, SFEBs were immunostained to detect stemness (Oct3/4) and neuroectoderm (Sox1) markers. Scale bars: 100 µm. Right panel shows a magnification of a representative SFEB. The white arrow indicate apoptotic area. Scale bars: 50 µm. **b)** 4 day differentiated SFEBs derived from miRNA KD ESCs were immunostained with apoptotic (Cleaved Caspase 3), stemness (Oct3/4) and neuroectoderm (Sox1) markers. Scale bars: 100 µm. The graph reports the percentage of SFEBs showing extensive apoptotic area. **c)** The apoptotic marker Cleaved Parp is measured by western blot analysis in 4 day differentiated SFEBs derived from miRNA KD ESCs. **d)** ESCs were tranfected with anti-miR-clusters and then induced to differentiate until 4 days of SFEB differentiation. SFEBs were stained with PI and percentage of hypodiploid PI-positive cells was measured by FACS analysis (n=6). Numbers over the bars indicate the P-Value.

Apoptosis starts after ESC to EpiSC transition during ESC differentiation

To investigate when apoptosis was triggered, we suppressed miRNAs in ESCs and evaluated apoptosis markers in undifferentiated state without finding any alteration in miRNA KD ESCs (Figure 14a). Then, we suppressed miRNAs in ESCs and analyzed the transition from ESCs to EpiSCs upon miRNAs suppression. As expected, in EpiSCs we found decreased levels of stemness marker Dax1, maintenance of Nanog and increased expression of the epiblast markers Fgf5, Otx2 and Dnmt3b (Figure 14b). In these conditions we did not find any alteration of Cleaved Caspase 3 expression upon miRNA KD, suggesting that the apoptotic event starts after the transition from ESCs to EpiSCs (Figure 14c).

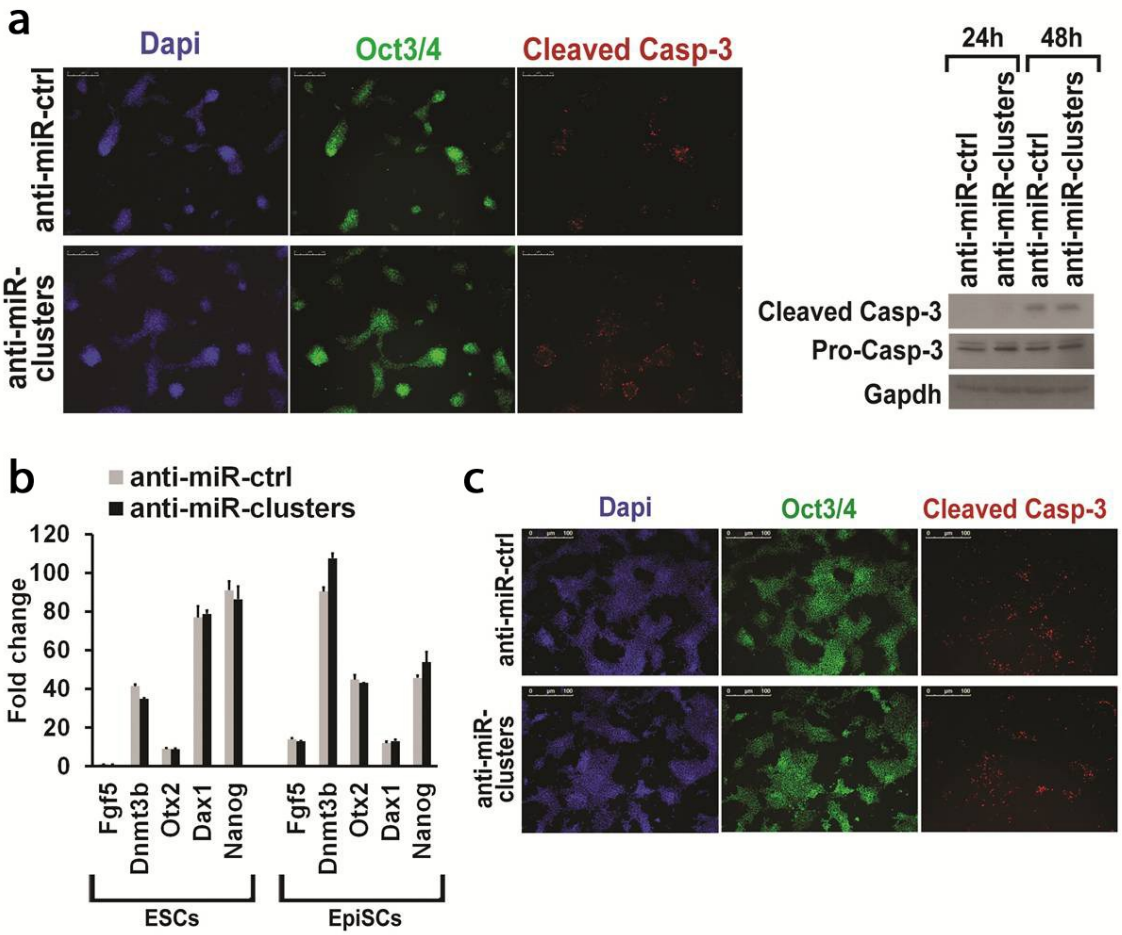

Figure 14. miR-23a and miR-23b cluster suppression do not impair ESC-EpiSC transition
a) ESCs were transfected with the indicated anti-miRNAs. Cleaved Caspase 3 was detected by immunostaining of undifferentiated cells after 24h from transfection (left panel) and by western blot after 24h and 48h from transfection (right panel). Scale bars: 100 µm. **b)** miR-23a and miR-23b clusters were suppressed in ESCs and EpiSC differentiation was induced. Stemness (Dax1 and Nanog) and epiblast (Fgf5, Dnmt3b, Otx2) markers were detected by qPCR in ESCs and EpiSCs. Data are presented as fold change relative to Fgf5 mRNA levels in control (anti-miR-ctrl) ESCs (fold change = 1). **c)** Indicated miRNAs were suppressed in ESCs and epiblast differentiation was induced. After 2 days of epiblast differentiation the presence of apoptotic cells was highlighted by staining with anti-Cleaved Caspase 3 antibody. Scale bars: 100 µm.

Thus, we investigated apoptotic events at 2 and 3 days of SFEB differentiation, when ESC to EpiSC transition is overcome. We found increased levels of Cleaved Caspase 3 upon miRNA KD already at 2 days of SFEB differentiation (Figure 15a). Also immunostaining analysis showed clumps of apoptotic cells in 2 day differentiated SFEBs, with a further increase of Cleaved Caspase 3 expression in the next 2 days (Figure 15b). To demonstrate that the apoptotic phenotype given by miRNA suppression during differentiation is not linked to specific differentiation conditions, we induced ESC differentiation with a methods that lead to a more homogenous population of neural precursors. This method consists in culturing ESCs as monolayer in presence of N2 and B27 supplements and in absence of serum and LIF. We used Sox1-GFP cells (kindly provided by A. Smith) that express the gene reporter Enhanced Green Fluorescent Protein (EGFP) under the control of Sox1 promoter, which is a specific marker of neuronal precursors. Also with this differentiation method, we found a significant percentage of GFP-positive cells undergoing apoptosis upon miRNA cluster suppression (Figure 15c). These results suggest that the proper levels of miR-23a and miR-23b clusters are required to balance apoptosis and survival of ESCs during neuroectodermal differentiation.

