EUROPEAN SCHOOL OF MOLECULAR MEDICINE

SEDE DI NAPOLI

UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

Ph.D. in Molecular Medicine – Ciclo VII/XXV

Molecular Oncology

"miRNAs in the regulation of Embryonic Stem Cell differentiation"

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Academic Year: 2012/2013

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Abstract

Embryonic Stem cells (ESCs) have the unique characteristics of self-renew and differentiate into all the cells derived from the three germ layers. These properties make them a limitless source of specialized cells for replacement therapies. However, the knowledge of the mechanisms controlling ESC differentiation into lineage-specific derivatives is necessary before using them for therapeutic purposes. Extracellular signalling, as that of bone morphogenetic protein 4 (BMP4), plays an important role in maintaining ESCs in undifferentiated state and in regulating the lineage commitment. Recently, it was identified a transmembrane protein, named Dies1, which suppression blocks ESC differentiation by interfering with the BMP4 signalling. Over the past few years, it has become evident the involvement of miRNAs in the ESC fate. Thus, we investigated whether a physiological modulation of Dies1 level by miRNAs could be a mechanism regulating ESC choice between pluripotency and differentiation. We demonstrated that miR-125a and miR-125b control Dies1 expression targeting its 3' UTR. Their overexpression impairs ESC differentiation, maintaining the cells in the epiblast state. This effect is due to a reduction of BMP4 signalling and a concomitant increase of Nodal/Activin pathway. This phenotype recapitulates that of Dies1 KD ESCs and is mediated by Dies1 suppression. Moreover we found that Dies1 is associated with BMP4 receptor complex and that BMP4 itself induces the expression of miR-125a at transcriptional level. This miRNA, in turn, controls BMP4 activity through Dies1 regulation. These results show that a feedback loop exists to set ESC sensitivity to BMP4, and it is mediated by miR-125a and Dies1. Interestingly, we found that miR-125b, opposite to miR-125a, is not directly regulated by Transforming Growth Factor-β (TGF-β) signals. These results demonstrate a new role of miR-125a and miR-125b in the regulation of the transition of ESCs to the epiblast stage, working on the control of TGFβ signalling.

Introduction

The discovery of Embryonic stem cell (ESC) potential to self-renew and to differentiate in all the three primary germ layer derivatives opens new hope for regenerative medicine. More recently, it was discovered that both mouse and human somatic cells can be reprogrammed to a pluripotent state that resembles that of ESCs, by ectopic expression of four transcription factors, Oct4, Sox2, c-Myc and KLF4 (Takahashi, et al., 2006). These ES-like pluripotent cells are known as induced pluripotent stem cells (iPSCs). This finding makes possible to take somatic cells from adults, reprogram them in vitro and differentiate in clinically relevant cell types. In this way the idea of cell transplantation-based regenerative medicine is closer to reality, with the big advantage of overcoming the immunogenicity and ethical controversy of ESCs (Watabe, et al., 2009). It is evident that ESCs represent a limitless source of cells useful for treatment of degenerative diseases and replacement therapies, but their plasticity makes them difficult to be manipulated. For these clinical applications, it is necessary to deeply understand the cellular and molecular mechanisms regulating ESC pluripotency and lineage commitment. Despite their complexity, ESCs have the big advantage to recapitulate *in vitro* the crucial steps of embryo development, allowing a more detailed study of cell fate decisions.

During pre-implantation development, the morula is made of two layers of cell: the trophectoderm (outside) and the inner cell mass (ICM) (inside). The trophectoderm gives rise to the placenta while the ICM will give rise to the developing embryo and associated yolk sack, allantois and amnion (Rossant, et al., 2004). These two cell populations express specific marker genes, such as Cdx2 and Eomes for trophoblast (Strumpf, et al., 2005) and Oct4 (Nichols, et al., 1998) and Nanog (Chambers, et al., 2003) (Mitsui, et al., 2003) for the ICM. At the blastocyst stage, the ICM is localized to one side of the cavity

known as the blastocoels, formed by fluids secreted by the trophectoderm. At this point, two lineages are segregated from the ICM: the primitive endoderm or hypoblast and the epiblast. The primitive endoderm will only contribute to extraembryonic tissues and it is distinguished by the expression of Gata4 and Gata6 markers. The epiblast is responsible for the generation of the three primary germ layers (Niwa, 2007) and it will express the pluripotency markers Oct3/4 and Nanog. During this process, epiblast cells egress through the primitive streak (PS), a transient structure that will form the posterior end of the embryo. The first mobilized epiblast cells pass through the PS and give rise the extraembryonic mesoderm, that will form the allantois, the amnion and the hematopoietic, endothelial and vascular smooth muscle cells of the yolk sac (Figure 1). In the following steps of gastrulation, cells migrate through the most anterior part of PS to generate the mesoderm, characterized by the expression of the specific marker Brachyury (T), and the definitive endoderm. The mesoderm will form the hematopoietic, vascular, cardiac, and skeletal muscle lineages, while the endoderm will develop towards organs like the stomach, the liver, the pancreas, the lungs etc. The ectoderm derives from the anterior region of the epiblast that doesn't enter the primitive streak, and will form the skin and the neural lineages (Murry, et al., 2008).

To further define all the processes and stimuli that specify ESC fate, it is possible to study, *in vitro*, the embryonic development, using ESCs derived from the inner cell mass (ICM) of the pre-implantation embryo. These cell retain their pluripotency *in vitro*, being able to generate cells of all lineages, including the germ line, after being introduced into host blastocysts (Keller, 1995). More recently, another pluripotent stem cell population was discovered. It was isolated from the post-implantation epiblast tissue and so named Epiblast stem cell (EpiSC) (Brons, et al., 2007). These cells are still pluripotent and can self-renew: indeed, they have the ability to generate derivatives of all three germ layers both during *in vitro* differentiation and *in vivo* teratoma formation, but they are inefficient in the formation of chimeras.

EpiSCs still express the pluripotency factors Oct3/4 and Nanog, but they have also a peculiar gene expression profile and a different dependence from extracellular signalling for maintenance. EpiSCs require high level of Activin and Fgf2 signalling, but not LIF and BMP4 as for ESCs. Moreover they have already undergone X-inactivation, suggesting a more advanced developmental stage (Chou, et al., 2008). Indeed, EpiSCs are said to be already 'primed' to differentiation, while ESCs are in a 'naïve' state.

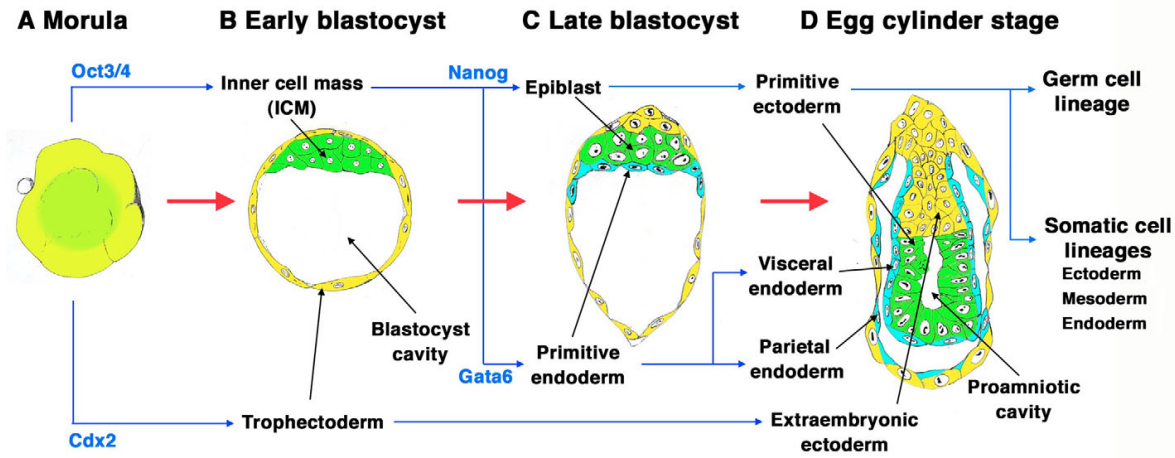

Figure 1. A schematic view of mouse preimplantation development.

(A) Pluripotent stem cells (green) are imaged in a morula as the inner cells, which (B) then form the inner cell mass (ICM) of the blastocyst. (C) After giving rise to the primitive endoderm on the surface of the ICM, pluripotent stem cells then form the epiblast and start to proliferate rapidly after implantation. (D) They then form the primitive ectoderm, a monolayer epithelium that has restricted pluripotency which goes on to give rise to the germ cell lineage and to the somatic lineages of the embryo. Certain key transcription factors (blue) are required for the differentiation of the various embryonic lineages (Niwa, 2007).

ESCs can differentiate going through an epiblast state. Three different protocols have been developed to control ESC differentiation in a specific lineage, using the proper culture conditions (Figure 2) (Keller, 2005). With the first method, ESCs are allowed to aggregate and form three-dimensional

colonies known as embryo bodies (EBs) (Doetschman, et al., 1985). They form a multidifferentiated structure in which the developmental program of ICM/epiblast cells is reactivated. Cellular differentiation proceeds on a schedule similar to that in the embryo but in the absence of proper axial organization or elaboration of a body plan (Doetschman, et al., 1985). With the second method, ESCs are cultured directly on stromal cells, and differentiation takes place in contact with these cells. It is also possible to use a medium conditioned by other cell types in which culture ESCs (Nakano, et al., 1994). The disadvantage of this method is that factors produced by supportive cells may influence the differentiation of ESCs to undesired cell types. The third protocol of differentiation is based on growing ESCs in a monolayer on extracellular matrix proteins (Nishikawa, et al., 1998). In this way, the influence of supportive stromal cells can be minimized.

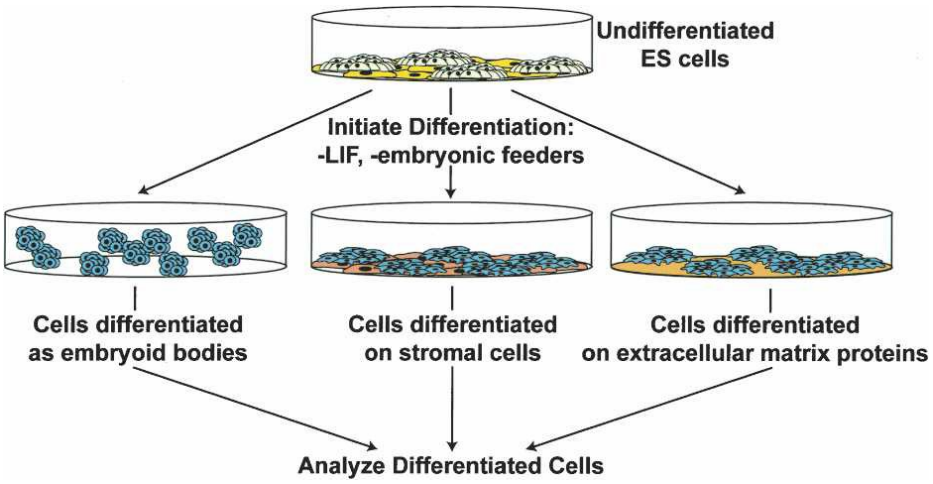

Figure 2. Three different protocols used for ESC differentiation.
(Keller, 2005)

Clearly, the ability of ESCs to give rise to a variety of different cell types suggests the complex network of regulatory mechanisms that control this process. To date a lot of studies have highlighted the mechanism of maintenance of ESC pluripotency. In particular, a strong contribution is given by the coordinated action of extracellular signalling, transcription factors, epigenetic modifications and, more recently, miRNAs.

1. Extracellular signalling

Initially, mESCs were derived and cultured on a layer of mitotically inactivated fibroblasts in serum-containing medium (Evans, et al., 1981). It was thought that fibroblasts support self-renewal providing trophic factors and acting as a feeder. To date, it is known that ESCs depend on the signalling of growth factors to maintain pluripotency. The most important extracellular signals controlling self-renewal and differentiation are LIF, TGF-β and WNT. However, the requirement for a particular signal may be context-dependent and a specific growth factor can have different role depending on the developmental stage of ESCs. For example, BMP4 normally induces differentiation of ESCs, but behaves as a self-renewal signal in the presence of LIF (Zhang, et al., 2010). It indicates that the pluripotency is a more complex mechanism controlled not simply by one or more extracellular signals, but also by their synergic action with transcription factors.

1.1 The LIF pathway and its connection to ERK1/2 signalling

The addition of leukemia inhibitory factor (LIF) to serum-containing medium is necessary to support self-renewal. LIF is an IL-6 family cytokine that signals through a receptor complex made of the transmembrane protein gp130 and the low-affinity LIF receptor β (LIFRβ) (Boulton, et al., 1994). Ligand binding induces the receptor dimerization and the activation of two main signalling pathways. Through its intracellular domain, gp130 recruits and activates the Janus-associated tyrosine kinase (JAK), which in turn phosphorylates tyrosine residues on gp130. This modification generates a binding sites for the signal transducer and activator of transcription 3 (STAT3) that is substrate for tyrosine phosphorylation mediated by JAK. Following phosphorylation, the transcription factor STAT3 dimerizes and translocates to the nucleus where it modulates transcription of target genes (Darnell, et al., 1994) (Figure 3). It was demonstrated that activation of STAT3 is required for self-renewal, since it can support stemness

maintenance also in the absence of exogenous LIF (Matsuda, et al., 1999). STAT3 exerts this function acting on target gene expression. A well characterized target of STAT3 is cMyc. Indeed, sustained cMyc expression can maintain stemness and inhibit ESC differentiation induced by LIF withdrawal (Cartwright, et al., 2005). Among the other identified targets of STAT3, a relevant role is played by Socs3 and Klf4. The first one was demonstrated to be a negative regulator of LIF signalling, acting through a negative feedback loop. Instead, Klf4 can promote ESC self-renewal, since its overexpression in ESCs leads to the block of differentiation (Li, et al., 2005).

On the other hand, phosphorylated gp130 can associate to SH2 domain containing proteins including Shp2 and Grb2. They, in turn, recruit SOS protein activating Ras and the mitogen activated protein kinase (MAPK) cascade. In particular, the effectors of this signalling are the extracellular signal-regulated protein kinase-1 and -2 (ERK1/2), that can enter in the nucleus where activate various TFs (Marais, et al., 1993). The role of ERK1/2 in ESCs is to negatively control self-renewal as demonstrated by the finding that preventing the activation of ERK1/2 by LIF, ESC self-renewal is enhanced (Burdon, et al., 1999). Moreover the ERK signalling promotes the transition of ESCs in a stage corresponding to the egg cylinder epiblast, responsive to inductive cues for germ layer segregation (Kunath, et al., 2007) (Stavridis, et al., 2007).

This result indicates that LIF signalling can activate two pathways giving opposite effects on self-renewal determining a balance between STAT3 and ERK1/2 signalling. The preferential activation of one way respect the other will define the choice between self-renewal and differentiation. Clearly, this is achieved by the cooperation of LIF signalling both with the other signalling and the transcriptional machinery.

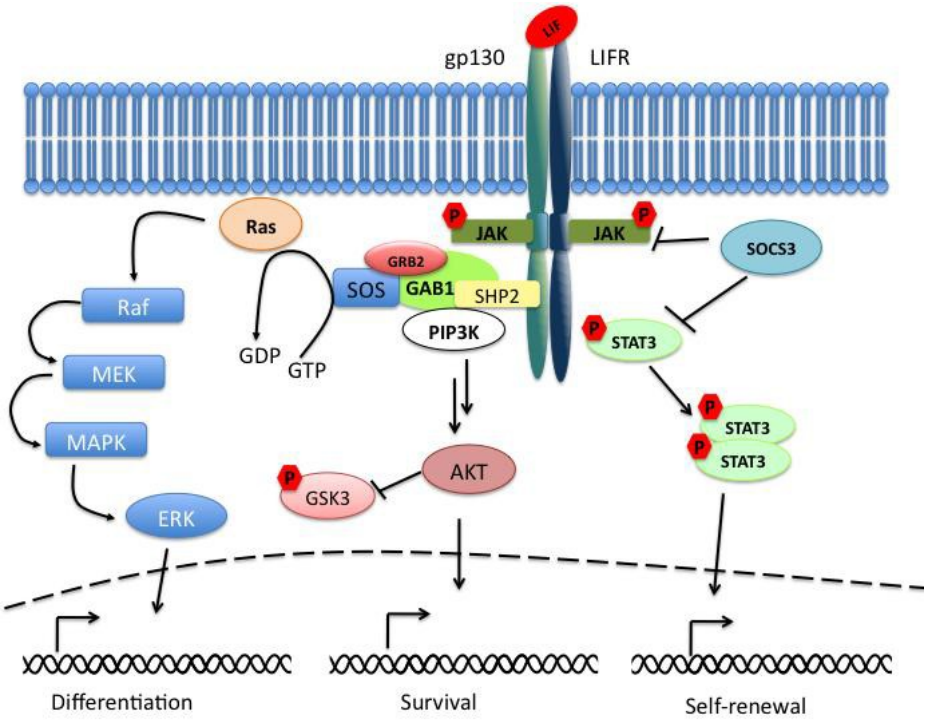

Figure 3. Schematic representation of the LIF-pathway
(Grafl, et al., 2011)

1.2 TGF-β superfamily signalling

Ying and collaborators observed that the presence of serum in the culture medium is necessary for self-renewal. Indeed, ESCs undergo neural differentiation simply by removing serum from the medium, even in presence of LIF. This finding suggests that other factors contained in the serum are crucial to inhibit neural differentiation sustaining self-renewal. In particular, they demonstrated that addition of BMP4 to serum-free medium containing LIF could support self-renewal (Ying, et al., 2003). BMP belongs to the transforming growth factor-β (TGF-β) superfamily, together with TGFs, Nodal, Activin. TGF-β ligands regulate different processes during embryogenesis, as the establishment of the body plan in the embryo (Wu, et al., 2009). They also play a central role in maintenance of ESC identity as proven by the observation that Smad4 deficient mouse embryos display

delayed outgrowth of the inner cell mass (Sirard, et al., 1998). At cellular level the TGF-β system is involved in the regulation of cell proliferation and growth arrest, pluripotency and differentiation, cell survival and apoptosis. These activities depend on cellular context, in particular on the stage of target cell, the local environment, and the identity and dosage of the ligand (Seuntjens, et al., 2009).

TGF-β ligands signal through serine/threonine kinase receptors, made of heterodimers of type I and type II receptors. In mammals, there are 7 type I receptors and 5 type II receptors, that exist as homodimers on cell surface in absence of the ligand. The binding of the ligand to one of the two types of receptors induces the association of type I and II dimers, forming an heterotetrameric complex. The ligand can bind preferentially one of the two receptors. For instance, TGF-βs and Activins bind to type II receptors, whereas BMPs associate to type I homodimers before forming a complex with type II receptors. However, following ligand binding, type I receptor is activated by phosphorylation from type II receptor and can now recruit receptor-regulated Smads (R-Smads). Each type I receptor can activate only a subset of R-Smads, and this peculiarity generate two distinct pathways among the TGF-β superfamily signalling. In particular, the ligands of BMP/GDF family signal through R-Smads as Smad1, Smad5 and Smad8, activated by Alk1, Alk2, Alk3 and Alk6 type I receptors. Instead, the ligands of TGF-β/Nodal/Activin family bind to Alk4, Alk5 and Alk7 type I receptors activating R-Smads as Smad2 and Smad3. Once recruited to the receptor complex, R-Smads are phosphorylated on serine residues by type I receptor. This modification allow them to form homodimers which complex with Smad4, the common mediator shared by both BMP and Nodal/Activin pathway. This complex then translocates to the nucleus where regulates the expression of target genes specific of each branch of TGF-β superfamily, so determining a peculiar cellular response (Schmierer, et al., 2007) (Figure 4).

This intricate signalling needs to be finely regulated. A first regulation occurs at level of ligand-receptor interaction. For example, some extracellular inhibitory proteins exist that sequester the ligands from the binding to their receptor (Follistatin, Chordin, Noggin, Caronte, Cerberus) (Massagué, 2000). In contrast, several co-receptors have been identified as necessary for ligand binding to the receptor acting as co-activator, such as Betaglycan, Endoglin, DRAGON and the EGF-CFC (Epidermal Growth factor–Cripto–FrL1–Cryptic) family member Cripto (Feng, et al., 2005). Dragon was identified as a BMP co-receptor, while Cripto is necessary for nodal signalling. The TGF-β superfamily pathways are modulated also through a negative feedback loop involving the induction of the inhibitory Smads (I-Smads), Smad6 and Smad7, by BMP and TGF-β (Schmierer, et al., 2007). Ubiquitin-proteasome mediated degradation of Smads is another way to control R-Smad levels and the sensitivity of cells to incoming signals. Indeed, Smurf1 (Smad ubiquitination-regulatory factor 1) and Smurf2, belonging to the family of HECT (homologous to the E6-AP carboxy terminus) E3 ubiquitin ligases, antagonize TGF-β family signalling by interacting with R-Smads and targeting them for degradation. Particularly, Smurf1 interacts with Smad1 and Smad5, thereby affecting BMP responses, whereas Smurf2 interacts with different R-Smads, allowing interference with BMP and TGF-β/activin signalling (Derynck, et al., 2003).

1.2.1 BMP

The relevance of BMP in ESCs is demonstrated by the suppression of its receptor Alk3 (also known as BMPRIA). Mice homozygous for this null allele died at embryonic day 8.0 (E8.0) without mesoderm formation. Mishina and collaborators proposed that the primary defect caused by this mutation could be in the regulation of epiblast-cell proliferation of egg cylinder-stage embryos (Mishina, et al., 1995).

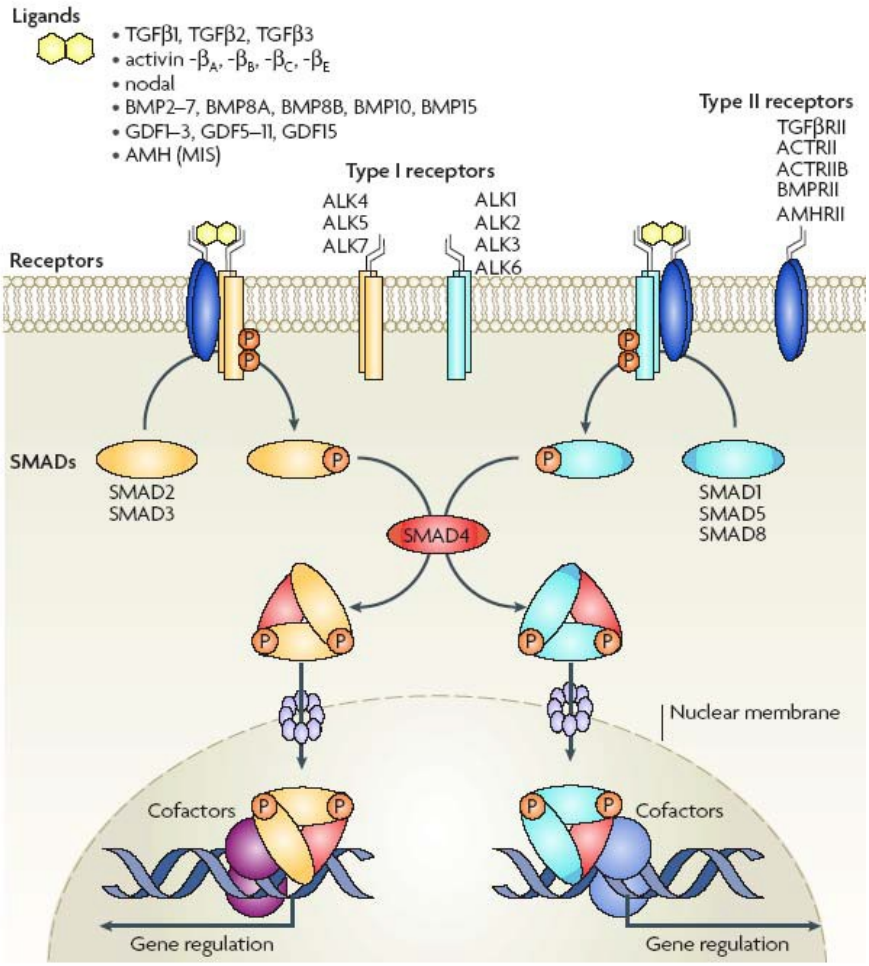

Figure 4. Core signalling in the mammalian TGFβ–SMAD pathways

Binding of ligands to type II receptors and recruitment of type I receptors activate the signalling. The phosphorylation of type I receptors allows the recruitment of R-SMADs that are in turn phosphorylated. TGF-β1, Activin and Nodal signal through type I receptors Alk4, 5 and 7 binding to induce SMAD2,3 phosphorylation, whereas BMPs bind to Alk1, 2, 3 and 6 that induce SMAD 1,5,8 phosphorylation. Once activated, R-Smads associate to the common mediator SMAD4 and translocate to the nucleus where modulate gene expression (Schmierer, et al., 2007).