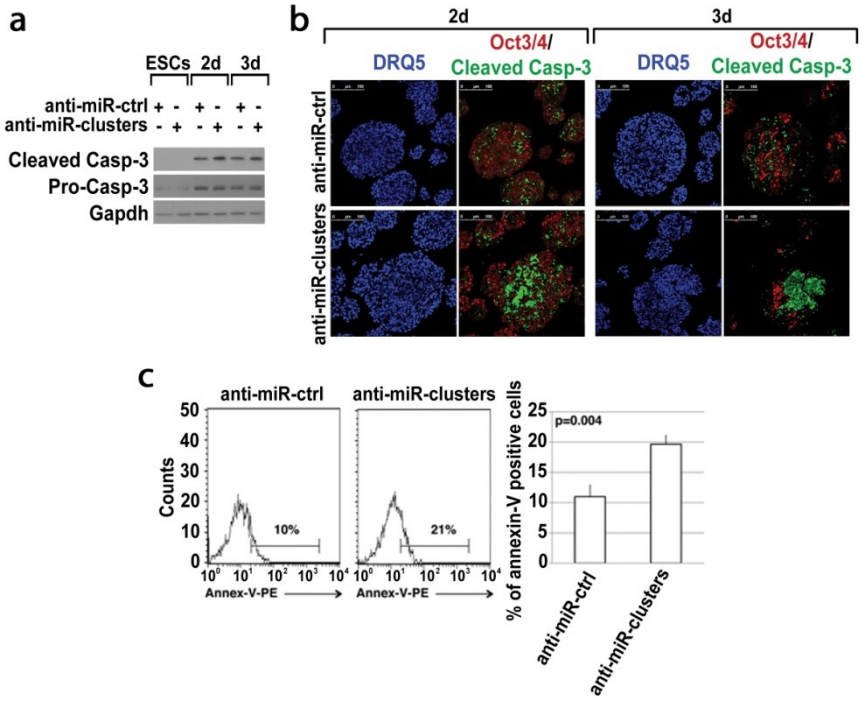

Figure 15. Apoptotic phenotype occurs after 2 days of SFEB differentiation
a) SFEBs were derived from miRNA KD ESCs and activation of Caspase 3 was detected by western blot in undifferentiated cells, 2 days and 3 days SFEBs. **b)** Cleaved Caspase 3 was detect by immunostaining of 2days and 3days SFEBs obtained from ESCs transfected with specific anti-miRNAs. **c)** Sox1-GFP cells were transfected with anti-miRNAs and differentiated into neuroectoderm precursors using monolayer protocol. Differentiated cells were stained with Annexin V. Annexin V positive cells were detected by FACS analysis and the percentage of positive cells is shown in the graph (n=3).

miR-23a and miR-23b clusters protect ESCs from apoptosis

To investigate whether these miRNAs have a more general effect to protect undifferentiated cells from apoptosis, we exposed ESCs to an apoptotic stimulus and evaluated the extent of apoptosis upon miRNA suppression. We transfected ESCs with specific anti-miRs and exposed these cells to 5 Gy of X rays. Cleaved Caspase 3 was undetectable by western blot in ESCs, whereas the exposure of ESCs to 5 Gy of X rays induced a slight activation of Caspase 3. The suppression of miRNAs in presence of an apoptotic stimulus resulted in a strong induction of apoptosis. All these results suggest an anti-apoptotic function of these miRNAs also in undifferentiated ESCs (Figure 16).

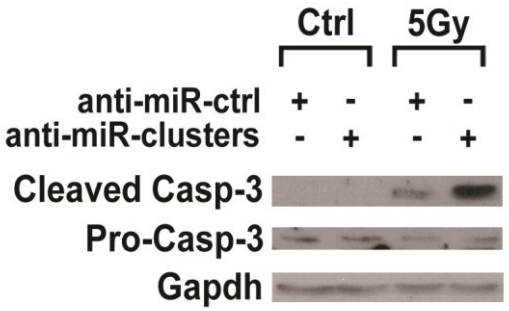

Figure 16. miR-23a and miR-23 clusters have anti-apoptotic function in ESCs
ESCs were transfected with indicated miRNAs and after 24h they were irradiated or not with 5Gy of X Rays. After 24h Cleaved Caspase 3 was detected by Wester blot analysis.

Suppression of miR-23a and miR-23b clusters mimics the treatment with low doses of BMP4

BMP4 signaling may have different effects on ESC differentiation also depending by the intensity of the signal. Indeed, high doses of BMP4 (10ng/ml) block ESC differentiation (Zhang et al., 2010), whereas low doses of BMP4 induce apoptosis of neural precursor cells during epidermal differentiation of ESCs (Gambaro et al., 2006). Accordingly, we have found that high doses of BMP4 block differentiation of ESCs toward neuroectoderm, whereas low doses of BMP4 activate Caspase 3 thus inducing apoptosis (Figure 17a-b).

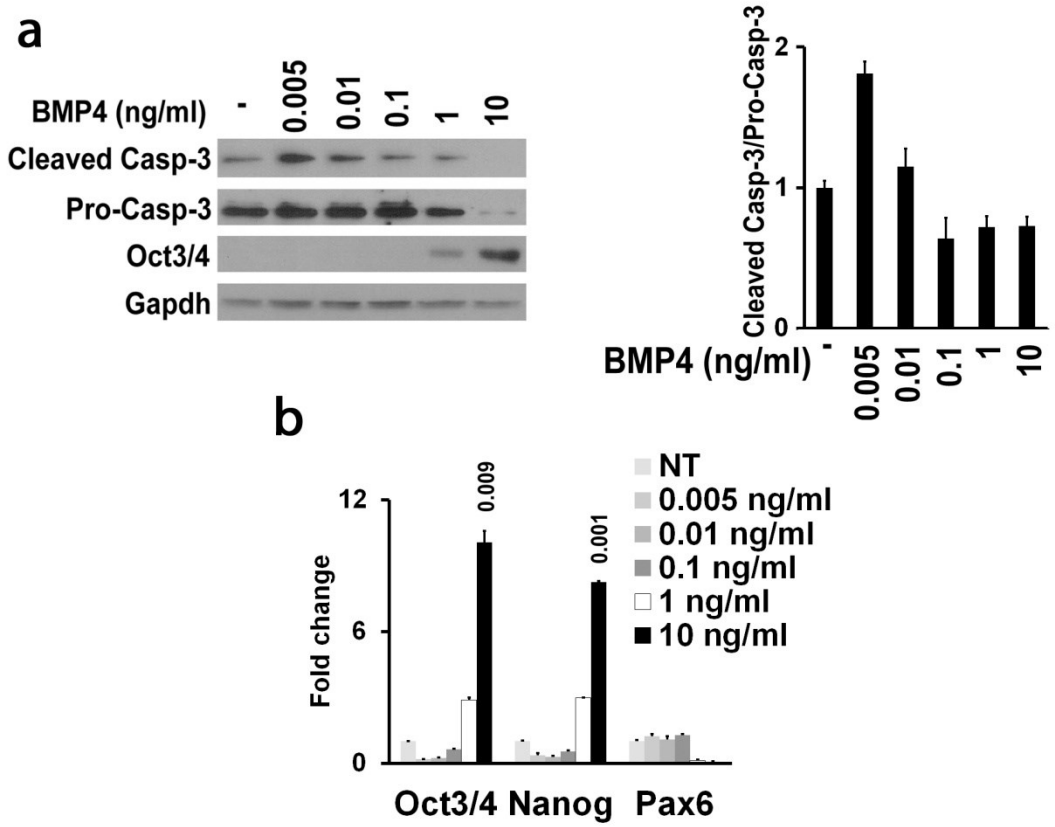

Figure 17. Different doses of BMP4 have different effects on ESC differentiation
a) ESCs were treated with indicated doses of BMP4 and differentiated as SFEBs. Cleaved Caspase 3 was detected by western Blot. Of note, high doses of BMP4 (10ng/ml) block differentiation, as indicated by the strong expression of stemness marker Oct3/4, resulting in reduction of pro-Caspase-3 expression. The graph reports densitometric analysis of Cleaved Caspase 3 compared to pro-Caspase 3. **b)** Stemness (Oct3/4 and Nanog) and neuroectoderm (Pax6) markers were measured by qPCR in 4 days SFEBs derived from ESCs stimulated with different doses of BMP4. Data are presented as fold change over untreated (NT) ESCs. Numbers over the bars indicate the P-Value.

Considering that we have found an apoptotic phenotype upon suppression of miR-23a and miR-23b clusters, we reasoned that the suppression of miRNAs could reinforce BMP4 signaling, thus miming the effects of low doses of BMP4. To investigate this possibility, we treated miRNA KD ESCs with the BMP4 antagonist Dorsomorphin. Indeed, if

apoptosis is due to an enhancement of BMP4 signaling, the block of BMP4 receptor should rescue the apoptotic phenotype. As shown in Figure 18, we have found that Cleaved Caspase 3 levels were reduced upon the contemporary suppression of miRNAs and BMP4 receptor, demonstrating that the apoptotic phenotype observed after miRNA suppression is due to a reinforcement of BMP4 signaling during differentiation.

To demonstrate that the rescue of apoptotic phenotype during differentiation is not linked to specific differentiation conditions, we differentiated Sox1-GFP cells with monolayer protocol and inhibited BMP4 and miRNAs. We found that also in these differentiation conditions apoptotic phenotype given by miRNA suppression was rescued by BMP4 signaling inhibition (Figure 19a).

Moreover, to further demonstrate that the apoptotic phenotype due to the suppression of miR-23a and miR-23b clusters is mediated by BMP4, we blocked BMP4 signaling at intracellular level by silencing Smad1 and Smad5, the two BMP4 effectors highly expressed in ESCs. Also in these experimental conditions, we found that the contemporary silencing of the miRNAs and Smads reverted the apoptotic phenotype (Figure 19b).

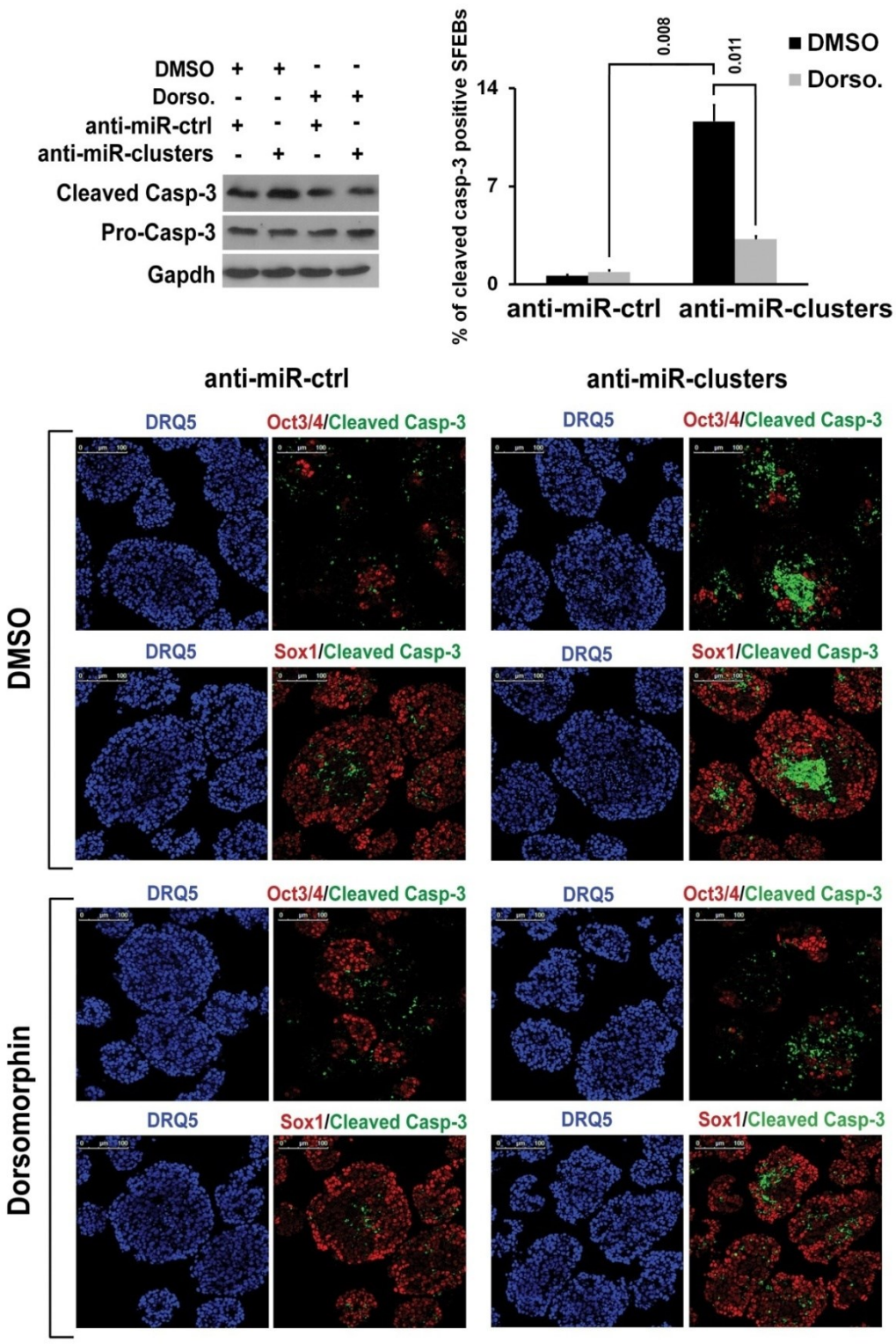

Figure 18. BMP4 inhibition rescues apoptotic phenotype given by suppression of miR-23a and miR-23b clusters

ESCs were transfected with indicated anti-miRNAs and treated with BMP4 inhibitor (Dorsomorphin) or DMSO as control. After 4 days of SFEB differentiation, activation of Caspase 3 was evaluated by western blot (upper-left panel) and by immunostaining analysis (lower panel). Proper SFEB differentiation was evaluated by immunostaining with stemness (Oct3/4) and neuroectoderm (Sox1) markers. Percentage of SFEBs containing diffused apoptotic areas is reported in the upper-right panel that shows a strong rescue of apoptotic phenotype upon Dorsomorphin treatment. Numbers over the bars indicate the P-Value.