BMP exerts its function inducing the expression of several target genes. In mouse ESCs and in various cell types, the classic BMP targets are the Inhibitor of differentiation (Id) family proteins. IDs suppress precociously expressed neurogenic basic helix-loop-helix (bHLH) transcriptional activators

and thus suppress neural differentiation, leading to strengthened self-renewal (Li, et al., 2013). Ectopic expression of Id1, Id2 and Id3 in ESCs maintains self-renewal in serum-free culture, even remaining LIF-dependent. This result indicates that the contribution of BMP/Smad in stemness maintenance is to induce Id expression.

BMP signalling cooperates with LIF and ERK pathway in determining ESC fate. In neuroepithelial cells, gene expression is regulated by a ternary transcription factor complex made of Smad, STAT3 and p300 (Nakashima, et al., 1999) (Sun, et al., 2001). Ying demonstrated that also in ESCs STAT3 associates with Smads, driving the specificity of target genes. He suggested that the formation of the STAT3/Smad1 complex may play a key role by limiting the availability of active Smad1 for partnering with other cofactors. Effective Smad action may be restricted by STAT3 to a subset of targets, notably Id genes, that are either receptive to STAT3/Smad complex or are inducible by low levels of Smad. In this scenario, withdrawal of gp130 stimulation would release active Smad to complex with transcriptional coactivators that drive recruitment to differentiation genes (Ying, et al., 2003). An evidence of cooperation between BMP and ERK pathways comes from the finding that BMP can induce the dual-specificity phosphatase 9 (DUSP9, also known as MKP-4), an ERK-specific phosphatase, inhibiting ERK activity. In this way, extrinsic BMP stimulus affects intrinsic ERK activity through DUSP9 (Li, et al., 2012).

Recently, it was found the existence of a BMP4-sensitive window during mouse ESC neural commitment. Cells at this stage correspond to the epiblast of the egg cylinder, and can be maintained as ESC-derived EpiSCs (ESD-EpiSCs). Moreover BMP4 was demonstrated to have an inhibitory role during ESC neural differentiation, acting at two different phases. First of all, it inhibits the derivation of ESD-EpiSCs (ESC derived-Epiblast Stem Cells) from mouse ESCs; and second, it suppresses the neural commitment of ESD-EpiSCs and promotes their non-neural differentiation (Zhang, et al., 2010).

1.2.2 Dies1, a new regulator of BMP4 signalling

In 2010, Aloia and colleagues, in the laboratory where I carried out my research project, discovered a new gene involved in BMP4 signalling. A screening based on RNA interference to find molecules regulating ESC fate allowed them to identify an unknown gene named Dies1 (Differentiation of ES cells 1) (Aloia, et al., 2010). Dies1 KD ESCs were not able to differentiate either toward neurons or cardiomyocytes. Even in forced differentiation conditions, Dies1 knockdown induces maintenance of the stemness markers of ESCs, strongly suggesting the important role of Dies1 in ESC differentiation. Dies1 encodes a transmembrane protein containing a signal peptide and a V-type Ig-like domain in the extracellular N-terminus region. In this region three possible Asn-glycosylation sites are present, indeed the glycosylation of Dies1 was addressed. Aloia et al. found that Dies1 downregulation reduces the induction of Id proteins (BMP4 pathway) increasing Nodal/Activin targets, whereas it doesn't affect LIF pathway. They concluded that this unbalance between BMP4 and Nodal/Activin is responsible for the phenotype observed upon Dies1 knockdown. This study demonstrated an involvement of Dies1 in the regulation of ESC differentiation and in the BMP4 signal transduction machinery, through the modulation of extracellular signalling (Aloia, et al., 2010).

Given the importance of BMP4 signalling in ESC fate, this work opens a new field of study aimed at unraveling of the molecular mechanisms in which Dies1 takes part together with BMP4 in the definition of ESC pluripotency.

1.2.3 Nodal/Activin

Nodal and activin contribute to the maintenance of mESC identity, as demonstrated by the reduction of pluripotent cell propagation in early embryogenesis of many Nodal-related mutant mice. For example, Nodal null mice display very little Oct3/4 expression and substantial reduction in the size

of epiblast cell population (Conlon, et al., 1994) (Robertson, et al., 2003). Smad2 knockout mouse embryos fail to form mesoderm and endoderm due to defects in primitive streak specification after implantation at 6.5 dpc, closely phenocopying Nodal mutants (Nomura, et al., 1998).

In cell culture, the importance of activin-Nodal-TGF-β signalling was highlighted by the observation that its inhibition by Smad7 expression or by the specific inhibitor SB-431542 dramatically decreases mESC propagation. In clonal cultures with serum free medium, supplementation of recombinant Nodal and activin increased the ESC proliferation ratio with maintenance of the pluripotent state. These findings indicate that Nodal and activin signalling promotes mESCs propagation with maintenance of pluripotent state in serum-free conditions (Ogawa, et al., 2007).

Nodal and Activin exert their function modulating the expression of a specific subset of genes. It was proposed that a graded Nodal/Activin signalling could determine a different intracellular response. In particular extracellular signalling gradients are translated into a gradient of Smad2 phosphorylation that can activate different target genes in a dose-dependent manner. An exchange of transcriptional co-partners allows the shifting of the pSmad2 transcriptional complex to different target gene subsets. The consequence is that a relatively modest stimulation with Activin leading to a physiological increase in Smad2 phosphorylation eventually drives mesendodermal differentiation, while the inhibition by SB-431542 resulting in a decrease of pSmad2 is able to promote trophectoderm cell fates (Lee, et al., 2011).

1.3 Wnt Signalling

Wnt signalling has been implicated in ESC fate, since it can interact with LIF, regulating STAT3 transcription (Hao, et al., 2006) and with BMP4, mediating cyclin D1 induction (Lee, et al., 2009). The central mediator of Wnt signalling is β-catenin. In absence of ligands, β-catenin is phosphorylated by two

kinases, casein kinase 1a (CK1a) and glycogen synthase kinase 3β (Gsk3β), which are associated with the so-called 'destruction complex', formed by APC and axin. Upon phosphorylation, β-catenin is ubiquitinated and degraded via the proteasome pathway (Wray, et al., 2012). The kinase responsible for β-catenin phosphorylation is GSK3β, so direct inhibition of GSK3β mimics Wnt signalling (Patel, et al., 2004). The identification of a novel GSK3β inhibitor, BIO, as a sustenance to stemness (Sato, et al., 2004) has given rise to the idea that Wnt signalling can support the self-renewal of pluripotent cells.

Activation of the signalling occurs when Wnt ligands bind to receptor complex, composed of a serpentine receptor of the frizzled family and a coreceptor of the low-density lipoprotein receptor related protein family, Lrp5/6. The interaction between the ligand and its receptor complex results in the dissociation of the destruction complex, allowing β-catenin to translocate into the nucleus. In the nucleus, β-catenin acts as a coactivator for transcription factors of the Tcf/Lef (T cell factor/ lymphoid enhancing factor) family (Wray, et al., 2012) (Figure 5). The member of this family, Tcf3, has a central role in Wnt signalling in ESCs: indeed, it can repress Nanog gene expression (Pereira, et al., 2006). A genome-wide analysis revealed that Tcf3 co-occupies the ESC genome with the pluripotency transcription factors Oct4 and Nanog. Moreover, it was found that, under standard culture conditions, Tcf3 may exist in an activating or repressive complex, but is predominantly in a repressive complex promoting differentiation. Following Wnt stimulation, the complex is converted to an activating form that promotes pluripotency. In this way the Wnt pathway, through Tcf3, influences the balance between pluripotency and differentiation by bringing developmental signals directly to the core regulatory circuitry of ESCs (Cole, et al., 2008).

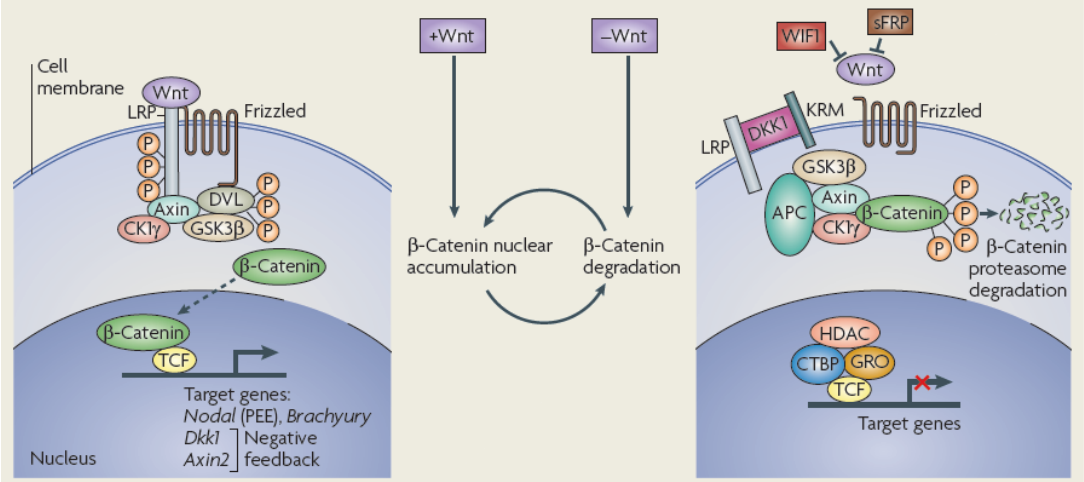

Figure 5. Schematic representation of Wnt pathway

In the absence of Wnt ligands (right panel), the presence of secreted inhibitors (Wnt inhibitory factor 1 (WIF1) and soluble frizzled-related proteins (sFRPs)) or the inhibition of LRP co-receptors (by secreted Dickkopf protein (DKK1)), the destruction complex, containing the core components axin, APC and GSK3β, recruits and phosphorylates β-catenin. Phosphorylated β-catenin is rapidly ubiquitinated and degraded by proteasome. When Wnt ligands bind to frizzled receptors (left panel), LRP co-receptors are phosphorylated by casein kinase 1γ (CK1γ) and glycogen synthase kinase 3β (GSK3β). The formation of a β-catenin 'destruction complex' is prevented and β-catenin can translocate to the nucleus. Nuclear β-catenin interacts with the T-cell-specific factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors to regulate target gene transcription (Arnold, et al., 2009).

2. Transcriptional networks

The property of pluripotency is conferred by the expression of a set of transcription factors, preventing differentiation. The master genes of pluripotency are Oct3/4, Sox2 and Nanog.

Oct3/4 (encoded by the Pou5f1 gene) is a member of POU-domain transcription factor family expressed in mouse blastomeres, epiblast cells and primordial germ cells (PGCs). Its crucial role in pluripotency was highlighted by the observation that mouse ESCs lacking Oct3/4 differentiate into trophectoderm, indicating that Oct3/4 is required to inhibit differentiation in

this specific lineage (Niwa, et al., 2000). Further experiments showed that this inhibition is due to the interaction of Oct3/4 with Cdx2 (Niwa, et al., 2005). Oct4 expression is continuously required for the maintenance of pluripotency but its expression is not sufficient, suggesting that interaction with other transcription factors is critical to Oct4 function (Niwa, et al., 2000). Among the Oct3/4 interactors, it was found Sox2. Sox2 has an high-mobility group box DNA-binding domain and it is expressed in the ICM and the extraembryonic ectoderm of pre-implantation blastocysts. It is known that Sox2 co-operates with Oct3/4 activating its target genes (Tomioka, et al., 2002). Moreover, Oct3/4 and Sox2 complex regulates their own expression (Chew, et al., 2005): Oct3/4 and Sox2 binding sites are located in the promoter regions of these two genes, generating a feedback loop to control the pluripotency state. To strongly support that pluripotency is the result of a network of transcriptional factors, it was demonstrated that Oct3/4 and Sox2 complex controls the expression of another key pluripotency gene, Nanog (Rodda, et al., 2005).

Nanog is an homeobox transcription factor essential for ESCs: indeed, Nanog-overexpressing ESCs are maintained in a pluripotent state even in absence of LIF (Chambers, et al., 2003). Moreover, Nanog-null ESCs were found to acquire a parietal endoderm-like morphology and express high level of Gata6 marker (Mitsui, et al., 2003), indicating that Nanog can act as Gata6 repressor. A putative Nanog-binding site has also been identified in the enhancer region of Gata6 but has yet to be demonstrated to bind Nanog (Mitsui, et al., 2003). The impairment of primitive endoderm differentiation is not the only effect of Nanog. It was found that also neuronal differentiation induced by BMP4 and LIF removal is impaired by Nanog (Ying, et al., 2003). Finally, Nanog can interact with Smad1 repressing Brachyury (T), a mesoderm-specific gene (Suzuki, et al., 2006). All together, these studies indicate the importance of Nanog in the maintenance of ESC self-renewal, since it blocks the primitive endoderm, neuronal and mesodermal

differentiation. Recent studies indicated that Nanog is not expressed homogenously in ESCs: there is a sub-population of pluripotent ESCs not expressing Nanog. The absence of Nanog doesn't affect the pluripotent state and it is dispensable for the maintenance of ES cells (Chambers, et al., 2007). Chambers suggested that low levels of Nanog represent a "window of opportunity" in which ESCs can differentiate, if subject to specific environmental or intrinsic perturbations.

To further explore the transcription network of pluripotency, chromatin immunoprecipitation (ChIP) experiments have been performed to identify transcriptional targets of the three transcription factors. Genome wide analysis revealed that a lot of promoters occupied by one transcription factor would also be occupied by the others (Loh, et al., 2006). This suggests that they cooperate in the positive and negative regulation of target genes controlling a cascade of pathways that are intricately connected to govern pluripotency, self-renewal, genome surveillance and cell fate determination (Loh, et al., 2006) (Figure 6). Nanog expression is positively regulated by the members of the Kruppel-like transcription factors family, Klf2, Klf4 and Klf5, controlling ESC self-renewal (Jiang, et al., 2008) (Hall, et al., 2009). Klf4 is one of transcription factors used by Yamanaka to reprogram somatic cells to a pluripotent state (Takahashi, et al., 2006), and Klf2 and Klf5 are able to substitute for Klf4 function in the reprogramming (Nakagawa, et al., 2008). This indicates a redundant function for Klf2, Klf4 and Klf5. In our laboratory, it was demonstrated that Klf5 has unique functions. In absence of LIF, Klf5 downregulation induces ESC differentiation, whereas its ectopic expression maintains ESC pluripotency (Parisi, et al., 2008). The evidence of a specific requirement of Klf5 in early embryogenesis is given by the finding that Klf5 knockout mice show developmental defects at the blastocyst stage (Ema, et al., 2008). To better define the contribution of Klf5 to stemness maintenance, Parisi and colleagues identified primary targets of Klf5 in ESCs, by combining genome-wide chromatin immunoprecipitation and microarray

analysis. They found that Klf5 controls genes essential in ESCs such as Tcl1, BMP4 and Nr0b1, and thus it may be required to maintain pluripotency by activating expression of these self-renewal promoting genes and by simultaneously inhibiting expression of differentiation promoting genes such as TGFβ2, Otx2, Pitx2 and GDNF. Moreover many Klf5 targets are not regulated by Klf2 and Klf4 indicating the specificity of Klf5 in ESC self-renewal (Parisi, et al., 2010).

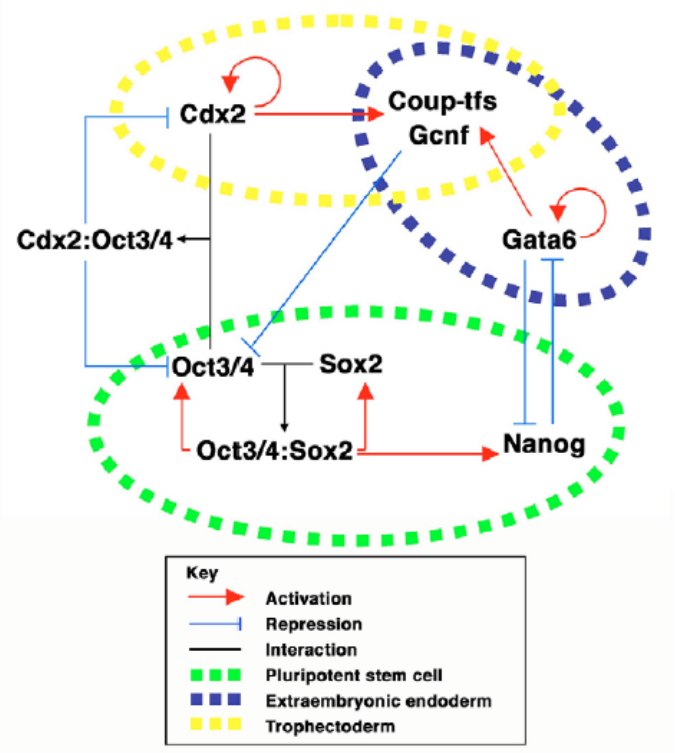

Figure 6. A transcription factor network to control ESC self-renewal and differentiation.

Transcription factor networks for pluripotent stem cells (green), trophectoderm (yellow) and primitive (extraembryonic) endoderm (blue). Positive-feedback loops between Oct3/4, Sox2 and Nanog maintain their expression to promote continuous ES cell self-renewal. Cdx2 is autoregulated and forms a reciprocal inhibitory loop with Oct3/4. A combination of positive-feedback loops and reciprocal inhibitory loops converts continuous input parameters into a bimodal probability distribution, resulting in a clear segregation of these cell lineages (Niwa, 2007).

3. Epigenetic modifications

Epigenetic regulation of gene expression is mediated in part by post-translational modifications of histone proteins, which in turn modulate chromatin structure (Jenuwein, et al., 2001). The core histones H2A, H2B, H3, and H4 are subject to many different modifications, including acetylation, methylation, and phosphorylation. The two more interesting histone modifications are histone H3 methylation on lysine 4 (Lys4) and lysine 27 (Lys27) since they are catalyzed, respectively, by trithorax and Polycomb-group proteins, which have key developmental functions. Lys4 methylation positively regulates transcription by recruiting nucleosome remodelling enzymes and histone acetylases, while Lys27 methylation negatively regulates transcription by promoting a compact chromatin structure (Bernstein, et al., 2006).

ESCs possess highly dynamic, decondensed chromatin (Meshorer, et al., 2006) and an interesting pattern of chromatin modifications. Large regions of the repressive histone mark, trimethyl histone 3 lysine 27 (H3K27me3), were found to harbour smaller regions of the active mark H3K4me3 and were termed 'bivalent domains' (Azuara, et al., 2006) (Bernstein, et al., 2006) (Figure 7). The coexistence of these marks suggests that lineage-specific genes are primed for expression in ES cells but are held in check by opposing chromatin modifications (Azuara, et al., 2006). Furthermore, the bivalent domains were enriched in regions encoding transcription factors that were not expressed or expressed at low levels. A recent study has demonstrated a link between the core transcription factors of pluripotency and epigenetic regulators (Loh, et al., 2006). Oct4 regulates the transcription of the H3K9 demethylases Jmjd1a and Jmjd2c. The first demethylates H3K9me2 while the second demethylates H3K9me3. Interestingly, Jmjd1a or Jmjd2c depletion leads to ES cell differentiation, which is accompanied by a reduction in the expression of ES cell-specific genes and an induction of lineage marker genes. Jmjd1a demethylates H3K9Me2 at the promoter regions of Tcl1,

Tcfcp2l1, and Zfp57 and positively regulates the expression of these pluripotency-associated genes. Jmjd2c acts as a positive regulator for Nanog, which encodes for a key transcription factor for self-renewal in ES cells. These experiments link the activity of Oct4 targets to modulation of the chromatin to facilitate expression of pluripotency-associated TFs (Niwa, 2007). It was found that ESCs can tolerate quite severe disruptions to their epigenetic machinery while retaining the characteristics of pluripotency (Pasini, et al., 2007) (Montgomery, et al., 2005). This observation induced Niwa to propose that epigenetic processes are likely to be responsible for the execution of the pluripotent program, which is itself established by the transcription factor network, rather than for the maintenance of pluripotency per se (Niwa, 2007).

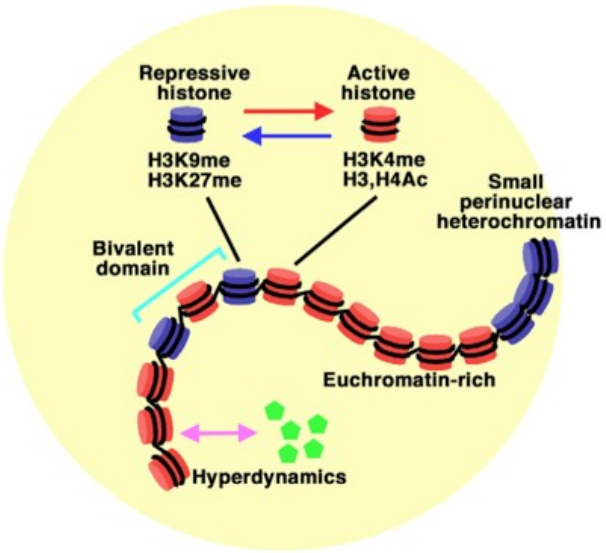

Figure 7. Characteristics of the pluripotent epigenome.

Small regions of perinuclear heterochromatin exist, but most of the chromatin exists as euchromatin, bearing histone marks associated with transcriptional activity. The hyperdynamics of chromatin proteins (green) might contribute to the maintenance of euchromatin. Bivalent domains are also a feature of the pluripotent epigenome, in which active histone marks (such as H3K4me) are flanked by transcriptionally repressive histone marks (such as H3K9me) (Niwa, 2007).

4. MicroRNAs as regulator of ESC fate

In the last years the role of miRNAs in regulating many biological processes became evident (Bushati, et al., 2008) (Houbaviy, et al., 2003). Since miRNAs can modulate at post-trascriptional level the expression of different target genes, they represent a fine mechanism to control ESC differentiation program.

Biogenesis of miRNAs is a multistep process initiated in the nucleus. miRNAs are transcribed by RNA polymerase II as long, capped and polyadenylated primary transcript (pri-miRNA) of 60-100 nucleotide in length, to form a stem loop structure. The pri-miRNAs are cleaved in the nucleus by the Microprocessor complex, containing the RNase III enzyme Drosha and the double-stranded RNA binding domain (dsRBD) protein DGCR8 at the base of the stem loop to produce 60-70 nt long precursors (pre-miRNA). The pre-miRNAs have to move to the cytoplasm by using a nuclear transport receptor complex, exportin-5–RanGTP. There, they are recognized by an heterotrimeric complex composed of the RNase Dicer, the double-stranded RNA-binding protein TRBP, and Argonaute (Ago) proteins. Dicer further cleaves pre-miRNAs 22 nucleotides from the Drosha cleavage site, thereby generating a mature miRNA duplex. This complex identifies the guide strand of the RNA duplex and separates the two strands. The guide strand of the miRNA remains associated with the Ago protein in the miRNA-induced silencing complex (miRISC), which in turn recognizes target mRNAs based on complementarity between the miRNA and the mRNA target. Nucleotides 2–7 (from the 5’ end) of the mature miRNA, also called the ‘‘seed’’ motif, form the critical region for target mRNA recognition that hybridizes nearly perfectly with the target (Martinez, et al., 2010). Without a perfect match between the two RNA molecules, the target is not cleaved, but its translation is repressed (Figure 8). Translational repressed mRNA-protein complexes can localize in cytoplasmic foci named P-bodies (Liu, et al., 2005). Here, translational repressed mRNA could stay in oligomeric structures for

storage or could form a complex with decapping enzymes and cap-binding proteins that trigger mRNA decay (Rana, 2007).