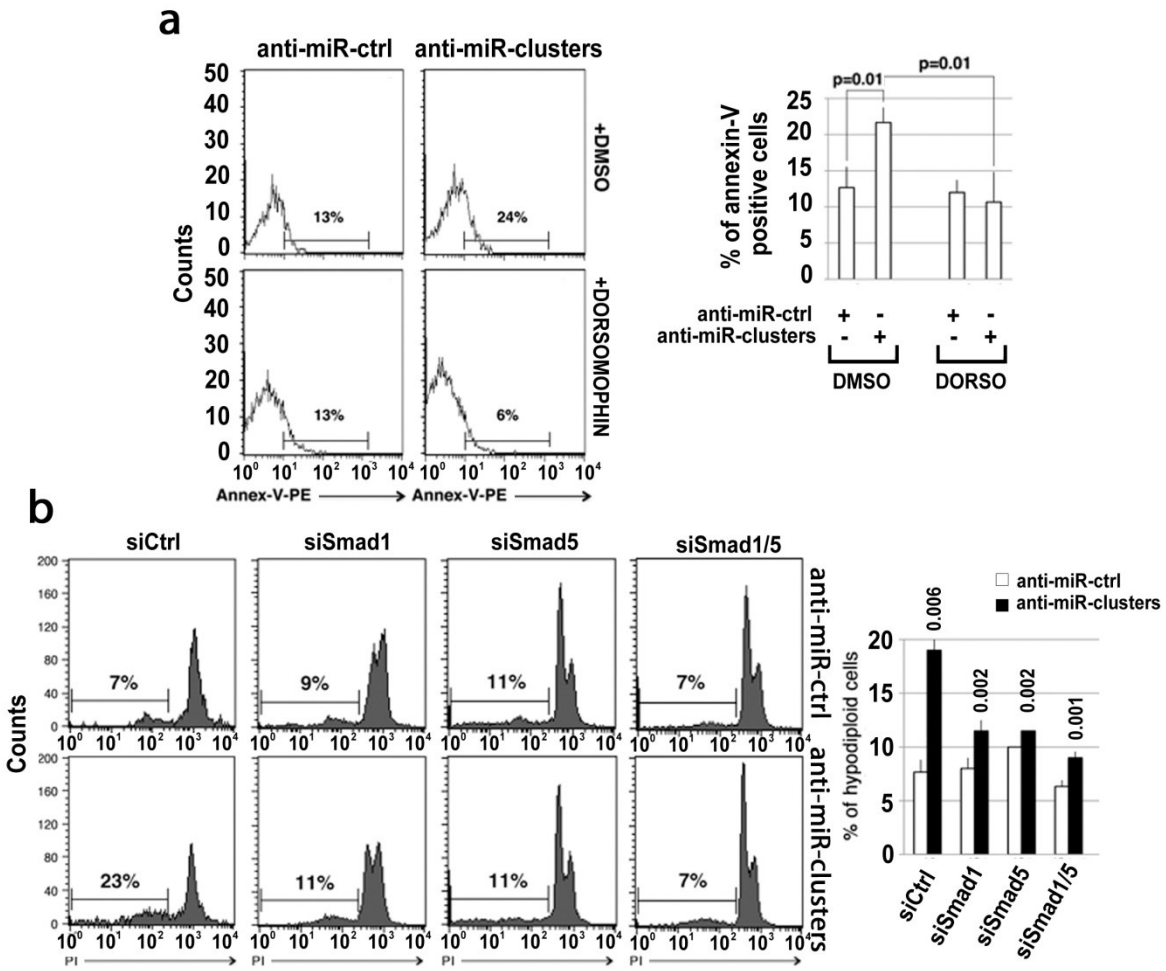

Figure 19. Both block of BMP4 receptor and BMP4 effectors rescues apoptosis induced by miRNA suppression.

a) Sox1-GFP cells were transfected with indicated anti-miRNAs, treated with Dorsomorphin and differentiated into neuroectoderm precursors using the monolayer protocol. After 4 days of differentiation, cells were stained with Annexin V and positive cells were counted by FACS analysis. Percentage of Annexin V positive cells were reported in graph (right panel). **b)** miR-23a and miR-23b clusters were suppressed with specific anti-miRNAs and Smad1 and Smad5 were silenced with specific siRNAs in ESCs. After 4 days of differentiation, SFEBs were stained with PI and PI-positive cells were analyzed by FACS analysis. Right panel shows percentage of hypodiploid cells. Numbers over the bars indicate the P-Value.

Smad 5 is a direct target of miR-23a and miR-23b clusters

In order to understand how the suppression of miR-23a and miR-23b clusters enhances BMP4 signaling, we searched for miRNA targets involved in BMP4 signaling pathway. Using bioinformatic tools, such as miRWalk and Targetscan, we have found several Smads among the predicted targets. Moreover, Rogler and co-workers have demonstrated that miR-23b cluster targets Smad3, Smad4 and Smad5 in liver stem cells (Rogler et al., 2009). To investigate whether our miRNAs could target Smads also in ESCs, we first examined the expression of Smads in these cells. We found that Smad3 is not expressed in ESCs and during the early steps of differentiation, whereas Smad4 and Smad5 were expressed in ESCs and their expression increased during differentiation (Figure 20a). Interestingly, we have already demonstrated that miRNA expression decreases during differentiation showing an expression profile opposite to that of Smads (Figure 10a,c). Thus, to investigate the possible regulation of Smad4 and Smad5 by miR-23a and mR-23b cluster, we inhibited miRNAs and measured Smad protein levels. We found that Smad4 protein levels were not impaired by miRNA suppression, whereas Smad5 accumulated upon miRNA down-regulation, demonstrating that miR-23a and miR-23b clusters regulate Smad5 expression in ESCs (Figure 20b).

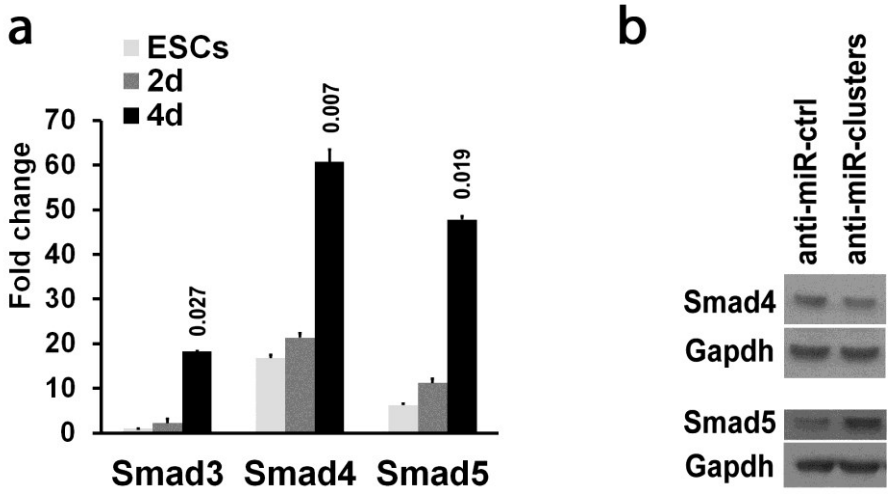

Figure 20. miR-23a and miR-23b clusters target Smad5
a) ESCs were differentiated as SFEBs and Smad mRNA levels were measured in undifferentiated cells (ESCs), 2 days (2d) and 4 days (4d) of SFEB differentiation. Data are presented as fold change relative to Smad3 mRNA level in undifferentiated cells (fold change = 1). **b)** ESCs were transfected with indicated anti-miRNAs and Smad4 and Smad5 protein levels were measured by western blot analysis. Numbers over the bars indicate the P-Value.

BMP4, miR-23a cluster and Smad5 generate a feedback loop regulating apoptosis during ESC neural differentiation

We have found that BMP4 controls the transcription of miR-23a cluster, that in turn regulates the expression levels of Smad5, finally maintaining the balance between apoptosis and survival during ESC differentiation. Considering these findings, we speculated that the transcription of miR-23a cluster is induced by BMP4 only when this signaling is strong enough to drive its transcriptional activation. Therefore, we measured the levels of pri-miRNA transcripts upon different doses of BMP4. We have found that only high doses of BMP4 induced the transcription of miR-23a cluster, the same doses that block differentiation of ESCs without inducing a significant apoptosis (Figure 21a). Moreover, we investigated the behavior of Smad5 in ESCs treated with different doses of BMP4. We did not find any changes in mRNA levels of Smad5 after BMP4 treatment (Figure 21b), whereas we found reduced Smad5 protein levels in ESCs treated with high doses of BMP4, thus showing an opposite trend compared with those of the miRNAs (Figure 21c).