The involvement of miRNAs in the control of ESC potential is demonstrated by the study of DGCR8 and Dicer mutant mice. DGCR8 knockout (KO) ESCs have an extended population doubling time compared to their wild-type and heterozygous counterparts, but are morphologically normal and continue to express ESC specific markers. They accumulate in the G1 phase of the cell cycle, indicating that DGCR8 is required for normal ESC proliferation and cell-cycle progression. Moreover DGCR8 KO ESCs cannot efficiently silence the ESC program, even under stringent differentiation conditions (Wang, et al., 2007). The role of miRNAs in ESCs is supported also by Bernstein work, in which he found that Dicer absence leads to embryonic lethality. Dicer KO pre-gastrulation embryos showed lack of Oct4-positive epiblast cells and could not undergo gastrulation. As expected, mESCs could not be derived from Dicer mutant embryos (Bernstein, et al., 2003). In 2005, Kanellopoulou and co-workers were able to generate Dicer-deficient ESCs using a conditional gene targeting approach. Surprisingly, these mutant ESCs are viable, hold an appropriate morphology, and express normal levels of pluripotency markers (Kanellopoulou, et al., 2005). But, consistent with *in vivo* finding (Bernstein, et al., 2003), Dicer-deficient ESCs failed to differentiate in multiple assays.

These works highlight the relevance of miRNA function in determining ESC fate.

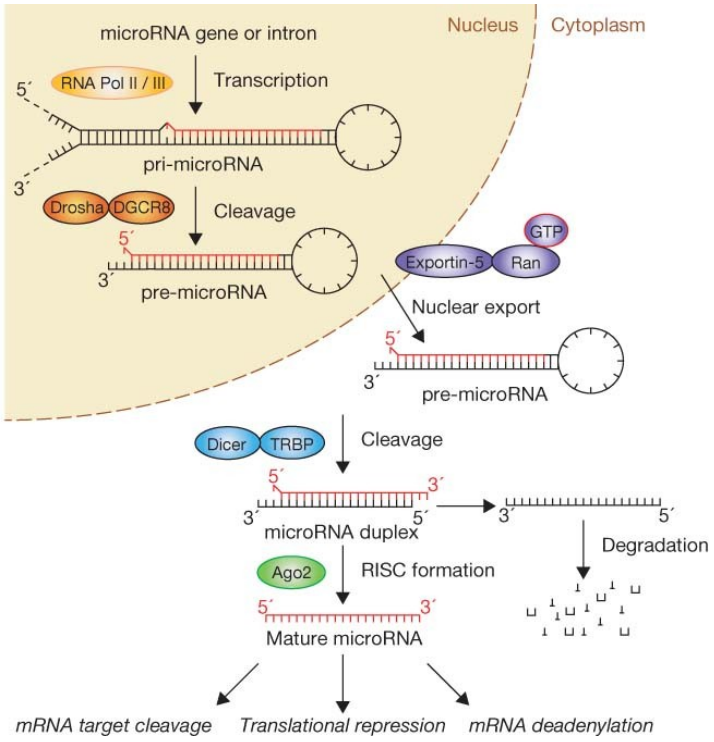

Figure 8. The miRNA processing pathway

This miRNA maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded (Winter, et al., 2009).

4.1 miRNAs in ESC function

ESCs express a specific set of miRNAs in undifferentiated and differentiated conditions as found by cloning and sequencing of small RNAs (Houbaviy, et al., 2003). Among miRNAs expressed in ESCs, there is highly represented the family of miRNAs with the AAGUGC seed sequence. Members of this family are organized in two major clusters. The conserved miR-302/367 cluster comprises four miRNAs (miR-302a, miR-302b, miR-302c and miR-302d)

and the unrelated miR-367. The second cluster is less conserved. In the mouse it is commonly referred to as the miR-290-295 cluster and includes six miRNAs (miR-290, miR-291a, miR-291b, miR-292, miR-294 and miR-295) and miR-293 (Rosa, et al., 2013). Further studies demonstrated that miR290 cluster regulates retinoblastoma like-2 (Rbl2) at the post-transcriptional level, leading to a transcriptional repression of Dnmt3a and Dnmt3b and the appearance of DNA-methylation defects, resembling those observed in Dicer KO cells (Benetti, et al., 2008).

Other miRNAs have been involved in ESC differentiation, as miR-134, miR-296, and miR-470, which target and down-regulate the core transcription factors Nanog, Oct4, and Sox2 (Tay, et al., 2008). Similarly, miR-200c, miR-203, and miR-183 repress Sox2 and Klf4 (Wellner, et al., 2009). It's interesting that the same transcription factors can in turn regulate the expression of specific miRNAs. Indeed, genome-wide mapping of binding sites for key ESC transcription factors revealed highly overlapping occupancy of Oct4, Sox2, Nanog and Tcf3 at the transcriptional start sites of miRNA transcripts, preferentially or uniquely expressed in ES cells (Marson, et al., 2008).

Among other miRNAs playing a role in ESC differentiation, there is the let-7 family. In mammals it includes several let-7 species (let-7a to let-7i) and other miRNAs, such as miR-98 and miR-202 [101]. Members of the let-7 family accumulate during development and differentiation, with a parallel reduction of their targets, and have a role in cancer (Büssing, et al., 2008). In ESCs, levels of let-7 miRNAs are regulated at the post-transcriptional level. The RNA binding protein Lin28 associate to the terminal loop of let-7 precursors blocking the production of the mature miRNA and targeting it for degradation (Hagan, et al., 2009) (Piskounova, et al., 2011). Coherently with its function, Lin28 is highly expressed in undifferentiated ESCs and is reduced during differentiation, when levels of mature let-7 increase. This mechanism is

regulated by a feedback loop, in which let-7 negatively regulates Lin28 (Rybak, et al., 2008).

Our laboratory also contributed to the characterization of other miRNAs working in the control of ESC differentiation. In particular, Tarantino and co-workers identified miRNAs differentially expressed in different steps of ESC differentiation. They clustered miRNAs in three groups on the basis of their expression profile: 1) miRNAs expressed in undifferentiated ESCs and that decreased during differentiation; 2) miRNAs already present in ESCs and increased in differentiating cells; 3) miRNAs completely undetectable in undifferentiated cells and increased on induction of differentiation. Focusing their study on a small group of miRNAs whose candidate targets are down-regulated on ESC differentiation, the group demonstrated that miR-34a, miR-100, and miR-137 are required for proper differentiation of mouse ESCs, and they function in part by targeting Sirt1, Smarca5, and Jarid1b mRNAs (Tarantino, et al., 2010).

These are only few examples of miRNA involved in ESCs. But a lot has still to be done, not only to identify novel miRNAs but also, and especially, to understand their contribution in governing ESC fate.

Methods

Reporter plasmid generation

The 3' untranslated region (UTR) of Dies1 (from nucleotide 2260 to 3268) was obtained by polymerase chain reaction (PCR) from ESC genomic DNA and cloned in pCAG-luc vector (Tarantino, et al., 2010) downstream of the firefly luciferase gene. The mutated 3' UTR of Dies1 carrying a scrambled seed sequence of miRNAs was obtained through a double round of PCR to insert the mutation. This fragment was cloned downstream of the firefly luciferase gene in *Eco*RI site in pCAG-luc vector.

For FRET analysis, EGFP was derived from pEGFP-N1 (Clontech, Mountain View, CA, USA) and then cloned downstream of Dies1 in pCAG vector by *Hind*III and *Not*I restriction. Alk3 cDNA was derived from pSport vector (U.S. National Institutes of Health Mammalian Gene Collection, Bethesda, MD, USA) by PCR and cloned in pCAG vector in *Bam*HI and *Hind*III restriction sites. mCherry was derived from pmCherry vector (Clontech) by PCR and cloned downstream of Alk3 by *Hind*III and *Not*I restriction. p75-mCherry vector was kindly provided by Dr. Simona Paladino (Universita`Federico II, Naples, Italy). The oligonucleotides used for cloning are reported in Appendix 1.

Cell culture, transfection and treatment

E14Tg2a (BayGenomics) mouse ESCs were maintained on feeder-free, gelatin-coated plates in the following medium: Glasgow Minimum Essential Medium (GMEM, Sigma) supplemented with 2 mM glutamine (Invitrogen), 100 U/ml penicillin/streptomycin (Invitrogen), 1mM sodium pyruvate (Invitrogen), 1X nonessential aminoacids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 10% FBS (Hyclone), and 10^3 U/ml leukemia inhibitory factor (LIF, Millipore).

C2C12 myoblasts were maintained in Dulbecco's Modified Eagle Medium supplemented with 2 mM glutamine (Invitrogen), 100 U/ml penicillin/streptomycin (Invitrogen), 10% FBS (GIBCO).

The cell lines were plated at $6x10^4$ cells/cm^2 16 h before transfection. Transfection of plasmids, pre-miRs, and anti-miRs (both from Ambion) in ESCs were performed using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. Short hairpin RNAs (shRNAs) in the pLKO.1-puro vector (Thermo Scientific) were used for suppressing Dies1 in ESCs. Transduced cells were selected with 2 μg/ml of puromycin (Sigma).

For cell treatment, ESCs and C2C12 were grown over night in knockout serum replacement (KSR) containing medium with LIF or DMEM plus 1% FBS, respectively, and then treated for the indicated time with 20 ng/ml of BMP4 (R & D Systems) and 20 ng/ml of activin (R&D).

ESC and C2C12 differentiation

Neural differentiation by monolayer (Parisi, et al., 2010) was induced plating ESCs onto gelatine-coated dishes at low density ($3x10^3$ cells/cm^2) in the following differentiation medium: GMEM supplemented with 10% knockout serum replacement (KSR, Invitrogen), 0.1mM β-mercaptoethanol, 2mM glutamine, 100 U/ml penicillin/streptomycin. Differentiation medium was changed on alternate days.

For serum free embryo body (SFEB) differentiation, $1x10^6$ ESCs were plated in 100-mm Petri dishes to allow spontaneous aggregation into SFEBs for at least 4 days, in the following differentiation medium: GMEM supplemented with 10% knockout serum replacement (KSR, Invitrogen), 0.1mM β-mercaptoethanol, 2mM glutamine, 100 U/ml penicillin/streptomycin (Watanabe, et al., 2005). Dorsomorphin (2 μM; Sigma) was added once in the differentiation medium when the cells were plated in Petri dishes to induce SFEB formation. DMSO was used as negative control.

For differentiating C2C12 myoblasts to myotubes, $20x10^3$cells/cm^2 were plated and, the following day, were transferred in a differentiation medium containing Dulbecco's Modified Eagle Medium with 2 mM glutamine (Invitrogen) and 2% horse serum (Sigma), for 3 days.

Luciferase reporter assay

For luciferase assay, ESCs were plated at 3x104 cells/cm2 16 hours before transfection in 24-well. Plasmids carrying the wild type or mutated Dies1 3'UTR, pre-miR-125a and 125b were co-transfected in ESCs by using Lipofectamine 2000 (Invitrogen). pRL-TK vector (Promega) expressing Renilla luciferase was co-transfected as an internal control. After 24 hours from transfection, the cells were lysed with 200µl of 1X Passive Lysis Buffer. Firefly and Renilla luciferase activities were measured with the dual-luciferase reporter system (Promega) by Sirius Luminometer (Berthold Detection Sistems). The data were expressed as relative to control transfected cells after normalization to Renilla luciferase reading. All transfection experiments were repeated in triplicate.

Alkaline phosphatase staining

For alkaline phosphatase staining, ESCs were cultured at clonal density (20-50 cells/cm^2). The cells were fixed in 10% cold Neutral Formalin Buffer (10% formalin, 110 mM Na2HPO4, 30mM NaH2PO4.H2O) for 15 minutes and then rinsed in distilled water for 15 minutes. The staining was obtained by incubation for 45 minutes at room temperature with the following staining solution: 0,1M Tris-HCl, 0,01% Naphthol AS MX-PO4 (Sigma), 0,4% N,N-Dimethylformamide (Sigma), 0,06% Red Violet LB salt (Sigma).

BrdU assay

For BrdU incorporation and detection, subconfluent ESCs were incubated in ESC medium containing BrdU for 2 hours and then the cells were fixed and processed for immunofluorescence with BrdU labelling and detection kit (Roche) following manufacturer's instruction. Fluorescence microscopy was performed as described in Immunostaining section.

RNA isolation, Real-Time PCR and TaqMan analysis

Total RNA from undifferentiated and differentiated ESCs and C2C12 was extracted by using TRI-Reagent (Sigma). The first-strand cDNA was synthesized according to the manufacturer's instructions (M-MLV RT, New England BioLabs). Real-time RT-PCR was carried out on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using Power SYBR Green PCR Master mix (Applied Biosystems). The housekeeping GAPDH mRNA was used to normalize the samples using $2^{-\Delta Ct}$ method. Gene specific primers used for amplification are listed in Appendix 2.

For TaqMan analysis, total RNA was isolated from undifferentiated and differentiated ESCs and C2C12with mirVana microRNA Isolation kit (Ambion) according to the manufacturer's instructions. From each sample, 10 ng of total RNA were used to synthesize single-stranded cDNA with TaqMan MicroRNA reverse transcription kit (Applied Biosystems) combined with the specific primer for miR-125a, miR-125b, miR-let7e, miR99b, or U6 as internal control. Expression level of miRNAs were measured by using TaqMan MicroRNA detection kit (Applied Biosystems) with the 7500 Real Time PCR System instrument and the Sequence Detection Systems (SDS) software version 1.4 (Applied Biosystems).

Protein extraction and western blot analysis

For protein extracts, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7,5) 150 mM NaCl, 1 mM EDTA, 1% Triton, 1% sodium deoxycholate and protease inhibitor cocktail (Sigma). The lysates were cleared by microcentrifugation at 14,000 rpm and then mixed with Laemli buffer. Proteins were resolved on SDS-PAGE, transferred onto PVDF membrane (Millipore) and incubated with indicated antibodies according to the manufacturer's recommendations. The following primary antibody were used: anti-Dies1 (Aloia, et al., 2010); anti-phospho-Smad1,5,8 (1:1000; Cell Signalling); anti-Smad1 (1:1000, Cell Signalling); anti-Lin28 (1:700, Abcam); anti-phospho-Erk1 (1:1000, Cell Signalling); anti-Erk1 (1:1000, Santa Cruz); anti-GAPDH (1:1000, Santa Cruz).

Chromatin immunoprecipitation (ChIP)-qPCR analysis

ESCs, transfected with pre-miRs or treated with BMP4 as indicated, were cross-linked with 1% formaldehyde for 10 minutes at room temperature and formaldehyde was then inactivated by the addition of 125 mM glycine. The chromatin was sonicated to an average DNA fragment length of 500-1000 bp. To immunoprecipitate soluble chromatin extracts were used anti-H3K4-3me (Millipore, Billerica, MA, USA), anti-H3K27-3me (Millipore) antibodies, anti-Smad1 (Cell Signalling) antibodies. Appropriate IgGs were used as negative control. Supernatant obtained without antibody was used as input control. The amount of precipitated DNA was calculated by real-time PCR relative to the total input chromatin, and expressed as percentage of total chromatin according to the following formula: $2^{\Delta Ct}$, where Ct represents the cycle threshold and ΔCt = Ct(input) – Ct(immunoprecipitation). Oligonucleotide pairs are listed in Appendix 3.

Immunostaining and microscopy

For immunostaining, monolayer differentiated ESCs were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in 10% FBS (Invitrogen)/1% BSA in PBS for 15' at room temperature. Thus the samples were incubated with the primary anti- bIII-tubulin 1:400 (Sigma–Aldrich) for 2 h at room temperature. Following primary antibodies incubation, the cells were incubated with appropriate secondary antibody (1:400, Alexa Molecular Probes) and counterstained with DAPI (Calbiochem). Images were captured with an inverted microscope (DMI4000, Leica Microsystems) (Parisi et al., 2010).

SFEBs were collected at day 4 of differentiation and fixed in 4% paraformaldehyde. After dehydration with increasing percentage of EtOH, samples were embedded in paraffin, sectioned in 7μm slices and mounted on glass slides. After rehydration and permeabilization with 0.2% TX-100, unmasking was performed in Citrate Buffer 1x. The non-specific block was performed by treating in 10% FBS/1% BSA/0.1% Tween 20/ 1x PBS for 2-3h at RT followed by primary antibodies incubation. Nuclei were counterstained with Dapi (Calbiochem). The following primary antibodies were used: anti-Oct3/4 (1:200, Santa Cruz), anti-Nanog (1:500, Calbiochem), anti-Sox1 (1:100, Santa Cruz). The appropriate secondary antibodies were used (1:400, Alexa Molecular Probes). Confocal microscopy was performed with an LSM 510 Meta microscope (Zeiss) using LSM 510 Meta software and LSM Image Browser (Zeiss).

Teratoma formation

ESCs transfected with pre-miR-125b or pre-miR-ctrl were differentiated as SFEBs for 3 days. Then SFEBs were dissociated and $2x10^6$ cells were used for subcutaneous injection in nude mice. Four weeks after the injection, tumors were surgically dissected from the mice. Samples were fixed in 4%

paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Derivation of epiblast stem cells (epiSCs) from SFEBs and cell culture

For derivation of EpiSCs, SFEBS at 4 and 5 days of differentiation were dissociated into a single-cell suspension with 0.05% Trypsin-EDTA at 37°C for 5 min. Individual cells were then seeded in 12-well plates coated with FBS at a density of 12,000 cells/well in epiSC medium as follows: 1 volume of DMEM/F12 combined with 1 volume of Neurobasal medium, supplemented with 0.5% N2 supplement, 1% B27 supplement, 2 mM glutamine (Invitrogen), 20 ng/ml Activin A (R&D Systems), and 12 ng/ml bFGF (Invitrogen). SB-431542 (10 μM; Sigma) was added once in the differentiation medium when the cells were plated to induce SFEB formation. DMSO was used as negative control. The medium was changed on alternate days.

Fluorescence resonance energy transfer (FRET)-fluorescence lifetime imaging microscopy (FLIM) experiments

For FRET analysis, ESCs were transfected with Dies1-GFP (donor) expression vector alone or in combination with Alk3-mCherry or p75-mCherry expressing vectors (acceptors. After 24 h from transfection, the cells were fixed in 4% paraformaldehyde and analyzed for FRET in PBS1X. FRET measurements were performed by using a Leica TCS SMD FLIM confocal microscope (Leica Microsystems). For each field, the lifetime (τ) was calculated with LAS AF software (Leica Microsystems), taking into account the instrument response function (IRF). The data were expressed as percentage of FRET efficiency (*E*), which was derived using the following equation:

$$E = 1 - (\tau_{FRET} / \tau) \times 100$$

where τ_{FRET} is the lifetime of the donor population that is interacting with the acceptor, and τ is the lifetime of non-interacting donor population (Llères, et al., 2007). The images representing the lifetime on the basis of a colour scale were obtained with Symphotime software (PicoQuant GmbH, Berlin, Germany) and brightness and contrast were adjusted in Photoshop CS2.

Statistics

Data are presented as the means ± SD of at least three independent experiments. Whenever necessary, statistical significance of the data was analyzed using Student's t test.

Results

1. Dies1 is a direct target of miR-125a and miR-125b in mouse ESCs

Dies1 was demonstrated to have a role in ESC differentiation, indeed its suppression impairs the differentiation program (Aloia et al., 2010). We decided to investigate whether a physiological modulation of Dies1 expression could be one of the mechanisms controlling ESC differentiation. To this aim, we searched for miRNAs targeting Dies1 3'UTR by using bioinformatic tools such as TargetScan and Miranda. Among the predicted miRNAs targeting Dies1, we choose to study miR-125a and miR-125b based on their conservation and identity in the seed sequence (Figure 9). These miRNAs belong to the same family and have an high homology in their sequence. They are encoded by two different genes: miR-125a gene is localized on mouse chromosome 17 clustered with miR-99b and Let-7e; miR-125b is encoded by two genes on chromosome 9 (miR-125b-1) and on chromosome 16 (miR-125b-2).

To check whether they could target Dies1 3'UTR, we overexpressed their precursors in undifferentiated ESCs and we analyzed Dies1 protein level by western blot assay. We found that Dies1 protein is strongly reduced in ESC overexpressing pre-miR-125a and pre-miR-125b, alone or in combination (pre-miR-mix). On the contrary, the suppression of miRNAs by anti-miR-125a and/or anti-miR-125b leads to an accumulation of Dies1 protein, supporting the hypothesis that these two miRNAs could regulate Dies1 expression (Figure 10). To confirm that this regulation occurs directly, we have performed a luciferase assay experiment. We have transfected ESCs with the specific pre-miR and with a construct encoding the luciferase gene fused to Dies1 3'UTR. As control, we have generated a construct carrying a form of Dies1 3'UTR mutated in the sequence recognized by the miRNAs. As you can see in the Figure 11, the expression of miR-125a and miR-125b reduced the luciferase activity of the construct carrying the Dies1 wt 3'UTR,

while with that carrying the mutated 3'UTR the luciferase activity was not affected. This result clearly demonstrates that the miRNAs 125a and 125b regulate Dies1 expression by binding directly its 3'UTR.

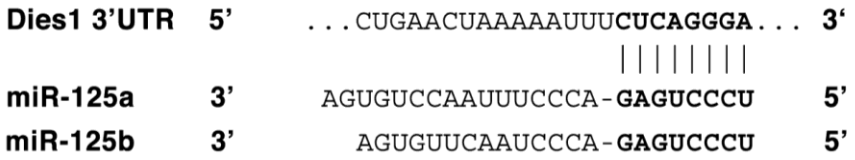

Figure 9. Predicted target site of miR-125a and miR-125b in the 3' UTR of Dies1.
miR-125a and miR-125b have the same seed region (in bold) and high sequence homology. They target the Dies1 3'UTR.

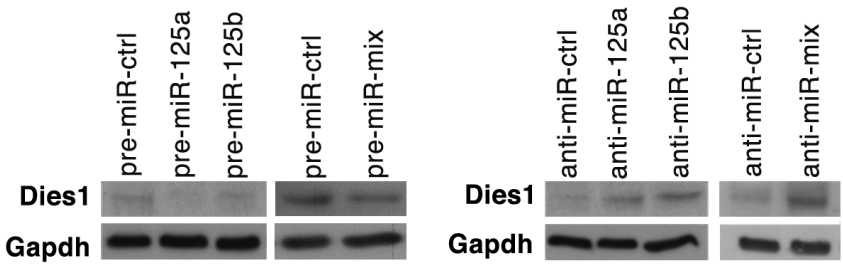

Figure 10. Dies1 protein level on miRNA modulation.
ESCs were transfected with the indicated pre-miR or anti-miR, alone or in combination (pre or anti miR-mix). 24h after transfection Dies1 protein levels were evaluated by western blot analysis.

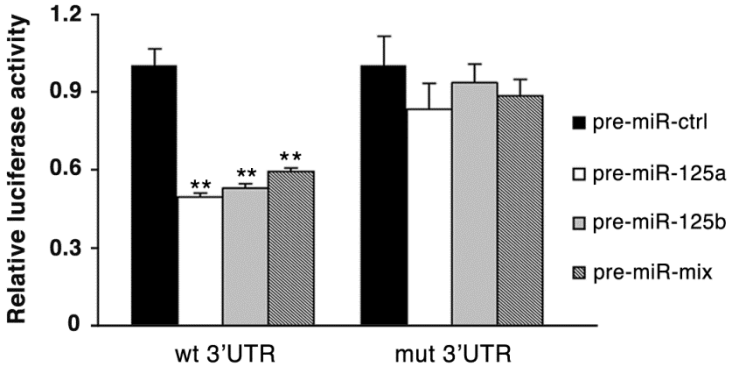

Figure 11. miR-125a and miR-125b directly regulate Dies1 expression by targeting its 3' UTR.