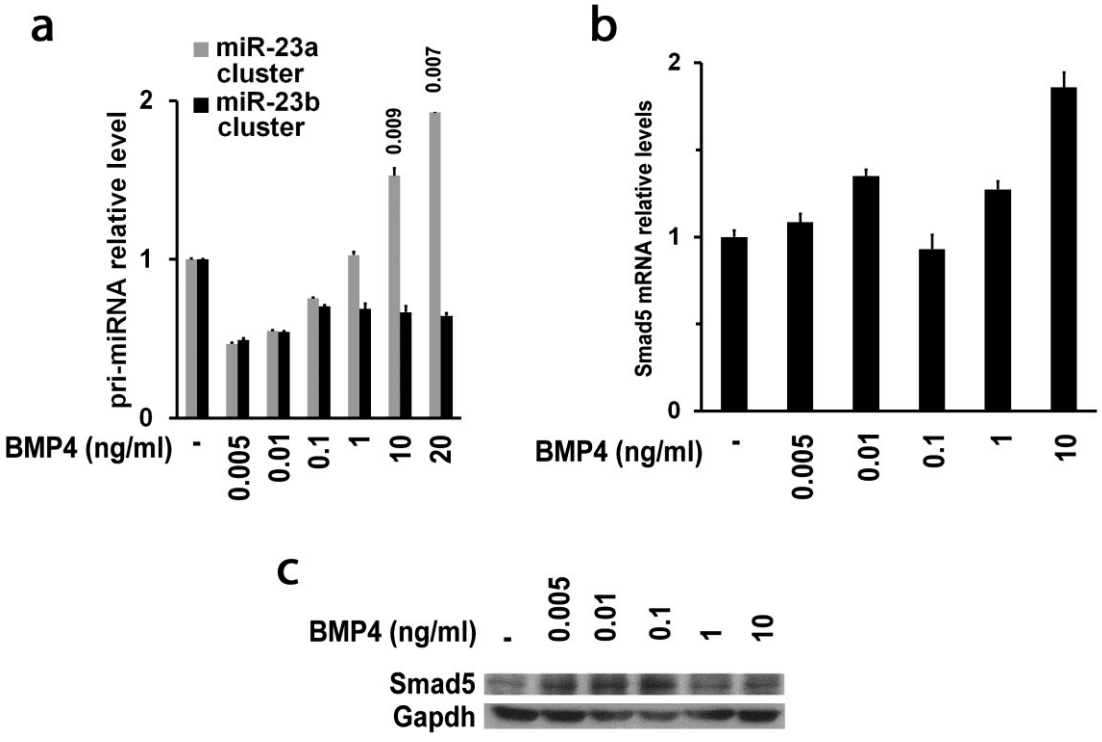

Figure 21. High doses of BMP4 induce miR-23a transcription and Smad5 post-transcriptional regulation

a) pri-miRNA levels of miR-23a and miR-23b clusters were measured in ESCs treated with indicated doses of BMP4. After 1h of BMP4 treatment, pri-miRNA levels were measured by qPCR.. **b)** Smad5 mRNA levels were measured in ESCs treated with indicated doses of BMP4 using qPCR. Data are shown as fold change relative to untreated cells (fold change = 1). **c)** Protein levels of Smad5 were measured in ESCs upon treatment with indicated doses of BMP4. Numbers over the bars indicate the P-Value.

Taken together these results demonstrate the existence of a feedback loop controlling the balance between apoptosis and survival: a strong stimulation of BMP4 induces the transcription of miR-23a cluster that targets Smad5. The reduction of Smad5 protein levels down-regulates the response of the cells to BMP4, resulting in the protection from apoptosis induced by BMP4 during ESC neuronal differentiation (Figure 23).

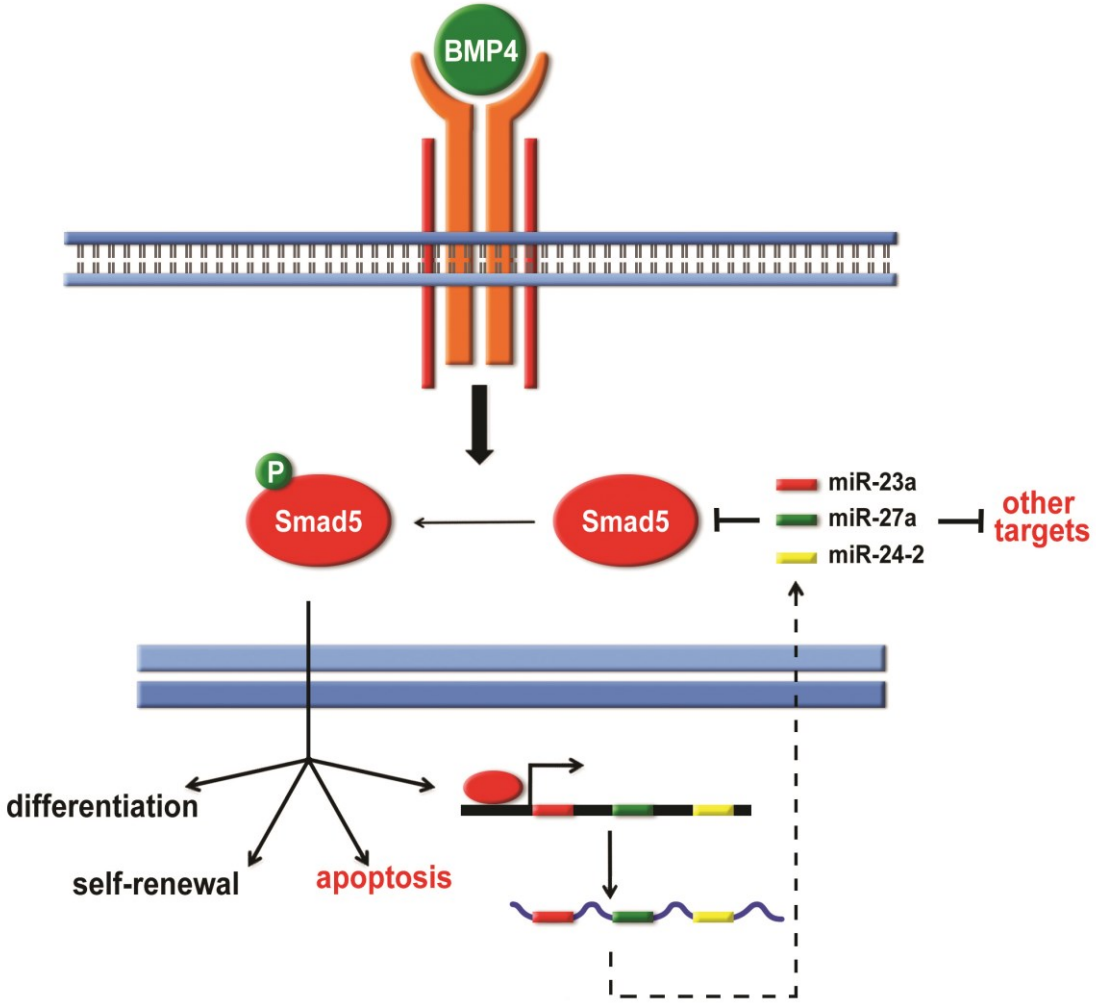

Figure 22. BMP4 and miR-23a cluster generate a feedback loop regulating apoptosis in ESC differentiation.
In ESCs, BMP4 regulates several processes such as differentiation, self-renewal and apoptosis through Smads activation. Moreover BMP4 induces the transcription of miR-23a cluster, that in turn targets Smad5. Smad5 suppression results in a reduced sensibility of the cells to BMP4. As a consequence, these miRNAs protect differentiated ESCs from apoptosis induced by BMP4. This effect could be also due to the direct targeting of pro-apoptotic genes by miRNAs.

DISCUSSION

Many papers have demonstrated that BMP4 has multiple and different roles in ESCs and during their differentiation. First, BMP4 contributes to maintain the undifferentiated phenotype of ESCs, mostly by blocking the transition from ESCs to EpiSCs (Zhang et al., 2010). Then, BMP4 is able to drive the differentiation of EpiSCs toward a mesendodermal fate by blocking the formation of neuroectoderm (Zhang et al., 2010). Moreover, BMP4 is a strong epidermal inducers of neuroectodermal precursors (Kawasaki et al., 2000), indeed it interferes with the survival of neural precursors though the induction of their apoptosis (Gambaro et al., 2006). However, the molecular mechanisms by which BMP4 fulfills these different functions are still poorly understood. We have recently demonstrated that the ability of BMP4 to block the transition of ESCs into EpiSCs depends on a feedback loop that involves miR-125a. Indeed, BMP4 signaling controls miR-125a expression at transcriptional level and in turn this miRNA targets the BMP4 co-receptor Dies1 and such regulatory loop sets ESC sensitivity to BMP4 (Parisi et al., 2012). We have hypothesized that the interplay between the BMP pathway and miRNAs may represent a more general regulatory mechanism modulating the response of ESCs to extracellular stimuli. Starting from this idea, we have searched for miRNAs directly regulated by BMP4 and that can mediate its function in ESCs. We have analyzed the changes in miRNA expression due to BMP4 pathway activation and we have found a miRNA cluster, i.e. miR-23a/miR-24-2/miR-27a, whose promoter is positively regulated by BMP4 signaling. The miRNAs of this cluster are highly expressed in ESCs and their expression, although slightly decreased, is maintained during the first phases of differentiation. We have unraveled a crucial function of these miRNAs that is the protection of differentiating ESCs from apoptosis. Indeed, we have shown that the suppression of these miRNAs leads to a dramatic apoptosis increase. Interestingly, we have found that this phenotype can be reverted by blocking BMP4 signaling at receptor level by using Dorsomorphin thus indicating that the apoptotic effect observed upon miRNA suppression is due to a reinforcement of BMP4 signaling. In agreement with these observations we have found that these miRNAs target the BMP4 effector Smad5. Therefore, we have described the presence of a feedback loop involving BMP4 signaling and miRNAs in ESCs. The biological function of this loop is to fine tune the sensitivity of ESCs to BMP4 stimuli. Indeed, when the levels of miR-23a cluster are normally high, the BMP4 endogenous signaling poorly acts and the differentiation into neuroectoderm, accompanied by a physiological apoptosis, can occur. In contrast, a

suppression of these miRNAs allows the reinforcement of BMP4 signaling, thanks to the accumulation of Smad5, and this in turn results in an aberrant apoptosis during early stage of differentiation. As described above, the apoptosis due to miRNA suppression is accompanied by a significant decrease of Oct3/4-positive pluripotent stem cells whereas the number of neuroectodermal cells is not affected. The death of pluripotent cells could be due to a direct and combined effect of other miRNA targets, beyond Smad5, in these cells or can be due to an influence of these miRNAs in lineage determination that we were not able to highlight because of the rapid occurrence of apoptosis. Another explanation can be the high sensitivity of pluripotent stem cells to the apoptosis that anyway physiologically occurs during differentiation. This hypothesis is supported by our observation that pluripotent stem cells, in which the miRNAs of the 23a and 23b cluster are suppressed, are more sensitive to apoptotic stimuli.

miR-23a/27a/24-2 is an intergenic miRNA cluster located on the chromosome 8. These miRNAs belong to three different families and are not related in sequence. Therefore, their control by BMP4 and their targeting of at least one common mRNA (Smad5) suggests a coordinate action that the cells can adopt to finely modulate their fate. Other targets of these miRNAs have been already described in different biological contexts (Chen et al., 2014; Cao et al, 2012) and of course, these miRNAs probably control multiple targets in addition to Smad5 in ESCs. Nevertheless, the rescue of the apoptotic phenotype obtained upon treatment of BMP4 antagonist Dorsomorphin demonstrated that the protective role of these miRNAs against apoptosis is mainly due to the targeting of Smad5 that results in a reinforcement of BMP4 signaling.

As mentioned above another cluster located on the chromosome 13 generates a transcript containing three miRNAs of the same families, miR-23b/27b/24-1. Also these miRNAs are expressed in ESCs and they presumptively control the same targets of miR-23a/27a/24-2. We have demonstrated that the suppression of all these miRNAs is necessary to observe the apoptotic phenotype due to the reinforcement of BMP4 signaling. However, our results indicated that BMP4 directly controls only the cluster of miR-23a/27a/24-2. This observation suggests that the BMP4 signaling during ESC differentiation undergoes different regulations that are dependent (miRNAs of 23a cluster) or independent (miRNAs of 23b cluster) by itself. We have already described a similar control mechanism of BMP4 pathway by the miRNAs of the 125 family. Indeed, we have demonstrated that while miR-125a is directly regulated by BMP4, miR-125b seems to not be regulated by this signaling (Parisi et al., 2012; Battista et al., 2013). This multiple controls of extracellular signaling

through different miRNAs can probably be due to the relevance of BMP4 in the control of the ESC fate, thus indicating that ESCs modulate in different ways a such important pathway.

The anti-apoptotic function of the miRNAs of 23a and 23b cluster in apoptosis was already suggested in other biological context by several reports. Indeed, Chen et al. have shown that mir-23a/b and miR-27a/b alleviate neuronal apoptosis by suppressing Apaf-1 (Chen et al., 2014). In hematopoietic cells exogenous expression of miR-24 induces cell survival whereas its suppression results in an apoptosis increase (Nguyen et al., 2013). On the other hand, also BMP4 has been described to have a role in developmentally regulated apoptosis (Councouvanis and Martin 1999; Furuta et al., 1997; Gambaro et al., 2006). Although all these independent reports indicated a role of both BMP4 and miRNAs of the miR-23a and 23b clusters in apoptosis, the link between these two actors was not revealed yet. Our findings have highlighted this connection by indicating that a low but inappropriate BMP4 signaling during ESC differentiation trigger apoptosis. This apoptotic signal cannot be counteracted by an increase of miRNAs of the-23a cluster because the transcriptional activation of this cluster by BMP4 itself occurs only when BMP4 signaling is strongly reinforced. On the other hand, when the BMP4 signaling is strong enough it drives the accumulation of the miRNAs that can protect from apoptosis and thus, BMP4 signaling leads to a differentiation block rather than to cell death. Finally, our results described a new loop involving BMP4 and miRNAs. ESCs employ this loop to adapt their response to BMP4 signaling on the basis of the strength of this morphogenetic signal.