ESCs were co-transfected with the indicated pre-miR and the reporter bearing wild-type (wt) or mutated (mut) Dies1 3' UTR. Luciferase activity was measured 24h after transfection and normalized with Renilla luciferase activity. **P < 0.01.

2. miR-125a impairs ESC differentiation

miR-125a is expressed in undifferentiated ESCs and its expression level stays almost constant up to four days of differentiation. Then it reaches high levels in terminally differentiated cells (Figure 12). Since Dies1 is involved in ESC fate and it's regulated by miR-125a at post-transcriptional level, we have decided to further investigate the role of miR-125a in ESCs. To this aim, we have overexpressed miR-125a in ESCs and cultured them in subconfluent condition in the proper medium, supplemented or not with LIF. After 7 days of culture, we have analyzed the maintenance of stemness phenotype performing an Alkaline Phosphatase (AP) staining. We didn't observed difference in the number of colonies positive for AP staining upon miR-125a overexpression in undifferentiated condition (Figure 13). Moreover, we evaluated the expression level of stemness genes as Oct3/4, Nanog and Rex1 by Q-PCR, but we didn't find difference between the ESCs overexpressing miR-125a and the control (Figure 14). However, when we removed LIF from

the culture medium, allowing ESCs to spontaneously differentiate, mir-125a ectopic expression induced an increase of colonies stained with alkaline phosphatase suggesting an impairment of differentiation(Figure 13).

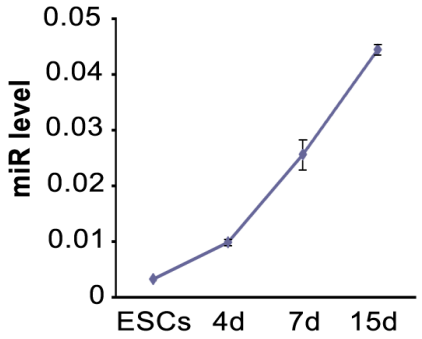

Figure 12. Expression profile of miR-125a during ESC neuronal differentiation.
miR-125a levels were measured by Taqman assay in undifferentiated ESCs and during different time points of neuronal differentiation.U6 RNA level is used to normalize.

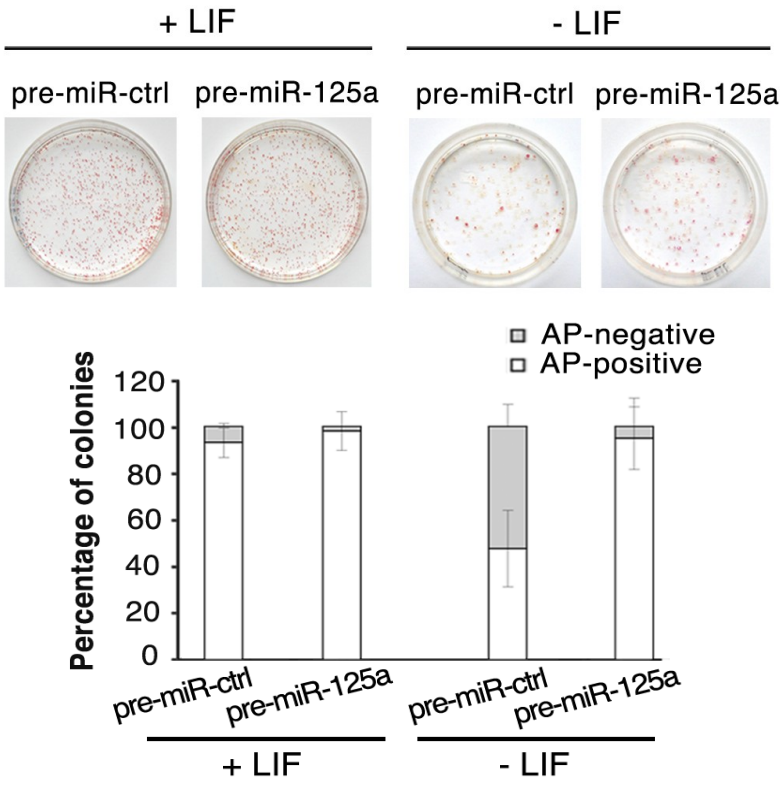

Figure 13. miR-125a maintains stemness in LIF removal induced differentiation.
ESCs overexpressing miR-125a or miR-ctrl were grown for 7 days in culture medium supplemented or not with LIF. The histogram reports the number of AP negative (grey) and positive colonies (white). In absence of the cytokine, miR-125a overexpressing cells have an higher number of stemness colonies (AP positive).

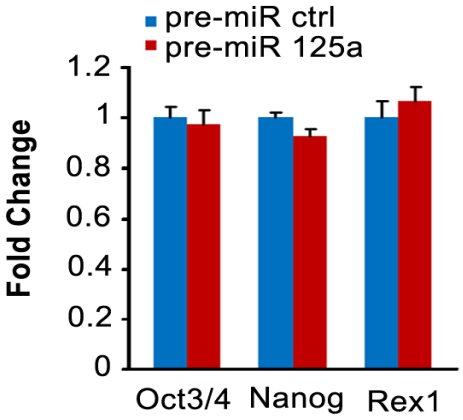

Figure 14. miR-125a expression doesn't affect stemness.

Analysis of the expression level of stemness markers (Oct3/4, Nanog, Rex1) in ESCs overexpressing miR-125a or miR-ctrl. The data are represented as fold change relative to the control.

To further analyze this phenotype, we grew the cells transfected with the control pre-miR or the pre-miR-125a as monolayer in chemically defined medium. At day 7, the immunostaining assay showed that the control cells differentiated mainly as neuroectoderm expressing the β-III tubulin marker and losing the stemness markers Oct3/4 and Nanog. The presence of miR-125a increases the number of the cells positive for stemness markers, reducing those of neuroectodermal origin (Figure 15A). We have also quantified the different expression of stemness (Oct3/4, Nanog, Sox2) and neuroectodermal (Map2) markers by Q-PCR analysis of pre-miR transfected ESC at 7 days of differentiation (Figure 15B).

To better define the identity of the single cell in the first steps of ESC differentiation, we decided to use a differentiation system based on the formation of serum free embryo bodies (SFEBs). We transfected pre-miR-125a or pre-miR-ctrl in ESCs and differentiated them as SFEBs. At four days of differentiation, we have analyzed the phenotype by immunofluorescence experiment. Almost all the control transfected cells expressed the neuroectodermal marker Sox1 and only few cells still expressed the stemness markers Oct3/4 and Nanog. Following miR-125a overexpression, the phenotype dramatically changed: a lot of cells maintain the expression of

Oct3/4 and Nanog and few cells are Sox1 positive (Figure 16A). The same result was confirmed by Q-PCR analysis of mRNA level of stemness (Oct3/4, Nanog, Sox2) and neuroectodermal (Pax6, Sox1) markers, indicating that miR-125a impairs ESC differentiation maintaining the expression of stemness factors (Figure 16B). This phenotype is very similar to that observed upon Dies1 suppression in ESCs, in agreement with the finding that miR-125a controls Dies1 expression (Figure 17).

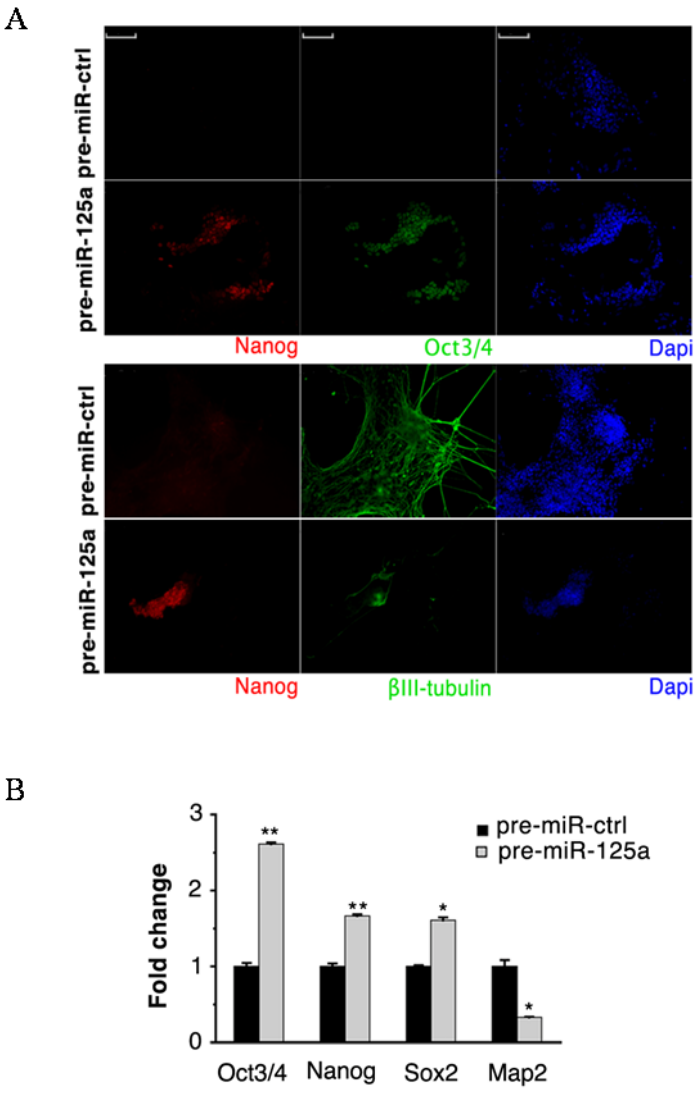

Figure 15. miR-125a overexpression impairs ESC neuronal differentiation.
ESCs were transfected with the indicated pre-miR and induced to differentiate in monolayer. After 7 days of differentiation, stemness (Oct3/4, Nanog, and Sox2) and neuronal markers (βIII-tubulin, Map2) were analyzed by immunostaining (A) and by qPCR analysis (B). Scale bars = 100 µm. The data in B are expressed as fold change relative to the control. * $P < 0.05$, ** $P < 0.01$.

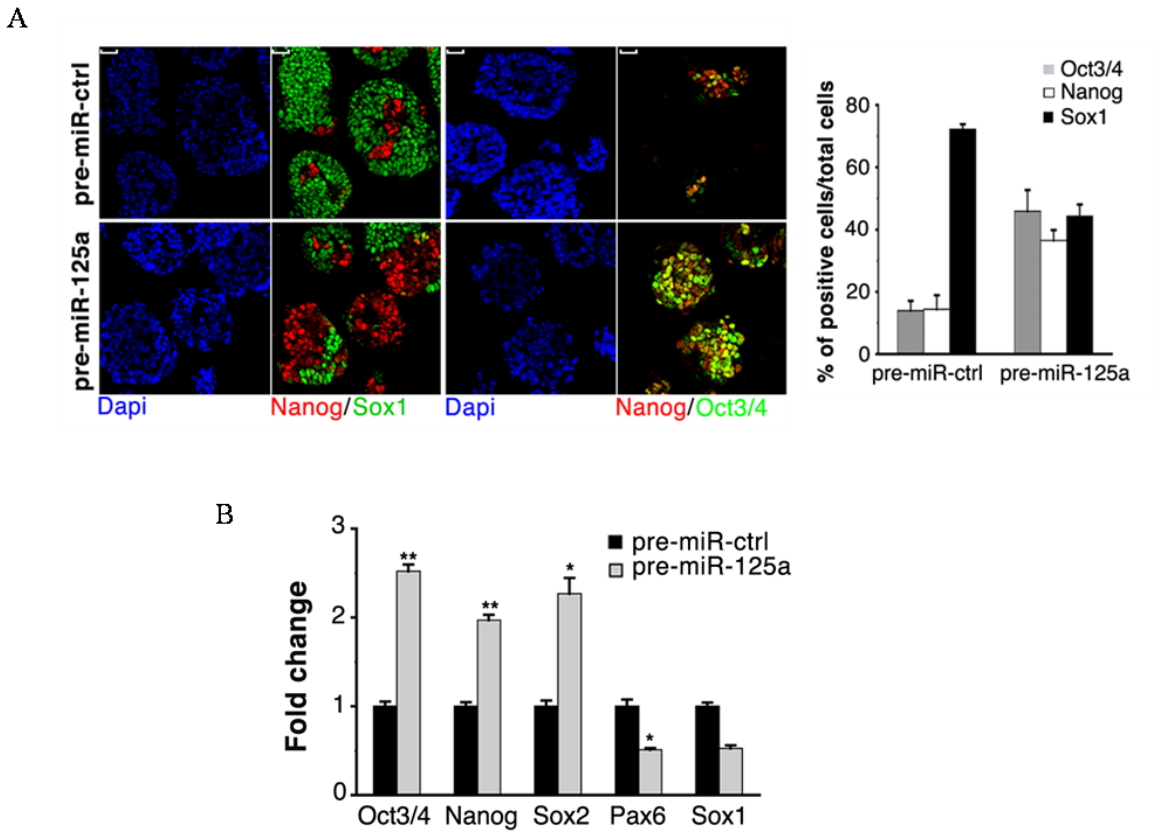

Figure 16. miR-125a maintains the expression of stemness markers during SFEB differentiation.

Following miR-125a overexpression, ESCs were differentiated for 4 days as SFEBs. The immunostaining analysis (A) shows an increased number of cells expressing stemness markers (Nanog, Oct3/4) and a reduced number of those positive for neuroectodermal marker (Sox1), in miR-125a transfected cells compared to the control . Scale bars = 20 µm. The percentage of positive cells on total cells for each marker was counted in ≥ 10 independent fields and was represented in the histogram. The same phenotype was confirmed by the qPCR analysis (B) of mRNA level of stemness (Oct3/4, Nanog, Sox2) and neuronal (Pax6, Sox1) markers. Data are expressed as fold change relative to the control. * $P < 0.05$, ** $P < 0.01$.

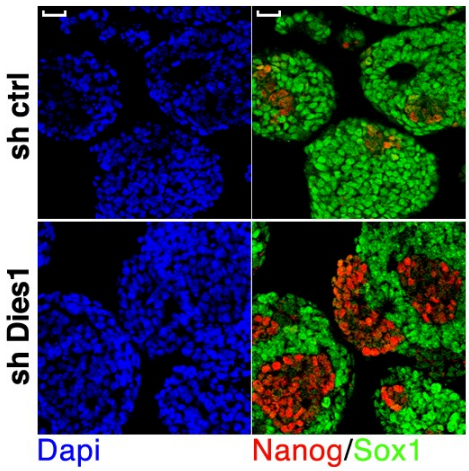

Figure 17. Dies1 suppression induces an impairment of SFEB differentiation, maintaining the expression of stemness markers.

ESCs were transfected with shRNA against Dies1 (sh Dies1) or a negative control (sh ctrl) and subjected to SFEB differentiation. After 4 days of differentiation, the expression of stemness (Nanog) and neuroectodermal (Sox1) markers was evaluated by immunostaining analysis. Scale bars = 20 μm.

Moreover, it is known that stemness genes have an epigenetic signature associated to their expression level. In particular they are characterized by high levels of histone H3 lysine 4 methylation (H3K4-3me) and low levels of histone H3 lysine 27 methylation (H3K27-3me), indicating an active transcription of these genes (Azuara, et al., 2006). Given the maintenance of stemness gene expression following miR-125a overexpression, we decided to check whether, at day 4 of SFEB differentiation, stemness genes lost the epigenetic signature typical of undifferentiated condition. To test this hypothesis, after 4 days of SFEB differentiation we immunoprecipitated the chromatin of ESCs overexpressing miR-125a and those transfected with the control, using antibodies able to recognize the marks H3K4-3me and H3K27-3me. Then the chromatin was amplified with specific primers for the region surrounding the transcriptional start site of stemness genes. We confirmed that miR-125a overexpressing cells retain high level of H3K4-3me and low

level of H3K27-3me at day 4 of differentiation, maintaining the characteristics of undifferentiated ESCs (Figure 18).

We wondered that the block of differentiation observed upon miR-125a overexpression could be associated to an alteration of cell proliferation. To exclude this possibility, we performed a BrdU incorporation assay in ESCs overexpressing miR-125a or a control miR. But, at 2h from the BrdU incorporation, no significant changes in the proliferation rate were observed (Figure 19).

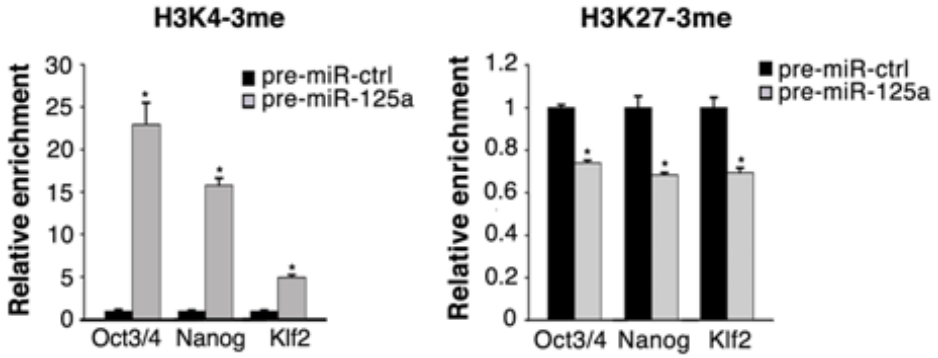

Figure 18. miR-125a ectopic expression alters the methylation state of histone H3 during SFEB differentiation.

ESCs transfected with the indicated pre-miR were induced to differentiate through SFEB formation. At day 4 of differentiation, the cells were subjected to chromatin immunoprecipitation with antibodies against H3K4-3me and H3K27-3me. Immunoprecipitated DNA was amplified by qPCR with primers designed on the transcriptional start sites of the indicated genes. Data are expressed as fold enrichment relative to the control. * $P < 0.05$.

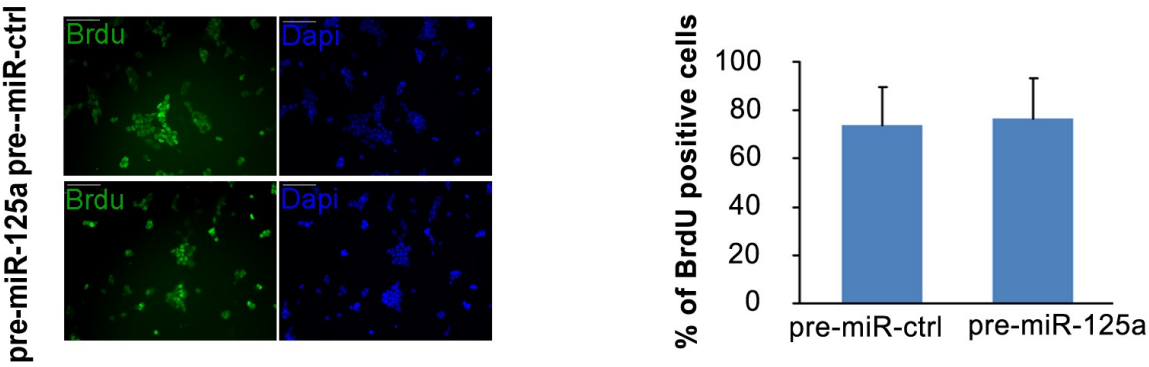

Figure 19. miR-125a overexpression doesn't affect cell proliferation.

ESCs were transfected with the indicated pre-miR. 24h after the transfection, BrdU was incorporated for 2h. Immunostaining with a specific antibody reveals the amount of BrdU incorporation. Histogram indicates the percentage of BrdU positive cells relative to the total cells.

3. The phenotype of miR-125a ectopic expression is dependent on Dies1

We found that miR-125a overexpression gives an impairment of ESC differentiation, resembling the phenotype of Dies1 suppression during differentiation. To explore whether the phenotype of miR-125a expression could be due to Dies1 downregulation, we performed a rescue experiment co-transfecting ESCs with pre-miR-125a or pre-miR-ctrl and a construct encoding Dies1 lacking its 3'UTR and thus insensitive to miRNA regulation. The cells were then differentiated as SFEBs for 4 days and the phenotype was analyzed by immunostaining and Q-PCR. We found that the number of the cells positive for Sox1 or Oct3/4 and Nanog is very similar to the control cells, when they are co-transfected with Dies1 and miR-125a (Figure 20). Thus, restoring Dies1 level in cells overexpressing miR-125a, the expression level of stemness and differentiation markers is re-established.

Aloia et al. previously demonstrated that Dies1 suppression in ESCs gives a reduction of BMP4 target genes, Id1 and Id3. The same effect was observed upon miR-125a overexpression and it was due to Dies1: indeed, co-transfection of Dies1 and miR-125a rescued the expression of Id genes

(Figure 21). These data demonstrated that the effects of miR-125a overexpression in ESCs are, above all, mediated by Dies1.

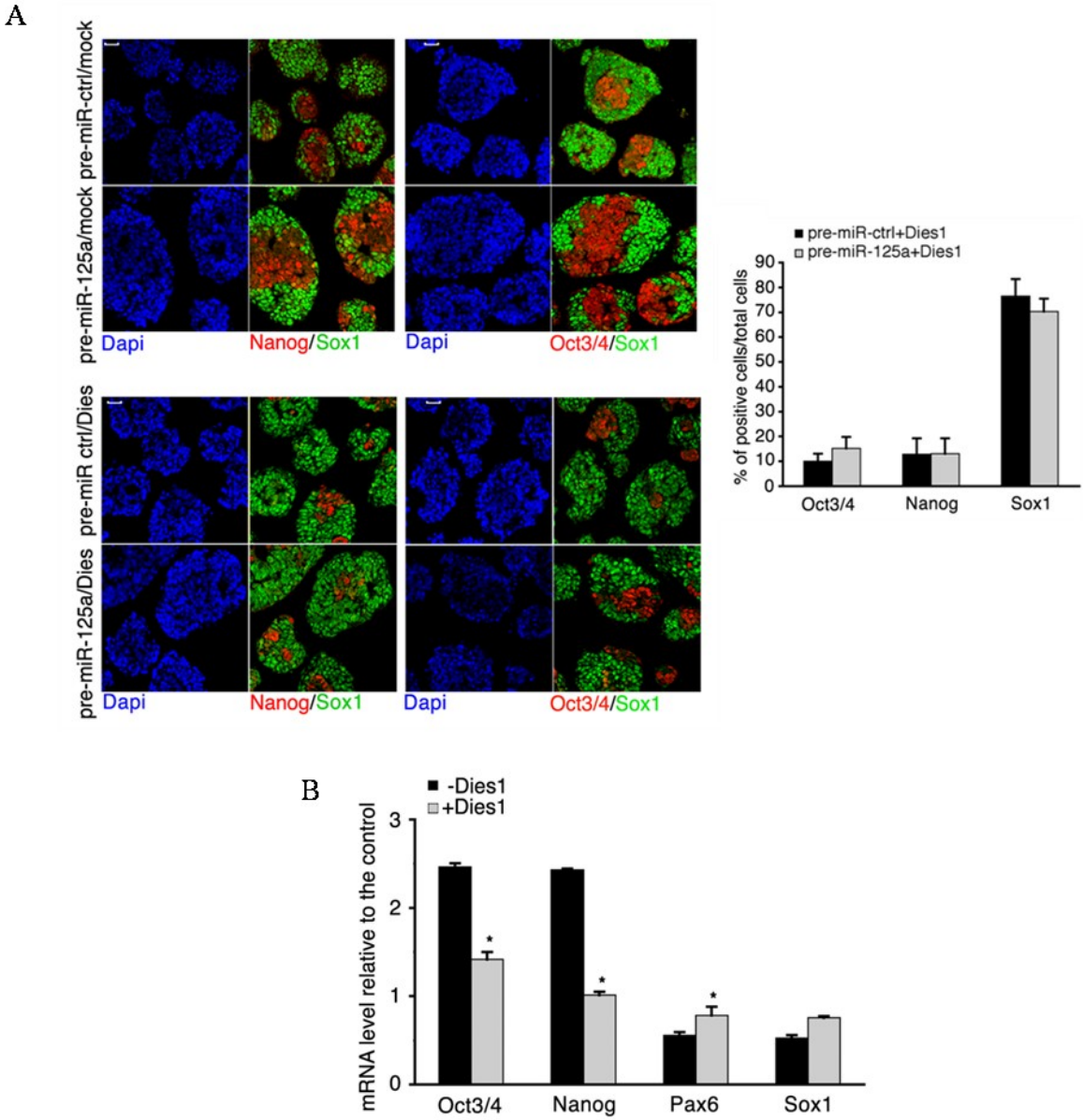

Figure 20. Dies1 re-expression rescues the phenotype induced by miR-125a overexpression.