MATERIALS AND METHODS

Cell culture, transfection and treatment

E14Tg2a (BayGenomics, San Francisco, CA, USA) mouse ESCs and Sox1-GFP ESCs25 were maintained onfeeder-free, gelatin-coated plates in the following medium: Glasgow MinimumEssential Medium (GMEM, Sigma, St. Louis, MO, USA) supplemented with 2 mMglutamine, 1 mM sodium pyruvate, 1X non-essential amino acids (all fromInvitrogen, Carlsbad, CA, USA), 0.1 mM β-mercaptoethanol (Sigma), 10% FBS(Hyclone Laboratories, Logan, UT, USA) and 103 U/ml Leukemia Inhibitory Factor(LIF, Millipore, Billerica, MA, USA).Transfection of plasmids and anti-miRs (Applied Biosystems, Foster City, CA, USA)was performed by plating 6x104cells/cm2 16 h before transfection and usingLipofectamine2000 (Invitrogen) following the manufacturer's instructions.For BMP4 treatment, ESCs were grown overnight in 10% Knock-out SerumReplacement (KSR, Invitrogen) containing medium with LIF and then treated withBMP4 (R&D Systems, Minneapolis, MN, USA) at the doses and for the time indicated.For apoptosis induction, ESCs were irradiated with 5 Gy of X rays by usingRS2000 Biological Irradiator (Rad Source) and incubated for 24 h before the analysis.

Differentiation of ESCs and generation of EpiSCs

ESC differentiationinto neuroectoderm was induced though SFEB formation.2,20 SFEBs wereinduced by placing 1x106 ESCs in 100-mm Petri dishes in the followingdifferentiation medium: GMEM supplemented with 2 mM glutamine, 1 mM sodiumpyruvate, 1 × nonessential amino acids, 0.1 mM β-mercaptoethanol and 10% KSR.Alternatively, the differentiation of Sox1-GFP ESCs25 into neural precursors wasdriven by plating 3x103 cells/cm2 on gelatin-coated dishes in differentiation mediumas previously described.24BMP4 (at the indicated doses) was added once in the differentiation medium whenthe cells were plated in Petri dishes to induce SFEB formation. Dorsomorphin (Sigma)was added at 2 μM to differentiating cells and DMSO was used as negative control.The formation of EpiSCs was induced adapting the methods of Hayashi et al.22 andNakaki et al. 23 In brief, ESCs were dissociated into a single-cell suspension with 0.05%Trypsin-EDTA at 37 °C for 5 min. Individual cells were then seeded in fibronectin-coateddishes at a density of 2.5x105cells/cm2 in ESC culture condition, and after 18 h themedium was switched to the following EpiSC medium: 1 vol of DMEM/F12 combined with1 vol of Neurobasal medium, supplemented with 0.5% N2

supplement, 1% B27supplement, 1% KSR, 2 mM glutamine (Invitrogen), 20 ng/ml Activin A (R&D Systems),and 12 ng/ml bFGF (Invitrogen). Within 2 days in these conditions the cells undergomorphological transformation (including flattening, diminished cell-cell interactions andformation of cellular protrusions) and express epiblast markers.

RNA isolation, miRNA profiling and quantitative real-time PCR (qPCR)

For quantitative PCR total RNA was extracted by using TRI-Reagent(Sigma). The first-strand cDNA was synthesized according to the manufacturer'sinstructions (M-MLV RT, New England BioLabs, Ipswich, MA, USA). qPCR wascarried out with the 7500 Real-Time PCR System instrument and the SequenceDetection Systems (SDS) 1.4 software (Applied Biosystems) using Power SYBRGreen PCR Master mix (Applied Biosystems). The housekeeping GAPDH mRNAwas used as an internal standard for normalization, using 2-ΔΔCt method. Genespecificprimers used for amplification are listed in Supplementary Table S2. For theanalysis of single miRNA sequence specific LNA primers were used for theindicated miRNAs or U6 as internal control (both from Exiqon, Vedbaek, Denmark).For miRNA profiling, small RNA was isolated from ESCs treated or not with BMP4with mirVana miRNA Isolation kit (Ambion, Austin, TX, USA). From each sample,40 ng of total RNA was used to synthesize single-stranded cDNA with UniversalcDNA Synthesis Kit (Exiqon). Expression level of all miRNAs of Sanger miRBasev17 database was measured by using Mouse&Rat Panel microRNA Ready-to-UsePCR (Exiqon) with the 7900HT instrument and the Sequence Detection Systems(SDS) v2.1 software (Applied Biosystems) using SYBR Green Master Mix (Exiqon).The miRNA profiling data were analyzed performing a comparative analysis byusing the comparative Ct method (2-ΔΔCt, RQManager 1.2 software; AppliedBiosystems) using U6 as normalizer.

Antibodies and western blot analysis

Undifferentiated and differentiatedESCs were lysed in a buffer containing 1 mM EDTA, 50 mM Tris-HCl (pH 7.5), 70mMNaCl, 1% Triton and protease inhibitor cocktail (Sigma), and analyzed by western blot. The following primary antibodies were used: rabbit Smad5 (1 : 1000Cell Signaling, Danvers, MA, USA), mouse Oct3/4 (1 : 2000 Santa CruzBiotechnology, Santa Cruz, CA, USA), rabbit Nanog (1 : 1000 Calbiochem-EMDBiosciences, La Jolla, CA, USA) mouse GAPDH (1 : 1000 Santa Cruz Biotechnology), goat Sox1 (1 : 100 Santa Cruz Biotechnology), rabbit CleavedCaspase-3

(1 : 1000 Cell Signaling), rabbit pro-Caspase-3 (1 : 1000 Millipore), rabbitanti-Parp (1 : 400 Abcam), rabbit phospho-Smad1/5/8 (1 : 1000 Cell Signaling),rabbit Smad1 (1 : 1000 Cell Signaling), rabbit Smad4 (1 : 1000 GeneTex, Irvine, CA,USA). Antibody protein complexes were detected by HRP-conjugated antibodiesand ECL (both from Amersham Pharmacia, Milan, Italy).

Chromatin immunoprecipitation (ChIP)-qPCR analysis

For ChIPqPCRanalysis, ESCs were treated with 20 ng/ml BMP4 for 1 h and then werecross-linked with 1% formaldehyde for 10 min at room temperature and then with125 mM glycine. The chromatin was then sonicated to an average DNA fragmentlength of 500–1000 bp. Soluble chromatin extracts were immunoprecipitated usingan anti-Phospho-Smad1,5,8 (Cell Signaling) antibody. Appropriate IgGs were usedas negative control. Supernatant obtained without an antibody was used as an inputcontrol. After qPCR, the amount of precipitated DNA was calculated relatively to thetotal input chromatin and expressed as percentage of total chromatin or as foldenrichment relative to untreated samples. Oligonucleotide pairs are listed inSupplementary Table S1.

FACS analysis

Analysis of DNA content by propidium iodide incorporation was performed in permeabilized cells by flow cytometry. SFEBs at 4 days ofdifferentiation were dissociated and 2 × 104 cells were collected, washed in PBSand resuspended in 200 μl of a solution containing 0.1% sodium citrate w/v, 0.1%Triton X-100 v/v and 50 μg/ml propidium iodide (Sigma Chemical, Perth, WA,Australia). After incubation at 4 °C for 30 min in the dark, cell nuclei were analyzedwith a FACScan flow cytometer (Becton Dickinson, Milan, Italy). Cellular debris wasexcluded from the analysis by raising the forward scatter threshold, and the DNAcontent of the nuclei was registered on a logarithmic scale. The percentage of theelements in the hypodiploid region was calculated.Phosphatidylserine externalization was investigated by annexin V staining. In brief,SFEBs at 4 days of differentiation were dissociated and 1 × 105 cells were collectedand resuspended in 100 μl of binding buffer (10 mM Hepes/NaOH pH 7.5, 140 mMNaCl, and 2.5 mM CaCl2) containing 5 μl of annexin V-FITC (Pharmingen/BectonDickinson, San Diego, CA, USA) for 15 min at room temperature in the dark. Then,400 μl of the same buffer was added to each sample and the cells were analyzed witha Becton Dickinson FACScan flow cytometer.

Immunostaining, bromodeoxyuridine assay and microscopy

Undifferentiated ESCs and EpiSCs were fixed in 4% paraformaldehyde andpermeabilized with 0.2% Triton X-100 in 10% FBS (Invitrogen)/1% BSA in 1X PBSfor 150 at room temperature. Thus, the samples were incubated with primaryantibodies and with an appropriate secondary antibody reported below. SFEBs werecollected at the indicated differentiation day fixed in 4% paraformaldehyde anddehydrated with increasing percentages of ethanol. Samples were embedded inparaffin, sectioned in 7-μm slices and mounted on glass slides. After rehydrationand permeabilization, the samples were treated as previously described.20 Nucleiwere counterstained with DAPI (Calbiochem, 1 : 1000) or, for confocal analysis, withDRQ5 (Cell Signaling, 1 : 1000) as indicated in the Figures. The following primaryantibodies were used: anti-Oct3/4 (1 : 200), anti-Nanog (1 : 500; Calbiochem), anti-Sox1 (1 : 100) and anti-cleaved caspase 3 (1 : 300). Alexa Fluor 594 or488 secondary antibodies were used (1 : 400; Invitrogen). For BrdU experiments,subconfluent ESCs were incubated in ESC medium containing BrdU for 1 h, andthen the cells were processed for immunofluorescence with BrdU labeling anddetection kit (Roche, Basel, Switzerland). Images were captured with an invertedmicroscope (DMI4000; Leica Microsystems, Heidelberg, Germany) with LeicaApplication Suite Advanced Fluorescence (LAS AF) software (Leica Microsystems).Confocal microscopy was performed with a Leica TCSSMD FLIM microscope (LeicaMicrosystems) using LAS AF software (Leica Microsystems). When required, thebrightness, contrast and color balance of the images were adjusted in PhotoshopCS2 (Adobe Systems, San Jose, CA, USA).

Statistical analysis

All values represent the means±S.E. of at least threeindependent experiments. qPCR data are presented as fold change relative to theindicated reference sample. Whenever necessary, statistical significance of the datawas analyzed using Student's t -test and the P-Value was reported in the figures.

Name[a]	Forward primer	Reverse primer
miR-125a	GTTTCTGTCTCGCTTCCCCGTTC	CAAATGATGGTCAAGCATCAGG
miR-142	GTCCCTGGGAAGTTACACGG	GATTCTTCAGGCCACCTGCT
miR-187	CTGGGTACACAGCAGATGGG	TATCCTGTGAGCCCAGGGAG
miR-193b	CTGTCTCTAGGGCAAGGCTG	TCTCTGAAGTGAGCCCCTCT
miR-196a-1	TGGTTAACTAGCAGCGGACC	GTGAGCTAAAATGGCCACGC
miR-196a-2	TCCTCAGGGAGGTTGTAGGG	GGGCTTGGGGAAGGAAGAAA
miR-199a	AGAAGAGTTCAGGGTGCGTG	TGTTCAGTTCCTCAGCGTGG
miR-211	TAGCCTGAGCCAAGAGCAAG	CGTCAGGATCATGGCGACTT
miR-23a/24-2/27a	ACTGGTGCATTCGGAAACCT	GGAGCATTCTTGCTTGCCTG
miR-23b/24-1/27b	TGGGGGTGTGGTTCTTTGTC	ATCATCTTGCCAGCGACTGT
miR-29b-1	AGTGACCGACATGTCACACC	GAGGGAAGGTGAAGTCCGTG
miR-29b-2	TGTCATCTGCGTCTGACAGG	GCAGTCAGAAGAACCAGCCT
miR-302d	GTCTTTCACCCTCCGAGGAC	TCCTTTACCTTCGTGACCGC
miR-324	TTCGAGGCTTCCGACTTTGT	GCTAAATGCTGCCCAGATGC
miR-339	GCCGCATCTGTCATCTTTGTC	CCACCTAGGGATCCCCTTCT
miR-362	CAGAGCGCATCTCTCGTTCT	CCCTTCCTGTGTGTGGTCAA
miR-379	CACCGTGCAACCATTCAAGG	TCTGAGGCCTGATTTGGCAG
miR-503	CTCCTCCCCACTATCCACCA	GGATCTGCTGGTGAGGCAAA
miR-582	ACAAAGGAAGCTCCGCCAAT	GCACGGCTTGTGTTTGAGAA
miR-9-1	TCCCATCTTTTCGCCTCCAC	CTCCGTAGTCTCGAGTCGCT
miR-9-2	GCTTAAACGCGGCAAGTACC	CCTTCCTCTGGCTGAACTCC
miR-9-3	TCCGTGTGTCTGTGTGTCTG	GACTCCGCTCCCGCAG
1	CAACCCTGTAGCGACTCTGG	CCGGTATGGGGCCTAGTGTA
2	TTGGCACTCTGCATCTGAGG	ACTCAGGCGTTTACTCAGGC
3	TTTTGTTCCTGGCCCCCTAC	CCCTCCACCATCAGGAACAC
4	CTTGGCTCCCTGTACCTGTC	TTACCTTCTCAGGCCACCCT
5	GGTGTGTCTTCCTAGCTGGC	GCATGATGCTGTCCTTCCCT
6	GAAGAGTGGGCCATTAGCGA	AAGCAGCGAGGATCGGAAAA
7	TGTGAGCCGGGAAACCAAAT	ACAAGTTCAGGGCTGGAAAGA
8	TGCAGACAGAGACTTTGCCA	GTCAGCCTGCATGTGAGCTA
9	GAGCATCTTGTGGCTTCCCT	GCTTGTGTTGTCCCCAGGTA
10	CACTTGCTGGATCAGCTTGC	TGGGGCCTACCCAATGTAGA
11	ACTGGTGCATTCGGAAACCT	GGAGCATTCTTGCTTGCCTG
Dax1	AGATGGAGAAAGCGGTCGTA	AAGCCAGTATGGAGCAGAGG
Dnmt3b	CCAAGGACACCAGGACGCGC	TCCGAGACCTGGTAGCCGGAA
Fgf5	TCCATGCAAGTGCCAAATTTACGGA	TTCTGTGGATCGCGGACGCA
Gapdh	GTATGACTCCACTCACGGCAAA	TTCCCATTCTCGGCCTTG
Id1	TCCGCCTGTTCTCAGGATCA	GTAGCAGCCGTTCATGTCGT
Id3	CGACATGAACCACTGCTACT	CTCCTCTTGTCCTTGGAGAT
Nanog	TCAGAAGGGCTCAGCACCA	GCGTTCACCAGATAGCCCTG

Oct3/4	AACCTTCAGGAGATATGCAAATCG	TTCTCAATGCTAGTTCGCTTTCTCT
Otx2	TTCCGTCACTCCAAATCTACCCA	GCCGGACGGTCTCGATTCGCCTGGAGT
Pax6	AGTGAATGGGCGGAGTTATG	ACTTGGACGGGAACTGACAC
Rex1	GCAGTTTCTTCTTGGGATTTCAG	CTAATGCCCACAGCGAT
Smad3	CTCCAAACCTCTCCCCGAAT	GACTGGCTGTAGGTCCAAGT
Smad4	CCACAGGACAGAAGCGATTG	ACTAAGGCACCTGACCCAAA
Smad5	AGGAACCTGAGCCACAATGA	GAAAGGAGCGTTGTTGGGTT

[a]The primer name is reported as indicated in the Figures.

Table 2. Primers used for Real-Time PCR

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