ESCs were co-transfected with the indicated pre-miR and a construct encoding Dies1 lacking its 3'UTR (Dies1) or the empty vector (mock), and differentiated for 4 days as SFEBs. The expression of stemness (Oct 3/4 , Nanog) and neuroectodermal (Sox1) markers was analyzed by immunostaining. Scale bars = 20 μm. The percentage of positive cells on total cells for each marker was counted in ≥ 10 independent fields and reported in the histogram (A). The same samples were collected for qPCR analysis of stemness (Oct 3/4 , Nanog) and differentiation (Sox1, Pax6) markers (B). * $P < 0.05$.

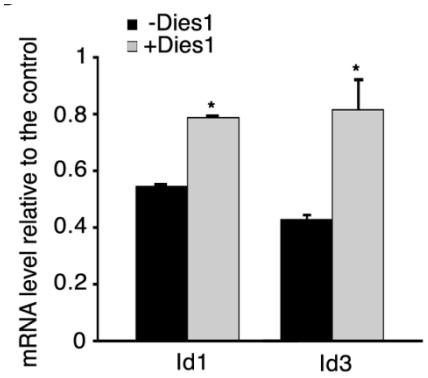

Figure 21. The reduction of BMP4 signalling upon miR-125a overexpression is due to Dies1 suppression.

The analysis of BMP4 target gene expression (Id1, Id3) by qPCR was performed on ESCs co-transfected with the indicated pre-miR and a vector expressing Dies1 lacking its 3'UTR (Dies1) or an empty vector (mock), at day 4 of SFEB differentiation. * $P < 0.05$.

4. BMP4 controls miR-125a and, in turn, Dies1

Dies1 has an important role in BMP4 pathway, acting as AlK3 co-receptor. To support this finding, we performed a FRET experiment, using the multiphoton FLIM technique. We transfected in ESCs a construct carrying Dies1 fused to EGFP (donor) and another carrying Alk3 fused to mCherry fluorescent protein (acceptor). In these cells we observed 6% of FRET efficiency, indicating that Dies1 and Alk3 were interacting at molecular level (Figure 22). As negative control, we used ESCs transfected with Dies1-EGFP fusion protein and the unrelated receptor p75-Cherry; in this condition we didn't find any significant molecular interaction. This result highlights the role of Dies1 in the Alk3 receptor complex, being necessary for BMP4 signalling.

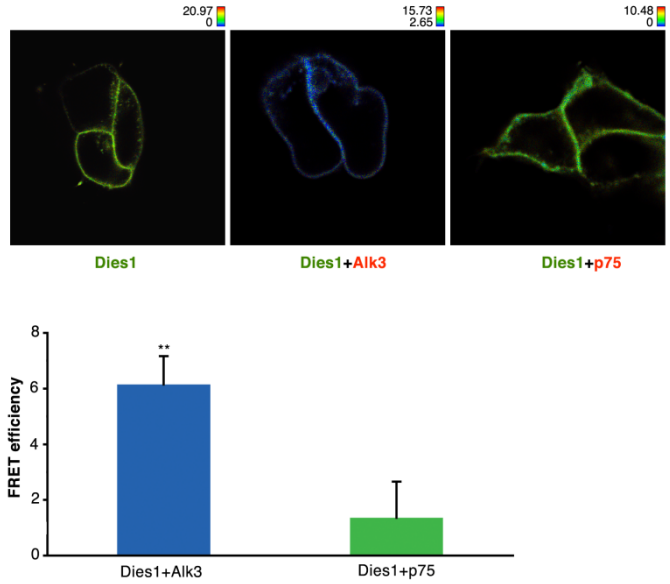

Figure 22. Dies1 and Alk3 interact at molecular level.

ESCs were transfected with a vector expressing Dies1 fused to EGFP (donor) alone or together with a vector expressing Alk3 fused to mCherry fluorescent protein (acceptor). ESCs co-transfected with Dies1-GFP and the p75 receptor fused to mCherry were used as negative control. Energy transfer was measured after 24 h. Colour of the images represents the time required for the energy transfer (lifetime) as indicated in the colour bar, where the values represent lifetime (ns). The more blue the colour, the closer are the donor and the acceptor. Histogram represents the efficiency of energy transfer (%) normalized to the control. **P < 0.01.

Moreover, we know that Dies1 expression is controlled at post-transcriptional level by miR-125a. So, we wondered whether the physiological level of miR-125a could be modulated by BMP4 itself. To verify this possibility, we treated ESCs with BMP4. First of all we controlled that BMP4 was working, analyzing the induction of BMP4 target genes (Id1 and Id3) and the phosphorylation status of Smad1,5,8 (Figure 23). Once assessed that BMP4 was correctly signalling, we analyzed the level of precursor and mature miR-125a, at 1h and 24h after the treatment respectively. The pri-miR-125a is highly increased after 1h of BMP4 treatment, as indicated by the Q-PCR performed with two oligonucleotide pairs annealing in two different region of precursor miR-125a (Figure 24A). The Taqman assay showed that this

increase is associated to an accumulation of the mature form of miR-125a 24h after the BMP4 stimulation (Figure 24B). To prove that miR-125a increase depends on BMP4 activation, we downregulated the BMP4 receptor Alk3 by siRNA, decreasing the activity of BMP4 pathway (Figure 25). We confirmed that miR-125a accumulates in Alk3 silenced ESCs compared to control ESCs, 24h after the transfection (Figure 26). Since this miRNA targets Dies1, we speculated that 24h following BMP4 induction, when the miR-125a level is increased, Dies1 protein should be decreased. For this reason we evaluated Dies1 protein level by western blot analysis and we found that it was reduced at 24h from the treatment (Figure 27A). Moreover its mRNA level didn't change at this time point, supporting the finding that miR-125a regulate Dies1 at post-transcriptional level (Figure 27B). These data indicate that BMP4 controls miR-125a level which in turn modulate Dies1 expression, regulating BMP4 signalling.

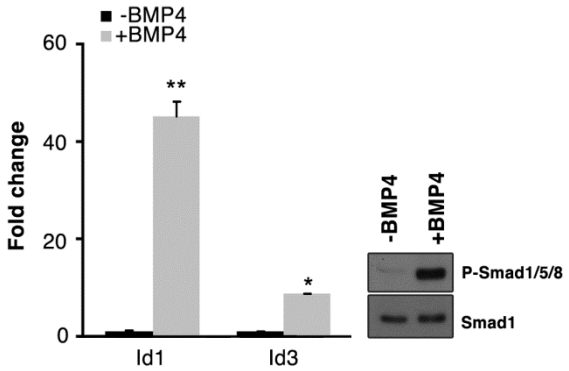

Figure 23. Activation of the BMP4 signalling.

ESCs were cultured overnight in chemically defined medium (KSR) and LIF. Then, the cells were treated with BMP4 for 1h. The activation of the pathway was checked by qPCR analysis of BMP4 target gene expression (Id1, Id3) and by western blot analysis of the phosphorylation status of Smad1,5,8. Data are expressed as fold change relative to the control. * $P < 0.05$, ** $P < 0.01$.

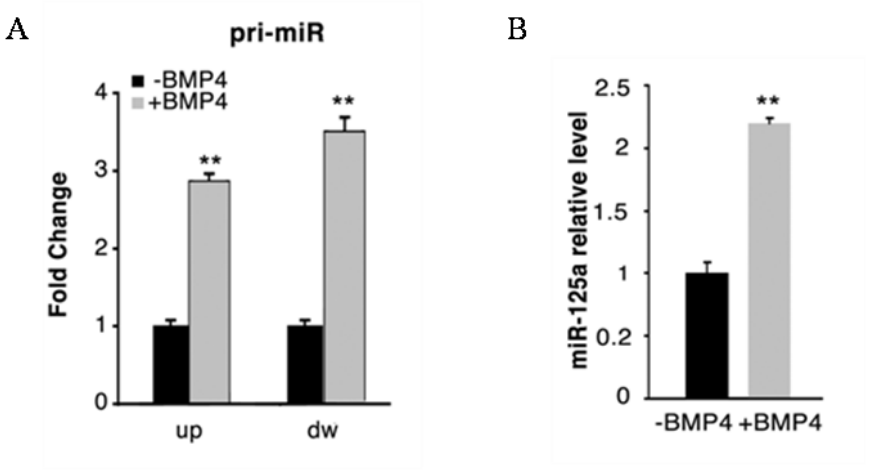

Figure 24. BMP4 affect the expression of miR-125a.

ESCs were cultured overnight in chemically defined medium (KSR) plus LIF and then treated with BMP4 for 1h and 24h. The level of pri-miR-125a was measured by qPCR 1h after the treatment (A). Primers used amplify the regions upstream (up) and downstream (dw) the sequence of the mature miR-125a. The analysis of mature miR levels was done at 24h from the BMP4 stimulation by TaqMan analysis (B). Data are expressed as fold change relative to control. ** $P < 0.01$.

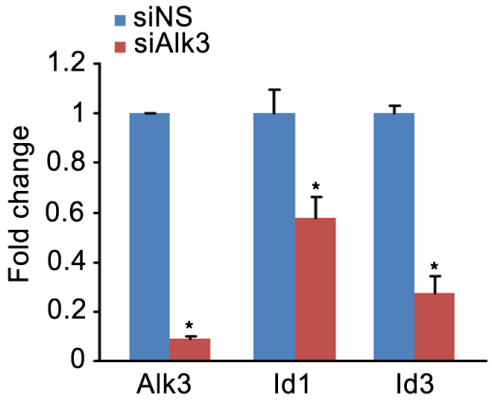

Figure 25. Alk3 suppression downregulates BMP4 signalling.

ESCs were transfected with a siRNA against Alk3 or a control siRNA. 24h after transfection, the expression level of Alk3 and BMP target genes (Id1, Id3) was measured by qPCR analysis. Data are expressed as fold change relative to control. * $P < 0.05$.

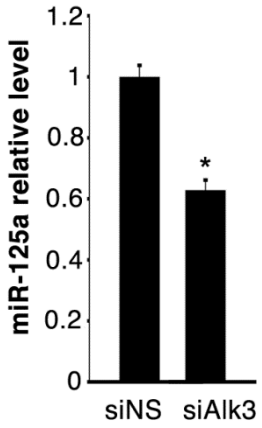

Figure 26. Alk3 suppression reduces the expression of miR-125a.

ESCs were transfected with a siRNA against Alk3 or a control siRNA. After 24 h from transfection, the level of mature miR was measured by TaqMan analysis. * $P < 0.05$.

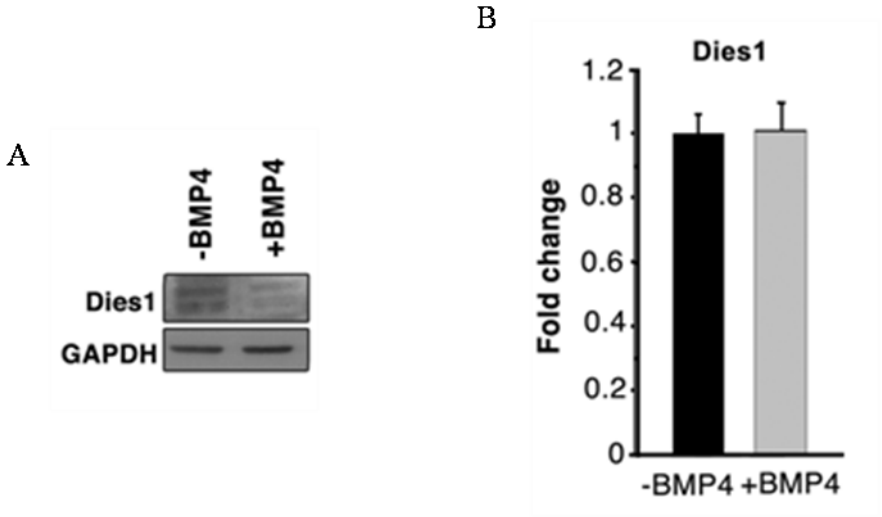

Figure 27. BMP4 stimulation modulates Dies1 expression at post-transcriptional level.

Dies1 protein level was measured by Western blot in ESCs treated with BMP4 for 24 h by using a specific antibody for Dies1 (A). Gapdh was used as loading control. The same samples were subjected to qPCR analysis of Dies1 transcript level (B). Data are expressed as fold change relative to control.

To verify that BMP4 regulates miR-125a directly, we immunoprecipitated the chromatin of ESCs treated or not with BMP4 for 1h, using an antibody against Smad1, the effector of BMP4 signalling. We analyzed, by Q-PCR, the immunoprecipitated chromatin with 10 primers designed to cover the region

upstream the miR-125a gene. Figure 28 shows that the regions 7 and 8 are strongly enriched after BMP4 treatment, indicating that they are possible binding regions for Smad1. It's interesting to note that these regions contain predicted binding sites for Smad1. These findings demonstrate that in ESCs BMP4 regulates directly miR-125a expression, recruiting Smad1 to the proper binding site on the miR-125a gene. This, in turn, controls Dies1 level modulating BMP4 signalling, generating a negative feedback loop.

Since miR-125a gene is in cluster with miR-99b and let-7e, we wondered whether BMP4 could control the expression of these miRNAs too. After 24h of BMP4 treatment, we observed different effects in the level of mature miRNA: let-7e was unaffected, while miR-99b increased (Figure 29). Given the regulation of miR-99b similar to that of miR-125a, we decided to analyze the role of miR-99b in ESC differentiation. But, at day 4 of SFEB differentiation, the expression of stemness markers was very similar between ESCs overexpressing miR-99b and the control cells, indicating that it hasn't a specific role in this context (Figure 30).

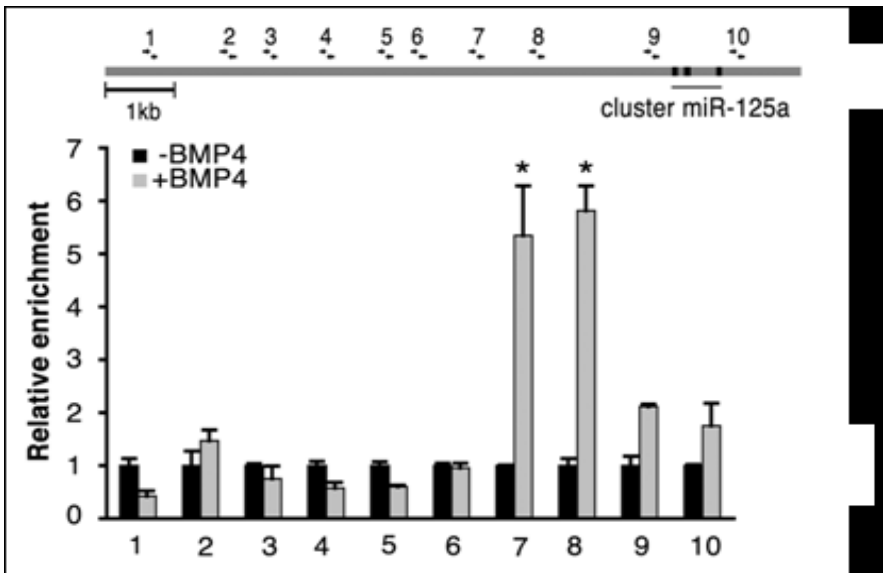

Figure 28. Upon BMP4 treatment Smad1 is recruited to miR-125a promoter regions. ESCs were treated with BMP4 for 1h and the samples were subjected to ChIP assay with the antibody against Smad1. The immunoprecipitated DNA was amplified by qPCR with primers detecting specific promoter regions denoted in the top panel. Data are expressed as fold enrichment relative to the untreated cells. * $P < 0.05$.

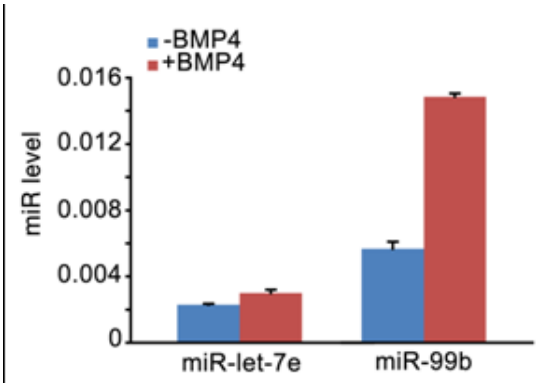

Figure 29. BMP4 regulates the level of miR-99b, without affecting miR-let7e level.
The level of mature miR-let7e and miR-99b were detected by Taqman assay in ESCs treated with BMP4 for 24h. The data are expressed as miR-level normalized with U6 RNA.

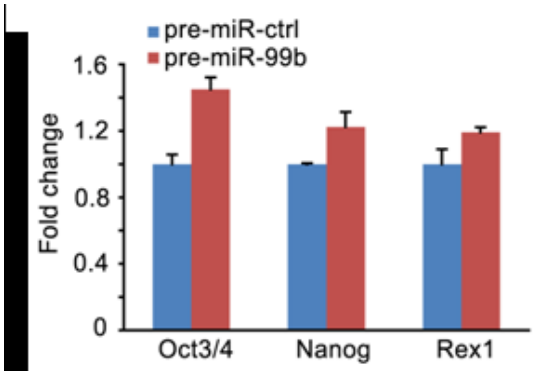

Figure 30. miR-99b ectopic expression doesn't affect the differentiation program.
ESCs transfected with the indicated pre-miR were differentiated as SFEBs for 4 days. The expression of stemness markers (Oct3/4, Nanog, Rex1) was analyzed by qPCR. The results are expressed as fold change relative to control.

5. miR-125a maintains the epiblast phenotype during ESC differentiation

We demonstrated that miR-125a overexpression maintains high levels of stemness markers at day 4 of SFEB differentiation, when in normal condition the cells have acquired a neuroectodermal phenotype (Sox1-positive) losing the stemness features. It is known that during SFEB differentiation the cells go through an intermediate stage, defined epiblast and corresponding to day 2 of differentiation, when they start to be committed to a specific fate but are still pluripotent. Indeed, Epiblast stem cells (EpiSCs) still express some

stemness markers as Oct3/4 and Nanog, losing some others like Rex1. Moreover they are characterized by the expression of specific genes such as Fgf5, Dnmt3b and Cerberus. On this basis, we speculated that the expression of stemness markers could be associated to an epiblast phenotype. So, we analyzed the expression profile of epiblast markers during SFEB differentiation. In normal condition, they reach high level between day 1 and 2 of differentiation. When miR-125a is overexpressed in ESCs, the expression of Fgf5, Dnmt3b and Cerberus is prolonged until day 4 of differentiation (Figure 31).

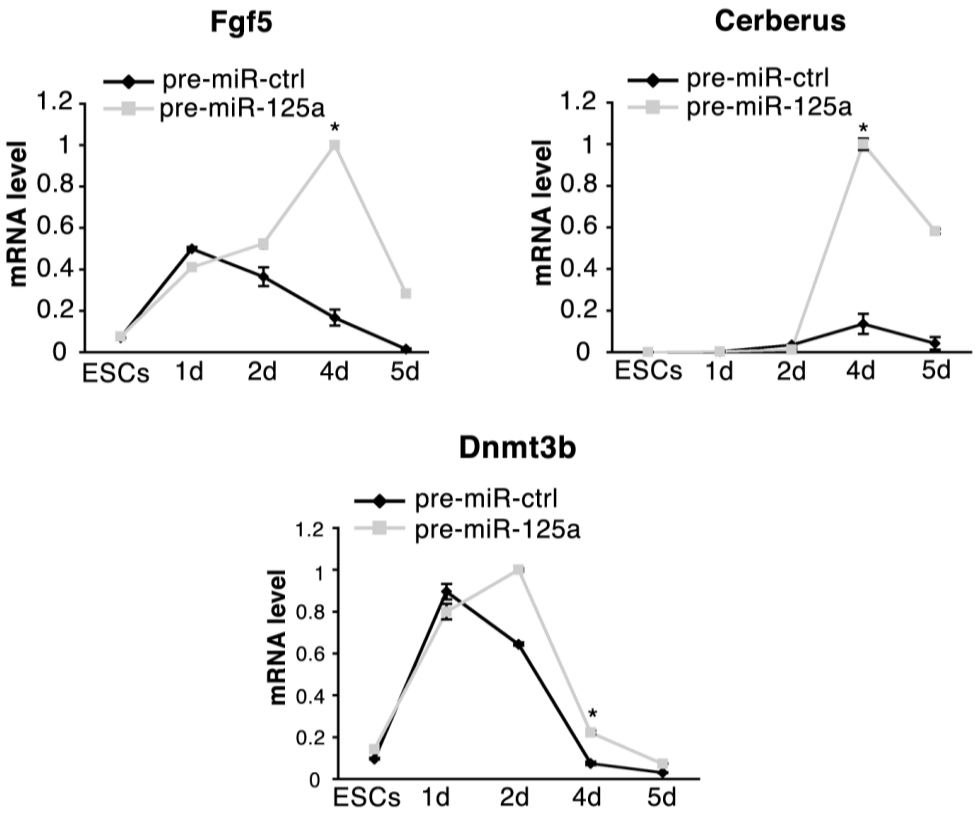

Figure 31. miR-125a overexpression induces a prolonged expression of epiblast markers.

ESCs were transfected with the indicated pre-miR and induced to differentiate through SFEB formation. Samples were collected at the indicated time points and analyzed by qPCR for the expression of epiblast markers (Fgf5, Cerberus, Dnmt3b). Data are expressed as fold change, calculated by assigning the arbitrary value, one, to the time point showing the highest amount of the indicated mRNA. * $P < 0.05$.

We analyzed also the epigenetic signature at Fgf5 transcriptional start site, after 4 days of SFEB differentiation, immunoprecipitating the chromatin with antibodies against H3K4-3me and H3K27-3me. We found that miR-125a overexpressing cells retain high level of H3K4-3me and low level of H3K27-3me on this site (Figure 32), indicating that Fgf5 gene is still transcriptionally active at day 4 of differentiation.

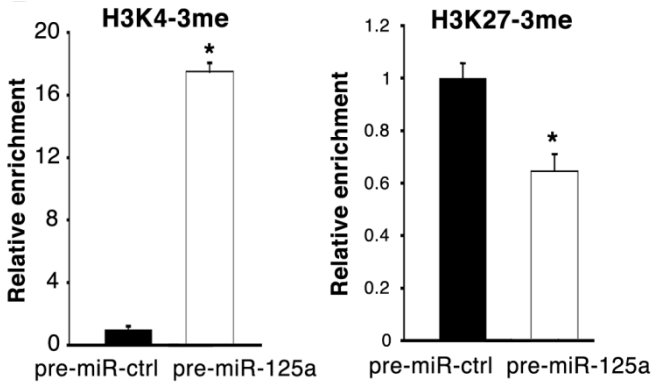

Figure 32. Fgf5 gene is still active at 4 days of SFEB differentiation upon miR-125a transfection.

ESCs transfected with the indicated pre-miR were differentiated through SFEB formation. At 4 days of differentiation, the cells were immunoprecipitated with an antibody against H3K4-3me and H3K27-3me. The DNA was then amplified by qPCR with primers designed in the region of the transcriptional start site of Fgf5. Data are expressed as fold enrichment relative to control. * $P < 0.05$.

6. miR-125a ectopic expression affects the transition through the epiblast stage during ESC differentiation

An important difference between ESCs and EpiSCs is their dependence on extracellular factors: while ESCs require LIF and BMP4 for maintenance, EpiSCs depend on Nodal/Activin and Fgf2 signalling. Moreover the literature reports that BMP4 hampers the transition from ESC state to EpiSC state. Since miR-125a overexpression induces an active transcription of epiblast markers, even after their physiological expression window, we analyzed the status of Nodal/Activin signalling, crucial to sustain an epiblast phenotype.

We found a strong induction of Nodal/Activin targets (Cripto, Lefty1, Lefty2 and Nodal) following miR-125a overexpression at day 4 of SFEB differentiation (Figure 33). Together with the previous observations, this result suggests that miR-125a could have a crucial role in the transition to epiblast state: following miR-125a overexpression, the reduced BMP4 signalling could allow this transition; in addition, the increased activity of Nodal/Activin pathway could sustain EpiSC maintenance in this condition. To verify this hypothesis, we decided to downregulate the BMP4 signalling using a specific inhibitor of BMP receptor, dorsomorphin. Differentiating ESCs in presence of dorsomorphin we obtained the same phenotype of miR-125a overexpression: in particular, the maintenance of stemness and epiblast markers, associated to a reduction of neuroectodermal markers. Also the Nodal/Activin signalling resulted upregulated upon dorsomorphin treatment, showing that this pathway is strictly and inversely related to that of BMP4 (Figure 34).

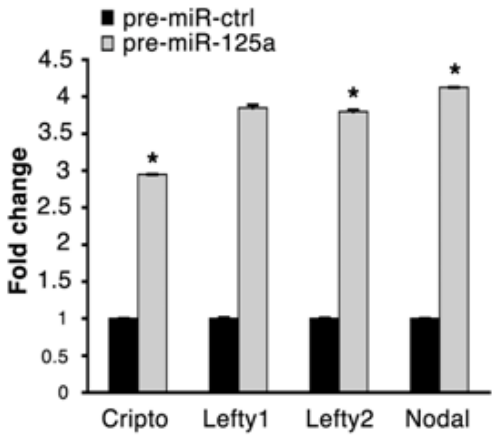

Figure 33. miR-125a overexpression increases the activity of Nodal/Activin pathways during differentiation.

The expression level of Nodal/Activin targets (Cripto, Lefty1, Lefty2, Nodal) was analyzed by qPCR in ESCs expressing miR-125a or miR-ctrl differentiated for 4 days as SFEBs. Data are reported as fold change relative to control. * $P < 0.05$.

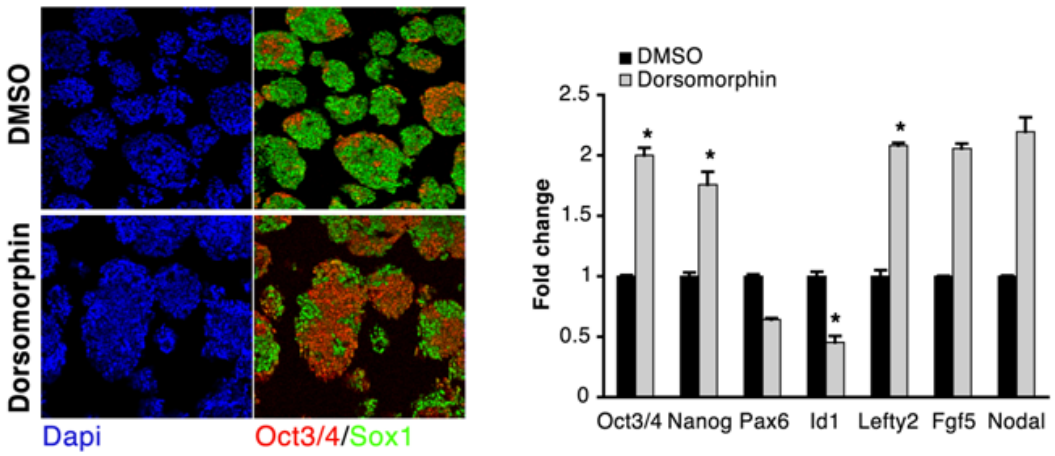

Figure 34. The inhibition of BMP4 signalling impairs differentiation, maintaining the epiblast phenotype.

ESCs were differentiated in presence of 2 µM dorsomorphin or DMSO as control. At 4 days of differentiation, the phenotype was analyzed by immunostaining for Sox1 and Oct3/4 expression. Scale bar = 20 µm. Expression levels of stemness (Oct3/4, Nanog), neuroectoderm (Pax6), and epiblast (Fgf5, Nodal) markers, as well as BMP4 (Id1) and Nodal/Activin (Lefty2) targets were measured in the same samples. Data are expressed as fold change relative to control. * $P < 0.05$.

To confirm that the persistence of epiblast markers means a permanence in the epiblast state, we transfected ESCs with pre-miR-125a or pre-miR-ctrl and differentiated them as SFEBs. We derived EpiSC-like cells dissociating the SFEBs and culturing the single cells in a specific epiblast medium for 5 days. Then we evaluated the number of colonies with an epiblast-like phenotype. Normally, the highest yield of EpiSC-like colonies is obtained at day 2 of SFEB differentiation, corresponding to the epiblast stage, and then it decreases. Since miR-125a overexpression maintains epiblast markers until day 4 of differentiation, we decided to derive EpiSCs at day 4 and 5 of differentiation, when in normal conditions it wasn't possible. The figure 35 shows that miR-125a overexpression gives an higher number of EpiSC-like colonies compared to the control, both at day 4 and 5 of differentiation. Moreover is evident that at day 5 of differentiation the yield of EpiSC-like

colonies strongly decreases also in miR-125a overexpressing cells, due to the lost of expression of miRNA, being transfected only transiently (Figure 36). We decided to check whether these EpiSC-like colonies have epiblast features. For this reason, we analyzed the expression of stemness and epiblast markers in EpiSC-like colonies derived from 4d differentiated SFEBs overexpressing miR-125a, compared to ESCs. We found that these colonies are actually EpiSCs, given the high expression of epiblast markers and the reduction (Oct3/4 and Nanog) or the lost (Rex1) of stemness markers (Figure 37). To demonstrate that the persistence in epiblast state is actually due to the increase of Nodal/Activin signalling, we transfected ESCs with pre-miR-125a or pre-miR-ctrl and differentiated them in presence of Nodal/Activin receptor inhibitor, SB-431542. We found that the presence of SB-431542 prevented to derive EpiSC-like colonies from 4 and 5 day SFEBs overexpressing miR-125a (Figure 38). So, the epiblast phenotype observed upon miR-125a overexpression requires Nodal/Activin signalling for maintenance.

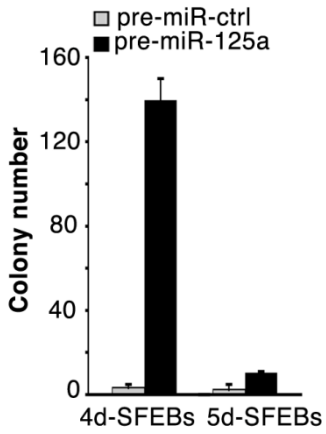

Figure 35. miR-125a overexpression allows to derive EpiSC-like colonies at later stage of SFEB differentiation.

ESCs were transfected with pre-miR-125a or pre-miR-ctrl and induced to differentiate as SFEBs. Then, 4 and 5 day differentiated SFEBs were dissociated, plated in epiblast medium and grown for 5 days in these conditions. Only the large colonies faintly stained for AP were counted.

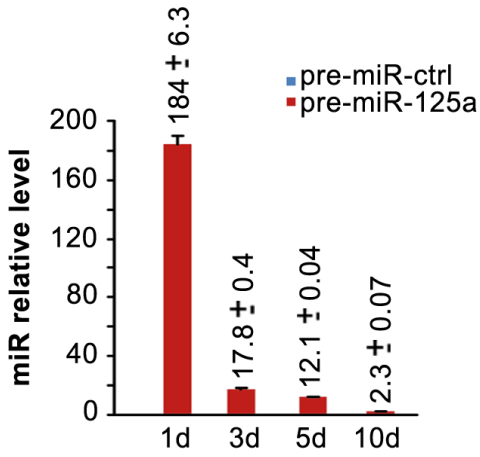

Figure 36. miR-125a overexpression during ESC differentiation.

ESCs transfected with pre-miR-125a were differentiated as SFEBs. Mir-125a levels were measured by Taqman analysis at the indicated time points of differentiation. Data are expressed as relative level compared to the control.

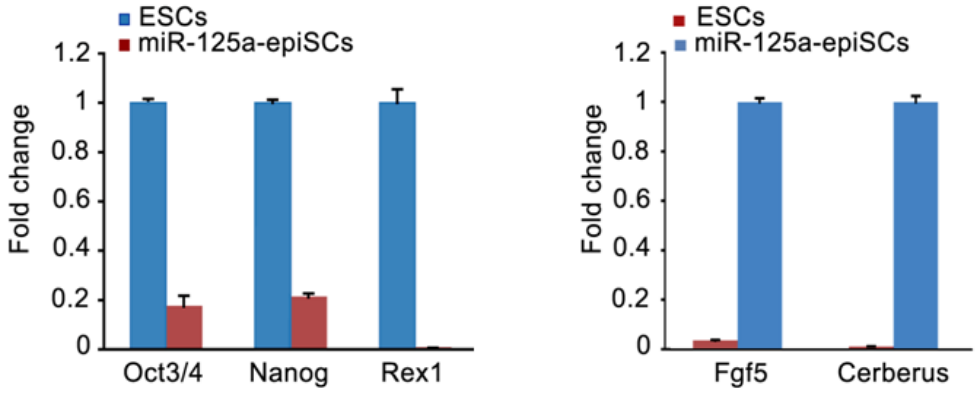

Figure 37. miR-125a overexpressing EpiSC-like colonies show epiblast features.

Four day differentiated SFEBs, derived from ESCs transfected with miR-125a, were dissociated and plated in epiblast medium. The cells were grown for 5 days in this condition and then collected to analyze mRNA level of stemness (Oct3/4, Nanog, Rex1) and epiblast (Fgf5, Cerberus) markers. The expression of these genes in miR-125a overexpressing EpiSC-like colonies was compared to that of undifferentiated ESCs.

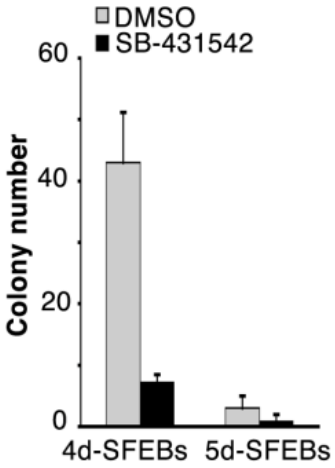

Figure 38. The epiblast phenotype depends on Nodal/Activin signalling for maintenance.

ESCs were transfected with miR-125a and induced to differentiate as SFEBs in presence of 10 μM SB-431542, the inhibitor of Nodal/Activin receptor, or DMSO as control. At 4 day of differentiation, SFEBs were dissociated and plated in epiblast medium. After 5 days of culture, the number of epiblast colonies was counted.

7. The effects of miR-125a overexpression on epiblast transition are due to Dies1 suppression

Being Dies1 a direct target of miR-125a, we analyzed the epiblast phenotype upon Dies1 suppression. As expected, we found that Dies1 suppression by shRNA strongly increases the expression of epiblast markers (Fgf5, Nodal, Otx2, Dnmt3b) and the activity of Nodal/Activin pathway (Lefty1, Lefty2, Cripto), at 4 days of SFEB differentiation (Figure 39). On this observation, we restored Dies1 level in ESCs overexpressing miR-125a and differentiated them for 4 days as SFEBs. We found that Dies1 was able to restore the proper level of epiblast markers and Nodal/Activin targets (Figure 40). This indicates that the epiblast phenotype induced by miR-125a overexpression is mediated by Dies1 suppression.

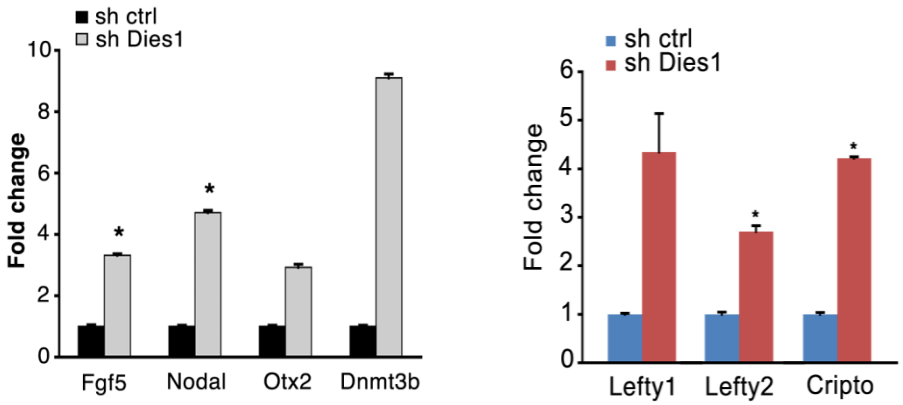

Figure 39. Dies1 suppression extends the epiblast stage during ESC differentiation.
The levels of epiblast markers (Fgf5, Nodal, Otx2, Dnmt3b) and Nodal/Activin targets (Lefty1, Lefty2, Cripto) were assayed by qPCR in ESC transfected with shRNA against Dies1 (sh Dies1) or a control one (sh ctrl), at day 4 of SFEB differentiation. Data are expressed as fold change relative to control. * $P < 0.05$.

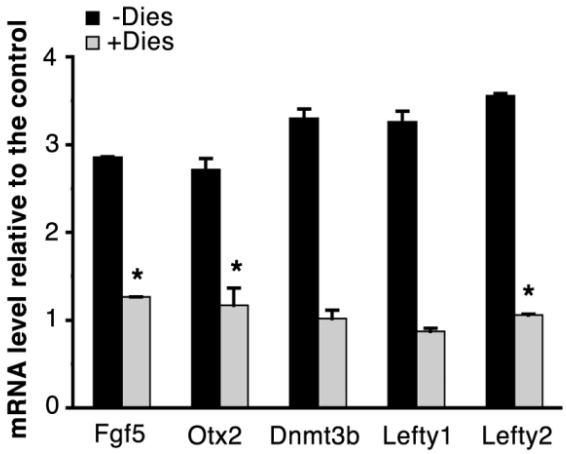

Figure 40. Dies1 is able to rescue the epiblast phenotype.
ESCs were co-transfected with the indicated pre-miR and vector expressing Dies1 lacking its 3' UTR (Dies1) or the empty vector (mock). After 4 days of differentiation, the expression of epiblast markers (Fgf5, Otx2, Dnmt3b) and Nodal/Activin targets (Lefty1, Lefty2) was measured by qPCR. Data are expressed as fold change relative to control.
* $P < 0.05$.

8. miR-125b overexpression impairs ESC differentiation maintaining an epiblast phenotype

MiR-125b belongs to the same family of miR-125a but it is expressed at lower levels in ESCs than miR-125a. Then, its expression increases during the first steps of differentiation and reaches high levels in differentiated cells (Figure 41). In agreement, it is highly expressed in many adult mouse tissues (Figure 42).

We have demonstrated that miR-125b, together with miR-125a, is able to regulate the expression of Dies1 modulating the BMP4 signalling, necessary in the differentiation fate of ESCs. So we decided to investigate whether also miR-125b could have a role in ESCs. First of all, we analyzed the expression level of stemness markers (Oct3/4, Nanog, Klf4 and Klf5) in ESCs transfected with pre-miR-125b or pre-miR-ctrl, but we didn't find difference in the undifferentiated condition (Figure 43).

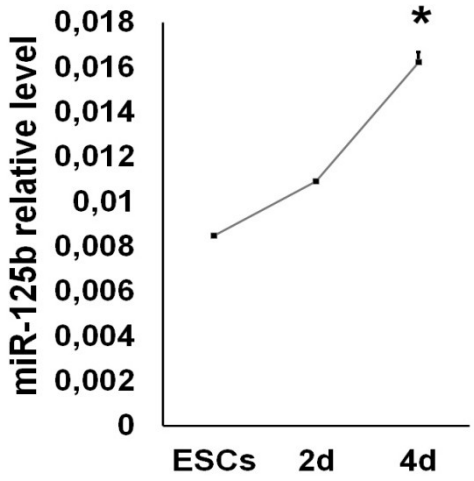

Figure 41. miR-125b expression in embryonic stem cells.

miR-125b expression level was measured by qPCR in undifferentiated ESCs and during SFEB differentiation. The data are normalized to the U6 internal control. * $P < 0.05$.

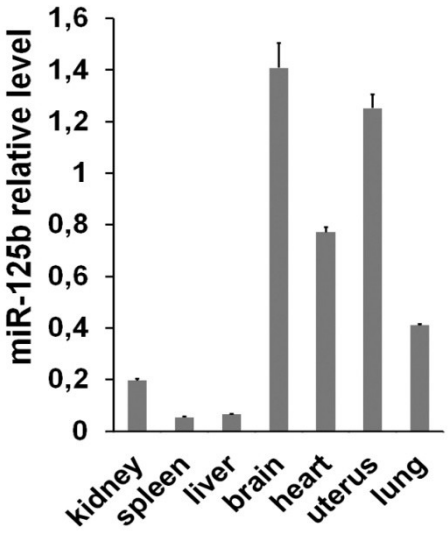

Figure 42. miR-125b expression in adult mouse tissues.

miR-125b expression level was measured by qPCR in the indicated adult mouse tissues. The data are normalized to the U6 internal control. * $P < 0.05$.

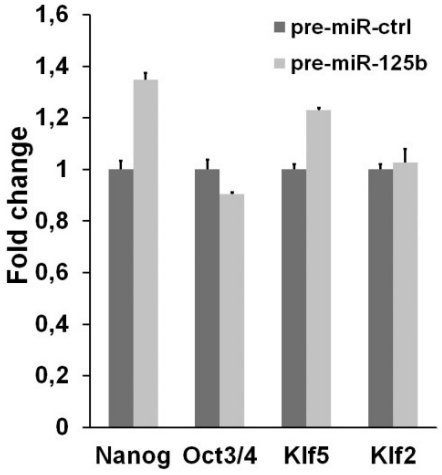

Figure 43. miR-125b overexpression doesn't affect the stemness of undifferentiated ESCs.

ESCs were transfected with pre-miR-125b or pre-miR-ctrl. The level of stemness markers (Nanog, Oct3/4, Klf5, Klf2) was analyzed by qPCR. The data are reported as fold change relative to control.

To study the involvement of miR-125b in the early phases of differentiation, we differentiated ESCs expressing miR-125b or miR-ctrl, as SFEBs for 4 days. At this time point, we found that overexpression of miR-125b, like of miR-125a, impairs differentiation, causing a reduction of Sox1 positive cell and an increase of Oct3/4 and Nanog positive cells (Figure 44). This result was confirmed by the analysis of expression profile of stemness (Oct3/4, Nanog) and differentiation (Pax6) genes (Figure 45). Moreover, since the differentiation program is signed by the activation of Erk signalling, we checked the phosphorylation status of Erk by western blot assay, at day 4 of SFEB differentiation. According to the observed block of differentiation, miR-125b overexpression reduced the phosphorylated form of Erk protein (Figure 46). To understand whether the impairment of differentiation occurs before or after the transition to the epiblast state, we evaluated the expression level of epiblast markers during SFEB differentiation. We found that miR-125b induces high level of Fgf5 at day 2 of SFEB differentiation, which remains significantly higher than control at day 4. The same trend was observed for Cerberus and Dnmt3b, whose levels were strongly increased following miR-125b overexpression, after four days of SFEB differentiation (Figure 47). To be sure that miR-125b blocks the differentiation in the epiblast stage, we analyzed the methylation status of epiblast markers. At day 4 of SFEB differentiation, Nanog, Klf2 and Fgf5 genes were actually active upon miR-125b transfection, as indicated by the increase of H3K4-3me and the reduction of H3K27-3me (Figure 48). Coherently with the maintenance of epiblast markers, miR-125b overexpression caused an unbalance between BMP4 and Nodal/Activin signalling: indeed, BMP4 target genes (Id1 and Id3) were decreased, while those of Nodal/Activin pathway were upregulated (Figure 49).

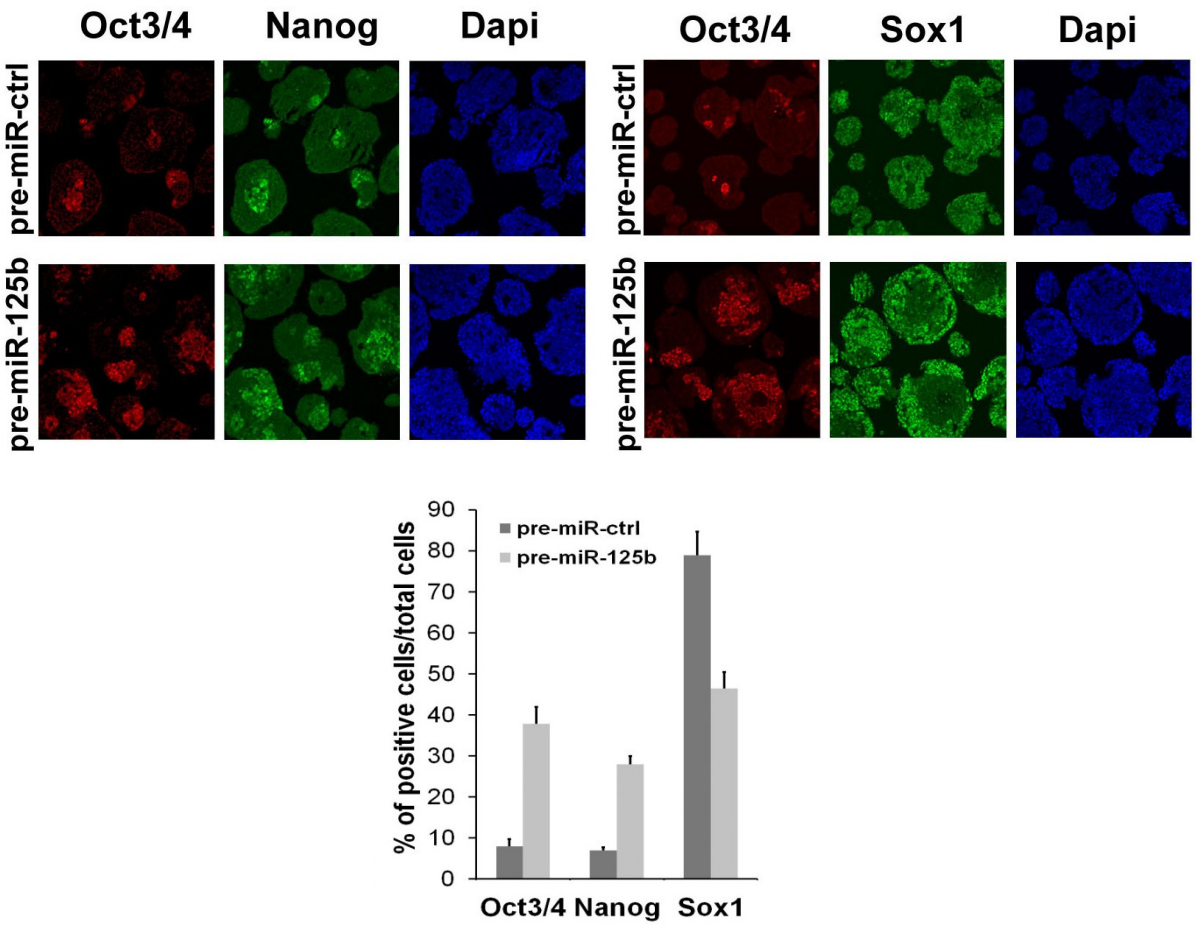

Figure 44. miR-125b ectopic expression alters ESC neuronal differentiation.

Markers of pluripotency (Oct3/4, Nanog) and neuroectoderm (Sox1) are shown in the immunofluorescence analysis of four-day differentiated SFEBs upon miR-125b overexpression. The percentage of positive cells on total cells is represented in the histogram. Scale bar = 50 μm.

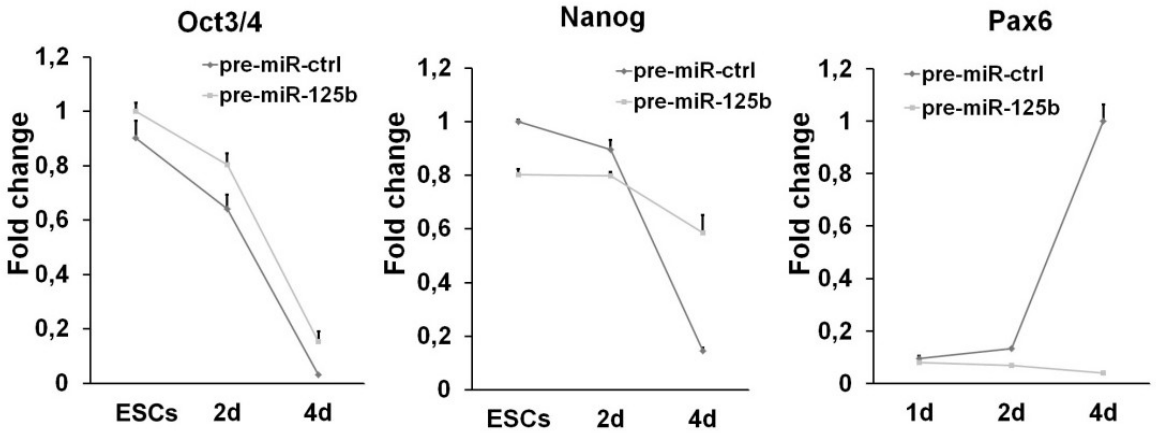

Figure 45. miR-125b overexpression maintains stemness gene expression, impairing ESC differentiation.

Expression profile of stemness (Oct3/4, Nanog) and neuronal (Pax6) markers was measured in undifferentiated ESCs and during SFEB differentiation, following pre-miR-125b or control pre-miR transfection. Data are expressed as fold change, calculated by assigning the arbitrary value, one, to the time point showing the highest amount of the indicated mRNA.

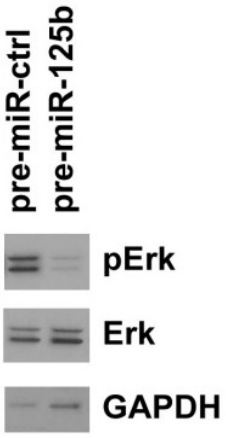

Figure 46. The activation of ERK signalling is impaired by miR-125b overexpression.

ESCs transfected with pre-miR-125b or the control pre-miR were differentiated through SFEB formation for 4 days. In these samples, the level of active ERK (P-ERK) was analyzed by Western blot. Gapdh was used as loading control.

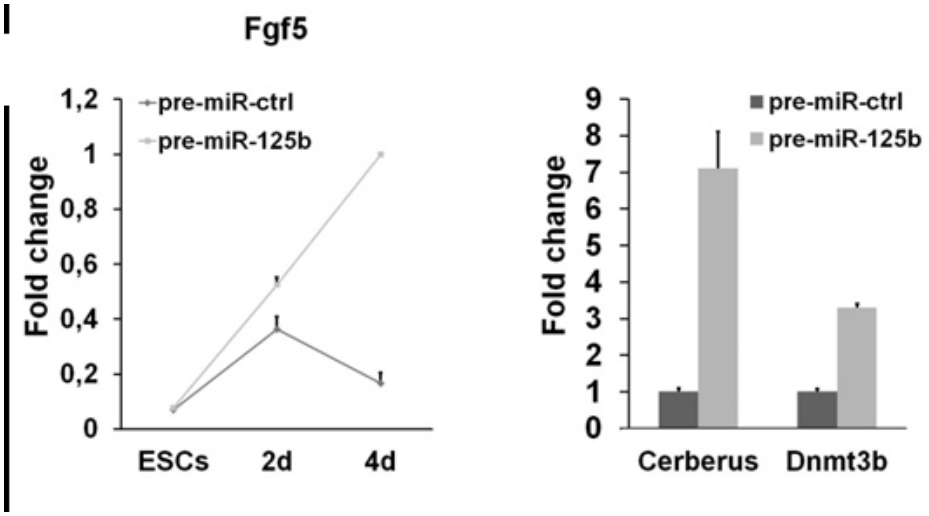

Figure 47. miR-125b expression maintains the epiblast phenotype.

Samples of cells expressing mir-125b were collected in undifferentiated condition or during SFEB differentiation (2d, 4d). By qPCR assay, the level of Fgf5 was measured at these time points, while that of Cerberus and Dnmt3b was analyzed at 4 days of differentiation. Data are expressed as fold change.

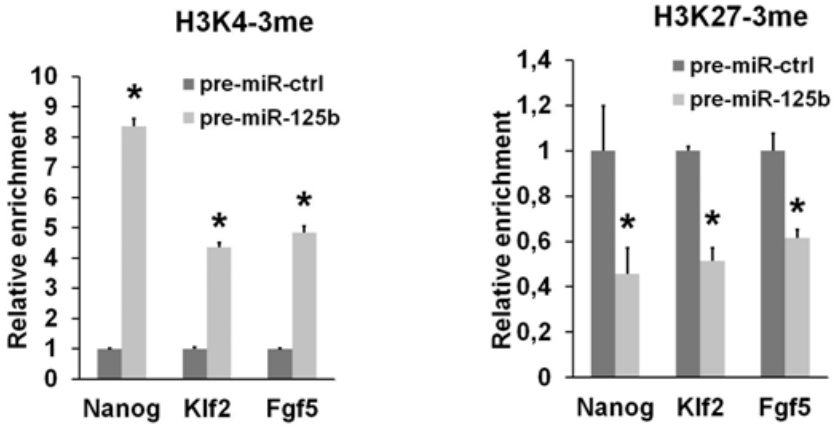

Figure 48. Stemness and epiblast marks are retained in miR-125b overexpressing ESCs.

Chromatin from ESCs transfected with the indicated pre-miR and differentiated for 4 days as SFEBs was precipitated with antibody against H3K4-3me and H3K27-3me. The qPCR analysis was done using primers designed in the region of the transcriptional start site of stemness (Nanog, Klf2) and epiblast (Fgf5) genes. Data are expressed as fold enrichment relative to control. * $P < 0.05$.

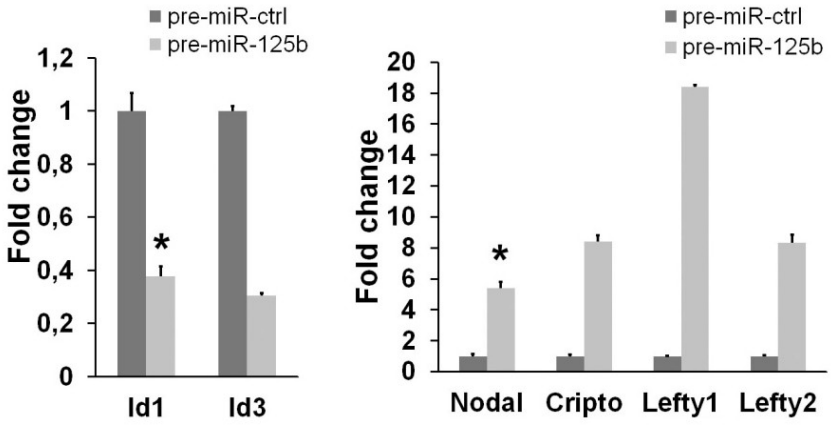

Figure 49. miR-125b expression induces an unbalance between BMP4 and Nodal/Activin signalling.

Upon miR-125b overexpression, the level of BMP4 (Id1, Id3) and Nodal/Activin (Nodal, Cripto, Lefty1, Lefty2) targets was analyzed by qPCR assay, at day 4 of SFEB differentiation. Data are expressed as fold change relative to the control. * $P < 0.05$.

9. miR-125b overexpressing cells still retain their pluripotency in differentiating conditions

We have demonstrated that miR-125b impairs differentiation maintaining the cells in an epiblast stage. To check whether these cells are actually pluripotent, we decided to perform a teratoma formation assay. To this aim, we transfected ESCs with pre-miR125b or pre-miR-ctrl and differentiated them as SFEBs. At day 3 of differentiation, the cells were dissociated and injected into immunodeficient nude mice. After one month, we found that miR-125b overexpressing cells pre-differentiated in vitro for three days were able to form a teratoma in four of five injected mice, while the control transfected cells give a small teratoma only in one mice over five injected (Figure 50A). Moreover, the tumors were explanted and subjected to histological analysis by ematossilin/eosin staining, to evaluated their morphology. Tumors derived from miR-125b overexpressing cells were well differentiated; on the contrary the only one tumor derived from control cells was not completely differentiated (Figure 50B). Therefore, miR-125b

overexpressing cells are able to differentiate *in vivo* and to form a teratoma, indicating that they maintain their pluripotency even after 3 days of *in vitro* differentiation.

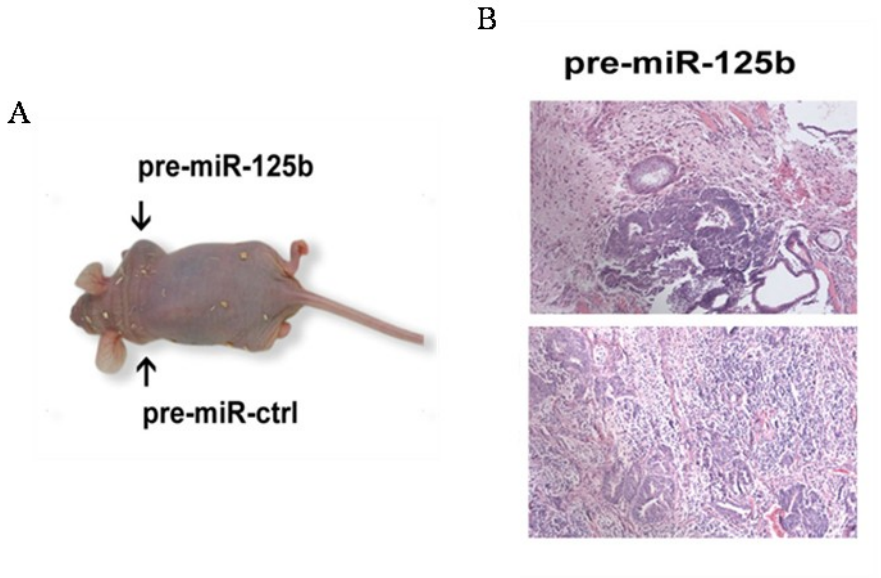

Figure 50. miR-125b expressing cells differentiated in vitro are able to form a teratoma in immunodeficient mice.

ESCs were transfected with the indicated miR and differentiated for three days in vitro. SFEBs expressing pre-miR-125b (right side) and pre-miR-ctrl (left side) were dissociated and injected into immunodeficient mice (A). Teratomas generated by cells overexpressing miR-125b were explanted after one month, and the tissues were analyzed after eosin-hematoxylin staining (B). * $P < 0.05$.

10. miR-125b phenotype on the ESC-epiblast transition is due to Dies1

We have previously shown that miR-125b regulates Dies1 expression in ESCs, modulating the BMP4 signalling. During SFEB differentiation, this results in the unbalance between BMP4 and Nodal/Activin signalling, facilitating the transition in the epiblast stage and the maintenance of this condition, impairing the neuroectodermal differentiation. We speculated that Dies1 could be responsible for the phenotype observed upon miR-125b overexpression. Thus, we transfected pre-miR-125b or pre-miR-ctrl together with a form of Dies1 insensitive to miR regulation. We found that at day 4 of

SFEB differentiation, Dies1 expression re-establishes the proper differentiation program (Figure 51), restoring the level of stemness and epiblast markers, as the activity of BMP4 and Nodal/Activin pathways (Figure 52).

Recently, it was demonstrated that miR-125b targets Lin28 in ESCs to regulate mesendodermal differentiation. So, we measured the expression level of Lin28 in miR-125b overexpressing cells by Q-PCR and western blot assays. But at four days of SFEB differentiation, following miR-125b overexpression, Lin28 mRNA and protein don't change demonstrating that they are not involved in the phenotype observed (Figure 53).

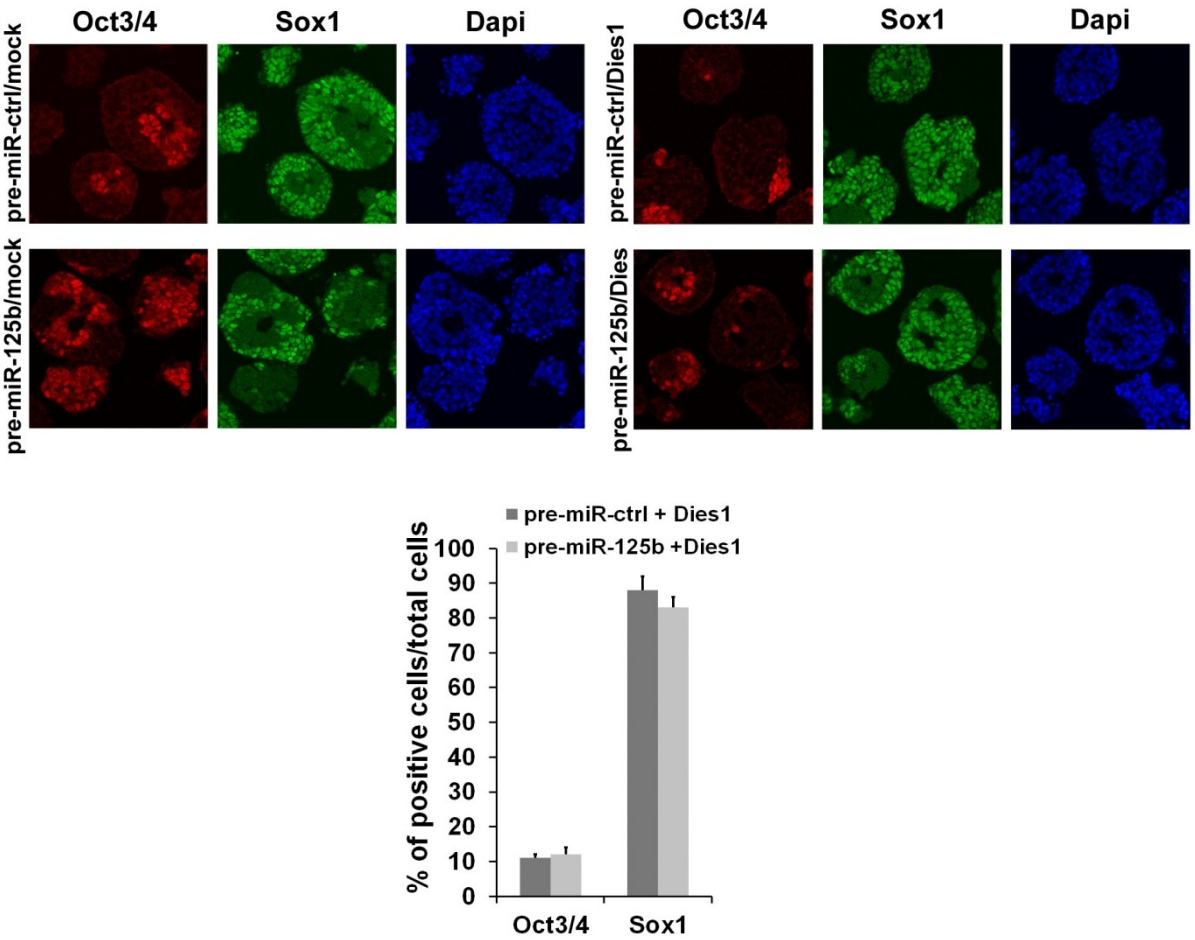

Figure 51. Dies1 rescues the phenotype of ESC differentiation induced by miR-125b overexpression.

Immunostaining of stemness (Oct3/4) and neuronal (Sox1) markers was performed on ESCs co-transfected with the indicated pre-miR and a vector expressing Dies1 lacking its 3' UTR (Dies1) and an empty vector (mock), at day 4 of SFEB differentiation. The histogram shows the percentage of positive cell for the indicated marker on total cells.

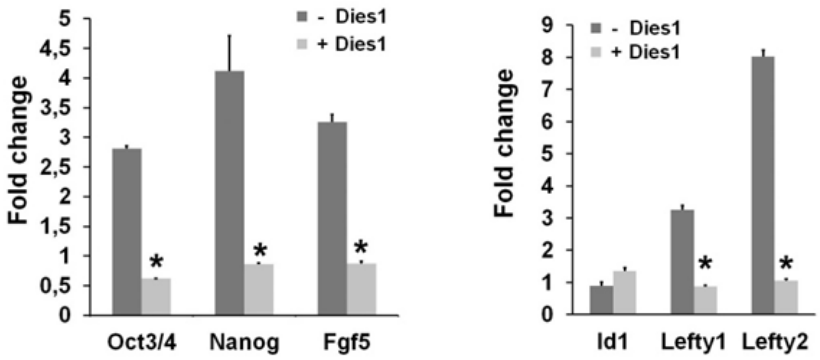

Figure 52. Dies1 restore the proper SFEB differentiation program.

The level of stemness (Oct3/4, Nanog) and epiblast (Fgf5) markers, such as the level of BMP4 (Id1) and Nodal/Activin (Lefty1, Lefty2) targets were measured by qPCR in ESCs co-transfected with the indicated pre-miR and a vector expressing Dies1 lacking its 3' UTR (Dies1) and an empty vector (mock), at day 4 of SFEB differentiation. Data are expressed as fold change relative to the control. * $P < 0.05$.

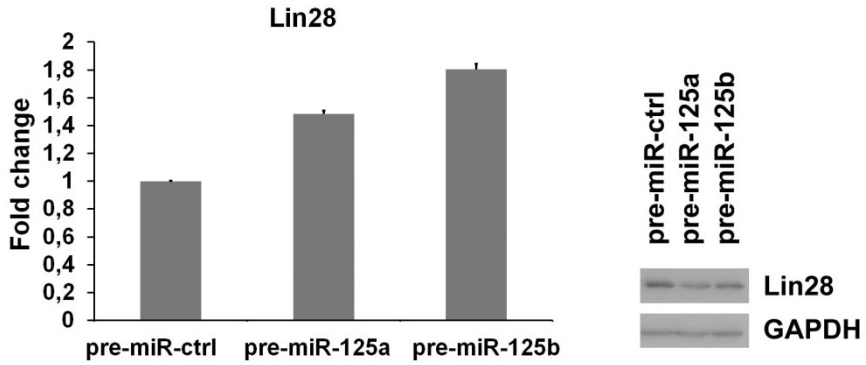

Figure 53. miR-125 isoforms do not target Lin28 protein in undifferentiated ESCs.

ESCs transfected with the indicated pre-miR were differentiated as SFEBs. Following four days, the level of Lin28 was checked in these samples by qPCR and Western blot assay.

11. Suppression of miR-125a and miR-125b promotes ESC differentiation

Given the phenotype induced by miR-125a or miR-125b in ESC differentiation, we asked whether their reduction could alter the differentiation program. Thus, we suppressed both miR-125a and miR-125b in ESCs using a mix of the specific anti-miR, to avoid that one of them could substitute for the other sharing the same targets. Then, the transfected cells were grown at low density in presence of LIF and assayed for AP staining after 7 days. We found that the suppression of both miRNAs reduces significantly the number of AP positive colonies compared to the control, suggesting that the miR-125a and b suppressed cells are losing the undifferentiated phenotype (Figure 54). To support this finding, we showed that, upon miR-125a and miR-125b suppression, Oct3/4 expression starts to decrease just at day 2 of SFEB differentiation and this is more evident at day 4 of differentiation, indicating that the reduction of miR-125a and miR-125b accelerates the differentiation program (Figure 55).

Since we demonstrated that Dies1 is a target of miR-125a and miR-125b, we speculated that Dies1 overexpression should phenocopy the miRNA suppression. Indeed, we showed that the ectopic expression of Dies1 in ESCs grown at low density in presence of LIF gives a lower number of the AP positive colonies than control transfected cells (Figure 56). This result confirms the strong correlation between Dies1 and miR-125a/miR-125b role in ESCs.

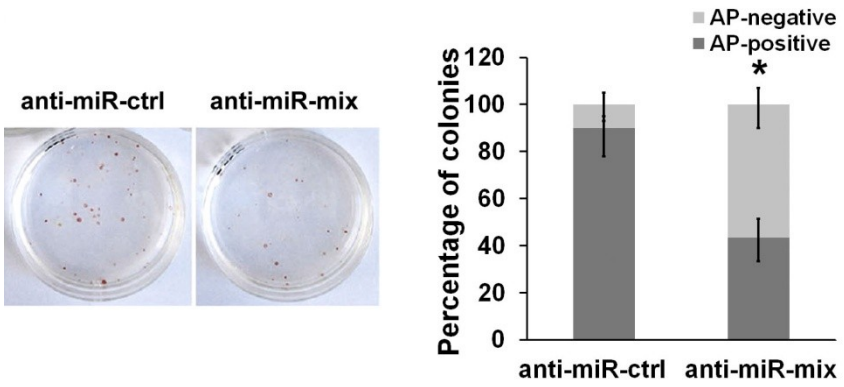

Figure 54. miR-125a and miR-125b suppression induces the lost of stemness phenotype.

miR-125a and miR-125b downregulated ESCs (anti-miR-mix) were grown in clonal condition in presence of LIF for 7 days. The stemness was assayed by staining for Alkaline Phosphatase (AP). The histogram reports the percentage of positive and negative colonies. * $P < 0.05$.

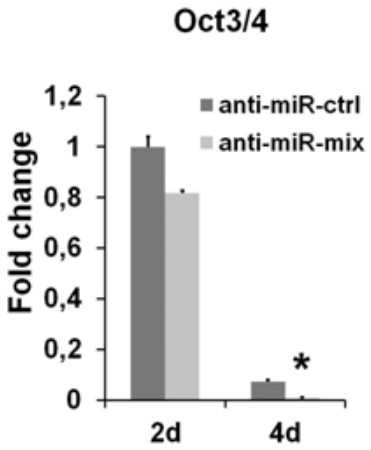

Figure 55. The expression of Oct3/4 is lost earlier during differentiation, following suppression of miR-125a and miR-125b.

Upon miR-125a and miR-125b suppression, ESCs were differentiated as SFEBs. Oct3/4 expression was measured by qPCR on samples collected at day 2 and 4 of differentiation. Data are expressed as fold change relative to control. * $P < 0.05$.

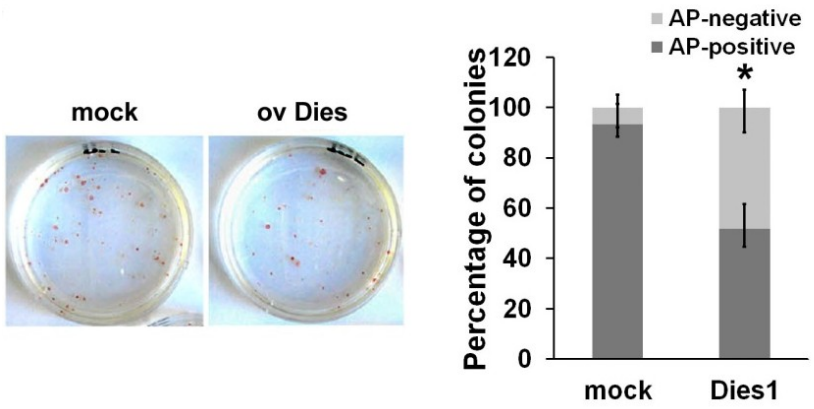

Figure 56. Dies1 ectopic expression allows ESCs to spontaneously differentiate.
ESCs overexpressing Dies1 after seven days of culture at clonal density in the presence of LIF were stained with Alkaline Phosphatase (AP). The percentage of AP positive and negative colonies is reported in the graph. * $P < 0.05$.

12. The regulation of miR-125b expression is independent from TGFβ signalling

We have demonstrated that miR-125a expression is under the transcriptional control of BMP4 signalling. In ESC differentiation, miR-125b gives the same phenotype of miR-125a, targeting Dies1 and affecting the same signalling pathway. The presence in ESCs of two miRNAs belonging to the same family and sharing the role in ESC fate determination, seems to be an unnecessary redundancy. But we supposed that a difference should exist and it could concern the miRNA regulation. To test this hypothesis, we analyzed the level of miR-125b in ESCs upon BMP4 treatment. Contrary to miR-125a, miR-125b precursor and mature levels were not affected following the treatment. The same result was obtained treating ESCs with Activin indicating that miR-125b is not regulated by these pathways (Figure 57).

Moreover, we decided to investigate whether this independence from TGFβ signalling was restricted to ESCs or it can be a general mechanism. We used the C2C12 cell line, where miR-125b was demonstrated to play a role in the differentiation program (Ge, et al., 2011), as the BMP4 pathway (Dahlqvist, et al., 2003). We found that miR-125b was actually expressed in these cells and

at 4 days of differentiation (Figure 58). Then, we evaluated the level of mature miR-125b but we didn't find any difference following BMP4 stimulation (Figure 59). This observation indicated that miR-125b controls BMP4 signalling, through Dies1, but is not regulated by this cytokine.

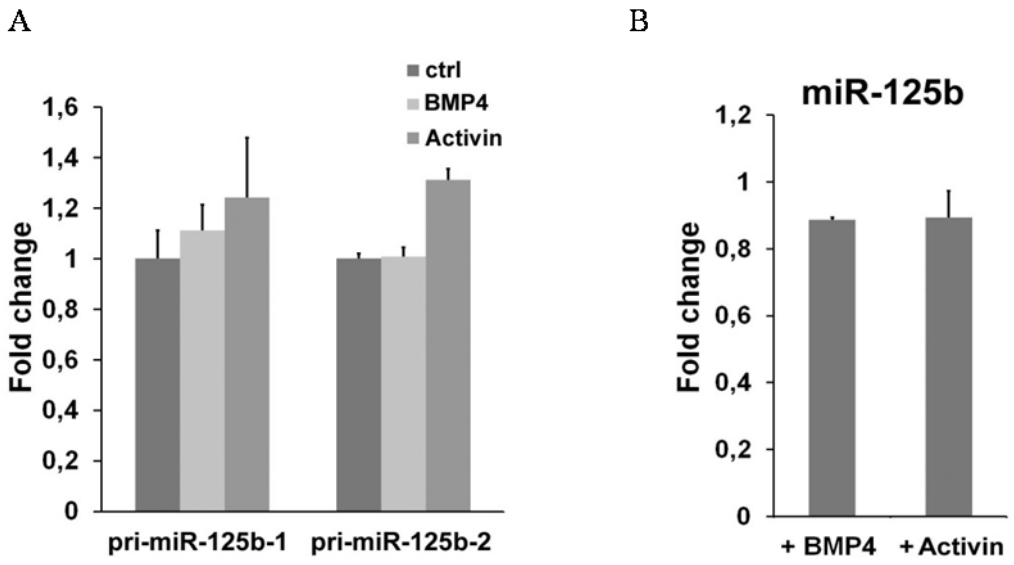

Figure 57. miR-125b regulation is independent from TGF-β signalling.

ESCs were treated with BMP4 or Activin. 1h after the stimulation, the level of pri-miR was analyzed by qPCR using specific primers that distinguish between the transcripts deriving from the two miR-125b genes (pri-miR-125b-1 and pri-miR-125b-2) (A). The mature form of miR-125b was measured by Taqman assay at 24h from the induction (B). Data are expressed as fold change relative to control.

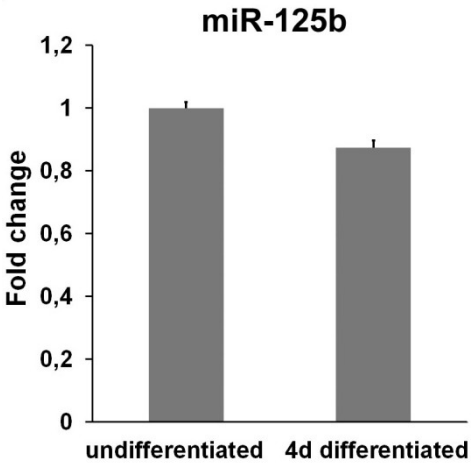

Figure 58. miR-125b expression in C2C12 myoblasts.

Expression level of miR-125b was evaluated in undifferentiated and 4d differentiated C2C12 by Taqman analysis. Data are expressed as fold change relative to control.

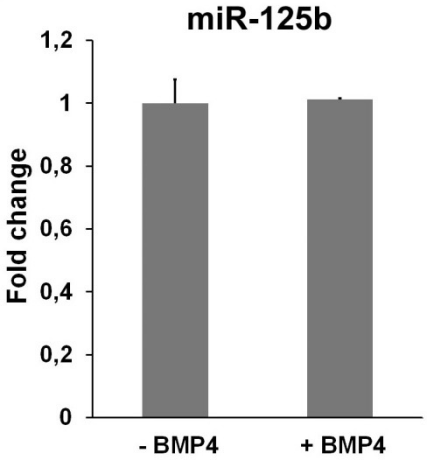

Figure 59. miR-125b level is not controlled by BMP4 in C2C12 myoblasts.

miR-125b expression was analyzed in C2C12 treated with BMP4 for 24h. Data are expressed as fold change relative to control.

Discussion

To unravel the mechanisms controlling ESC fate is an important goal for ESC application in regenerative medicine. Despite the growing number of studies in this field, a lot has still to be discovered and clarified.

Recently, it was identified a new gene involved in BMP signalling and in ESC differentiation, named Dies1 (Aloia, et al., 2010). Considering its role in ESC differentiation, we searched for Dies1 regulators that could physiologically modulate its expression during ESC fate decision and thus the cell sensitivity to BMP4 response. We speculated that this could be one of the regulatory mechanisms of ESC differentiation. So, we decided to explore the possibility of a post-transcriptional regulation of Dies1 by miRNAs, given their established role in the regulation of protein expression at post-transcriptional level. Here we demonstrate that Dies1 is a direct target of both miR-125a and miR-125b. These miRNAs are expressed in undifferentiated ESCs, where the level of miR-125b is lower than miR-125a. Their expression remains almost constant until day 4 of SFEB differentiation, and reaches higher level in differentiated cells. The involvement of these miRNAs in ESCs was previously demonstrated by other groups. In particular, miR-125a was found to down-regulate the RNA binding protein Lin28, after 6 days of differentiation as embryo bodies, when the concentration of miR-125a is very high and that of Lin28 goes down (Zhong, et al., 2010). Wang and collaborators showed that also the miR-125b negatively regulates mesendodermal commitment of ESCs through direct targeting of Lin28, but without affecting ectodermal lineage (Wang, et al., 2012). The role of these miRNAs was investigated also in human ESCs. miR-125 isoforms were demonstrated to promote neural differentiation of hESCs by avoiding the persistence of non-differentiated stem cells and repressing alternative fate choices. This phenotype was associated with the regulation by miR-125 isoforms of SMAD4, a key regulator of pluripotent stem cell lineage commitment (Boissart, et al., 2012). The region of Smad4 mRNA targeted by

miR-125a and miR-125b in hESCs is not conserved in the mouse gene, indicating that the phenomenon observed in hESCs is species-specific.

Here, we demonstrate that miR-125a and miR-125b ectopic expression doesn't affect the undifferentiated condition of mouse ESCs, while it impairs the early steps of mouse ESC neuronal differentiation, as Dies1 suppression does. Moreover the phenotype observed can be rescued at least in part by Dies1 re-expression in ESCs, indicating that miR-125a and miR-125b can participate at ESC differentiation program acting at different level, modulating the expression of specific target at specific time point.

The target of miR-125a and miR-125b, Dies1, has an important role in BMP4 pathway, acting as AlK3 co-receptor. This observation is supported by our FRET/FLIM data showing that Dies1 can interact at molecular level with the BMP4 receptor, Alk3. It is known that this signalling depends on different co-receptors for working, and Dies1 could represent a new regulatory co-receptor involved in the signal transduction in ESCs. More recently, another group have proposed a new function of Dies1 (that they called VISTA). They found that Dies1 is mostly expressed on hematopoietic tissues (spleen, thymus, and bone marrow) or tissues with ample infiltration of leukocytes (lung). Weak Dies1 expression was also detected in non-hematopoietic tissues (heart, kidney, brain, ovary). Analysis of several hematopoietic cell types revealed expression of Dies1 on peritoneal macrophages, splenic CD11b+ monocytes, CD11c+ dendritic cells, CD4+ and CD8+ T cells, but a lower expression level on B cells (Wang, et al., 2011). They have suggested Dies1/VISTA as a new member of the Ig superfamily network, which exerts immunosuppressive activities on T cells both *in vitro* and *in vivo* and could be an important mediator in controlling the development of autoimmunity and the immune responses to cancer (Wang, et al., 2011). This function could seem unrelated to that in ESCs. Actually, some data indicates that ESCs are able to inhibit T-cell activation and the immune response *in vivo*, at least in part due to the

production of TGF-β (Koch, et al., 2008). A possible involvement of Dies1 in this context needs to be deeply explored.

It's known that BMP4 have different roles in different stage of ESC differentiation (Zhang, et al., 2010). In undifferentiated ESCs, BMP4 contributes to stemness maintenance, mostly by blocking the transition of ESCs to the epiblast stage. In EpiSCs, it prevents the differentiation toward the neuroectodermal fate, favouring mesodermal lineage.

We have shown that miR-125 isoforms downregulate the BMP4 signalling, inducing a concomitant increase of Nodal/Activin pathway, as Dies1 suppression does. The alteration of these signal transduction pathways leads to an impairment of ESC differentiation. In particular, miR-125a and miR-125b overexpression facilitate the transition of ESCs to epiblast state, due to the reduction of BMP4 signalling, which normally opposes this progression. At the same time, the increase of Nodal/Activin pathway sustains the permanence of the epiblast phenotype, until miR-125a and miR-125b ectopic expression levels are maintained. The role of BMP4 pathway in the epiblast transition is supported by data obtained from the ESC treatment with dorsomorphin, a BMP4 receptor inhibitor. In this condition, ESCs cannot properly differentiate, giving a phenotype very similar to that observed upon miR-125a and miR-125b overexpression, like the persistence of epiblast state.

It is known that EpiSCs can be derived and cultured in a medium containing Nodal/Activin, and their expansion depends on this signalling (Brons, et al., 2007). Moreover, Zhang and co-workers demonstrated that it is possible to derive an epiblast population from ESCs differentiated as SFEBs. The higher yield of EpiSC-like colonies is obtained at day 2, corresponding to the epiblast stage, while later during differentiation this is strongly reduced (Zhang, et al., 2010). We demonstrated that EpiSC-like colonies can be derived from miR-125a overexpressing ESCs differentiated for 4 and 5 days as SFEBs. Moreover, we confirmed the literature data indicating that the derivation of EpiSC-like colonies depends on the Nodal/Activin signalling.

Indeed, the EpiSC-like colony derivation from miR-125a overexpressing ESCs differentiated for 4 and 5 days as SFEBs is impaired by the addition of Nodal/Activin receptor inhibitor, SB-431542, suggesting that it is necessary for the maintenance of the epiblast phenotype. All these data indicate that a fine balance between BMP4 and Nodal/Activin pathways is necessary to correctly induce the progression to the epiblast stage. As other studies demonstrated, EpiSCs still retain their pluripotency. We demonstrated that mir-125b overexpressing ESCs, pre-differentiated *in vitro*, have the ability to induce, *in vivo*, the formation of a fully differentiated teratoma. This result confirm that the overexpression of miR-125a and miR-125b actually impairs differentiation, blocking EpiSCs progression toward the various differentiation fate.

Given the effects of miR-125 isoforms on ESC differentiation, we decided to investigate also the suppression of both miRNAs. Coherently we found that miR-125a and miR-125b depletion in ESCs induces a loss of undifferentiated state in presence of LIF, such as an early turn off in the expression of stemness markers during differentiation. It's interesting that also this aspect is phenocopied by Dies1 ectopic expression, further supporting the correlation between this gene and miR-125a and miR-125b in ESCs.

Dies1 plays an important role in BMP4 signalling and its expression is regulated by miR-125 isoforms at post-transcriptional level. So, we wondered whether the miRNA expression could be controlled by BMP4 signalling. We demonstrated that BMP4 stimulation induces the expression of miR-125a, recruiting Smad1 on its promoter. The miRNA, in turn, targets Dies1 mRNA modulating BMP4 signalling and generating a feedback regulatory loop which sets the cell sensitivity to BMP4. This could represent an important mechanism in the ESC environment where the extracellular signalling play a key role in determining the cell identity. miRNA 125a gene is localized on mouse chromosome 17 clustered with miR-99b and Let-7e. Since they have a common promoter region, we checked whether also these others are regulated

by BMP4. We found that Let7e is not responsive to BMP4 stimulation, while miR-99b expression is increased following the treatment. Nevertheless, its overexpression in ESCs didn't induce any obvious change in ESC differentiation.

miR-125b belongs to the same family and gives the same phenotype of miR-125a in ESC differentiation. Even if it could appear as an unnecessary redundancy, we found that the BMP4 regulation demonstrated for miR-125a was not observed for miR-125b. Indeed, this gene is not responsive to BMP4 stimulation, neither to Nodal/Activin one. The independence from TGF-β signalling regulation is not restricted to ESCs, but is a more general event, since the same was confirmed in C2C12 myoblasts, where miR-125b has a function during myogenic differentiation.

In summary, our data demonstrate that in the first steps of ESC differentiation, in particular in the transition to the epiblast stage, the BMP4 signalling undergoes different regulations that are dependent (miR-125a) or independent (miR-125b) by BMP4 itself, but both are mediated by Dies1 function (Figure 60). This finding unveils the presence of different ways that can participate in the modulation of BMP4 signalling in ESCs. Moreover, the close link between Nodal/Activin and BMP4 pathways is strengthened as one of the mechanisms governing the timing and the execution of the early steps of ESC and EpiSC differentiation.

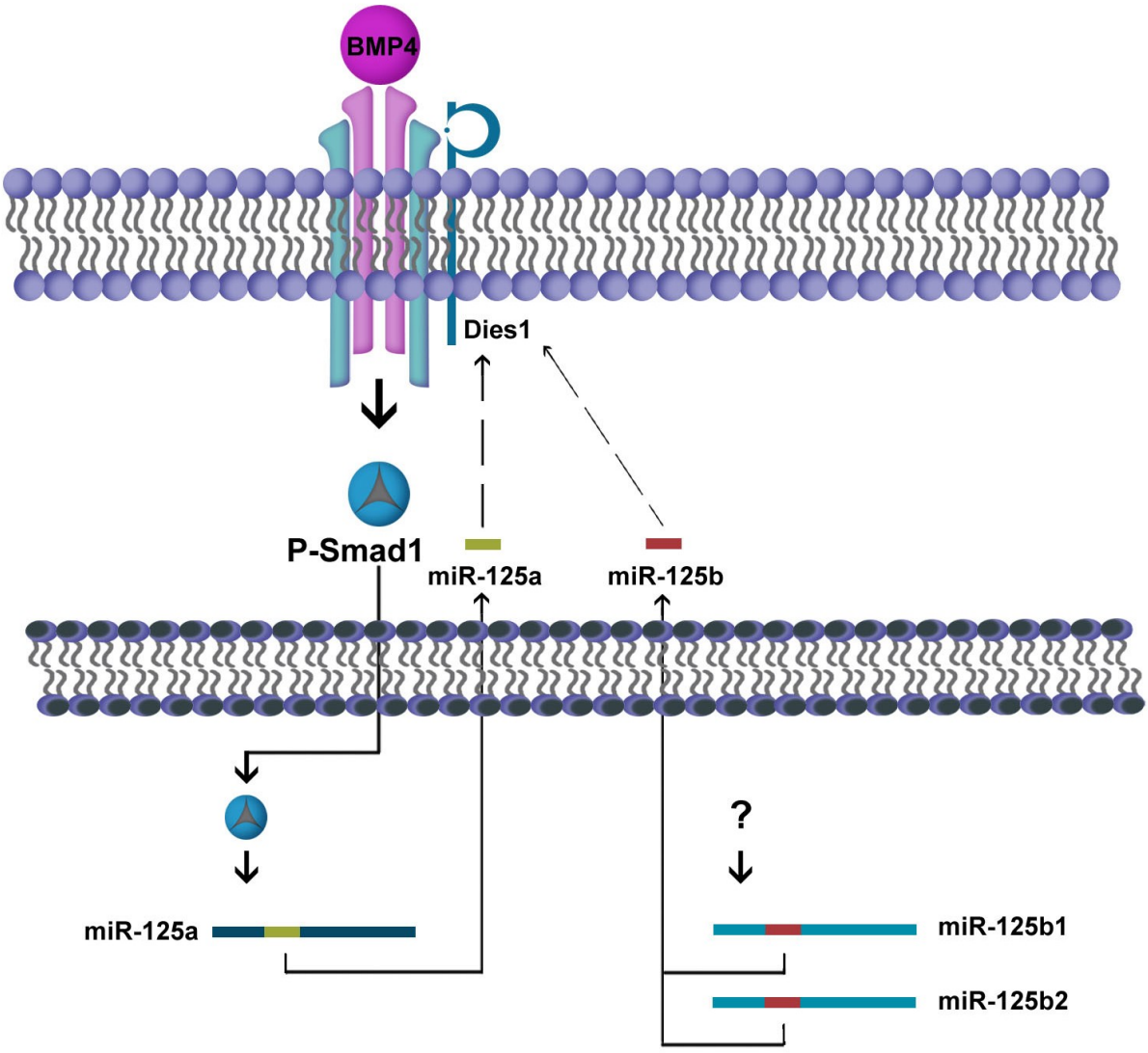

Figure 60. Schematic representation of the BMP4 regulation by miR-125a and miR-125b through Dies1.

In ESCs, the activity of BMP4 signalling is modulated by miR-125a and miR-125b, both regulate Dies1 expression. At the same time, miR-125a level is under the control of BMP4 pathway itself, generating a negative feedback loop. Instead miR-125b expression is independent from TGF-β signalling.

Appendix 1. Primers used for cloning

Name	Forward primer	Reverse primer
Dies1 3'UTR	GCCTGACCTGTCTCCAGCCCTAAGC TTCAGACCTCACCACTCAG	GCACTCGGTACCTTATCAACTGTATC CTTAGCAGAATTCCTC
Dies1 mut 3'UTR	GGGAGCTGAACTAAAAATTTGACA CGTGACTAAAATAGGCAAAAGAGG	CCTCTTTTGCCTATTTTAGTCACGTGT CAAAT TTTTAGTTCAGCTCCC
Dies1-EGFP	GCCGAAGCTTGGCGGAGGTGTGAG CAAGGGCGAGGAGCTGTTCACCGG GGTGGTGCCC	GCGTGAGCGGCCGCCCGCTTTACTTG TACAG CTCGTCCATGCCGAGAGTG
Alk3	CAGCGGATCCGCCGCCATGACTCA GCTATACACTTACATCAGATTAC	GCCAAGCTTAATCTTTACATCCTGGG ATTCAA C
Alk3-Cherry	GCCGAAGCTTGGCGGAGGTGTGAG CAAGGGCGAGGAGGATAACATGGC CATCATCAAGG	GAGTCGCGGCCGCTACTTGTACAGCT CGTCC

Appendix 2. Primers used for Real-Time PCR

Name	Forward primer	Reverse primer
Alk3	CTCATGTTCAAGGGCAGAATCTAG	TTTCTGGCTTCTTCTGGTCCAA
Cerberus	ACTGTGCCCTTCAACCAGACCATTG	TGCCCCTTCTCCGGGAAAACGA
Cripto	ATCCAGTGTGGTTTTGCTTGTG	TCTCTGATGGCAAGGTCTCTCC
Dies1	GCAGGCAAAGGCTCGGGGTC	CCGCAGCCGTGATGCTGTCA
Dnmt3b	CCAAGGACACCAGGACGCGC	TCCGAGACCTGGTAGCCGGAA
Fgf5	CCTCATCTTCTGCAGCCACCTGATC	GTTCCGAGCCGCTTCCTTGGCTGCC
Gapdh	GTATGACTCCACTCACGGCAAA	TTCCCATTCTCGGCCTTG
Id1	GAGCAGCAGGTGAACGTCCT	TCCTTGAGGCGTGAGTAGCA
Id3	GTAAGAGCCCGTCGACCGA	GCAGTGGTTCATGTCGTCCA

Klf2	CCAACTGCGGCAAGACCTAC	CAATGATAAGGCTTCTCACCTGTGT
Klf5	GGTCCAGACAAGATGTGAAATGG	TTTATGCTCTGAAATTATCGGAACTG
Lefty1	CTCGGGTCACCATTGAATGG	TGGACACGAGCCTAGAATCGA
Lefty2	GTCACCATTGAATGGCTGAGAG	GTGGATGGACACGAGCCTAGAG
Lin28	GTTCGGCTTCCTGTCTATGACC	CTTCCATGTGCAGCTTGCTCT
Map2	AACGGGATCAACGGAGAGCT	TTGACTACTTGAACTATCCTTGCAGAT
Nanog	TCAGAAGGGCTCAGCACCA	GCGTTCACCAGATAGCCCTG
Nodal	CCTCCAGGCGCAAGATGT	ACCAGATCCTCTTCTTGGCTCA
Oct3/4	AACCTTCAGGAGATATGCAAATCG	TTCTCAATGCTAGTTCGCTTTCTCT
Otx2	CATGATGTCTTATCTAAAGCAACCG	GTCGAGCTGTGCCCTAGTA
Pax6	AGTGAATGGGCGGAGTTATG	ACTTGGACGGGAACTGACAC
pri-miR-125b-1	GAGTCTGCAACCGAAATTGCCTG	GTTCCTTCAGCGATGCAAAGGC
pri-miR-125b-2	GCTGTCCGTTTACCTGGAAGAAG	CTGGTGGTTTATGCCGAGAATC
Rex1	GCAGTTTCTTCTTGGGATTTCAG	CTAATGCCCACAGCGAT
Sox1	CATCTCCAACTCTCAGGGCT	ACTTGACCAGAGATCCGAGG
Sox2	CTGCAGTACAACTCCATGACCAG	GGACTTGACCACAGAGCCCAT
up	GGTGACCCCTGGCAACCTTCCT	TCATTGTGGGGGAGGGGGAGC
dw	TGAGGCATCTCCTGGTTCCTTTCT	TCCCCAGAGGTGGGAACGGG

Appendix 3. Primers used for ChIP

Name	Forward primer	Reverse primer
1	ACCCGAAGGAGAATGCTCTGTGT	TCATCTCAGCAGCTTGCCCTGGGGA
2	CACTTGACGCCCAGGGGCTG	GCCTTGAAACTCAGGACCCAGCA
3	ACCAAGCCCTAGTGAGCTGAGGT	TGGAGTCAAAGTCAGGGCCTCGT
4	TGCTGAGTGATTTGCAGCTGCCT	GCCCACAGAGACAAGGAGAGGG
5	AACACGTTCGGGACTGCCCG	AGGGACCAAGAGACCGGAAGCT
6	GGGGAGGAACACTGCGCAGG	CCAGGGGCTGCATTTCCACCC
7	AGGGTCCCCAAGGGAGGAGG	CCACCACCACTTCGCTATCC
8	ACCAGGTTTCCCCCACCCC	TGGTCCCGCCCCCTTAACCC
9	TCTGCCGGGGAGGGCTATGG	CCCTGCCGCCTTGCACTCAA
10	TTCAGGGCATCCACGTGGGC	TGGCAGACACGGAGGCGTTC
Fgf5	AGGGACGGTCAAGATTCCTT	AGAACCAGCAGAGTCCCAGA
Klf2	TGCAGATCTTGAGGGCCTAGTTGT	TCCCATGGAGAGGATGAAGTCCAA
Oct3/4	GGACTAGAACCCAGAATTGCAAGA	GTACAGACAGTGATGGCATGAAGC
Nanog	TGTGAGCTCAGTGCTCCTTCCAAA	TTCAGACCTTGGCTCCAGATGCTA

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