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"Identification and characterization of genes involved in mouse embryonic stem cell differentiation into neurons"

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ABSTRACT

Mouse Embryonic Stem (ES) cells are pluripotent, as they have the ability to differentiate into all cell types of the embryo. Many protocols have been established to differentiate ES cells into the desired cell type *in vitro*. However, the identification of molecular machinery involved in ES cell biology will increase the knowledge of embryo development and will improve the use of ES cell for regenerative medicine. To identify genes involved in pluripotency and differentiation we developed a systematic approach based on the screening of a shRNA library. The results of the screening shed light on an uncharacterized gene, we named Dies1 (Differentiation of Embryonic Stem cells) given its effect on ES cell differentiation. Dies1 knock-down affected proper ES cell differentiation into neurons, astrocytes and cardiomyocytes. Dies1 knock-down cells, in fact, maintained the expression of undifferentiated markers such as Oct3/4, Nanog and Alkaline Phosphatase in conditions promoting differentiation. In addition, the growth of Dies1 knock-down cells was independent from LIF. Dies1 suppression exerted its effect on ES cell differentiation *in vivo* and *in vitro*, giving rise to teratoma smaller than controls when Dies1 knock-down cells are injected into nude mice. The *in silico* analysis of Dies1 sequence indicated that it possesses a trans-membrane domain and an Ig-like domain in the putative extra-cellular domain of the protein, like many membrane receptors. This analysis was confirmed by the staining of Dies1 on cell surface of mouse ES cell and blastocyst. The possible role for Dies1 as receptor was also supported by the dominant negative function of Dies1 extra-cellular domain, whose effect resembled Dies1 suppression. Thus, we investigated the involvement of Dies1 in the signaling pathways controlling ES cell pluripotency: LIF and BMP4. Dies1 knock-down did not affect the expression of undifferentiated markers such as Oct3/4, Nanog, Sox2 and Rex1 and of LIF down-stream targets such as Kruppel like factors in undifferentiated conditions. On the other hand, Inhibitory of Differentiation (Id) genes, which are BMP4 targets, were down-regulated in Dies1 knock-down cells. This down-regulation was dependent on a decreased response to BMP4, as shown by luciferase assays and occurred in a Smad dependent manner, as the amount of phosphorylated Smad 1/5/8 complex was decreased in Dies1 knock-down cells. Moreover, the involvement of Dies1 in BMP4 signaling was supported by the direct interaction between Dies1 and BMP4 *in vitro*. The down-regulation of BMP4 targets induced also the up-regulation of Nodal/Activin targets, such as Cripto and Lefty1/2. Such a regulation between Nodal/Activin and BMP4 was observed also in consequence of the suppression by RNA interference of the BMP4-type I receptor Alk3. This phenomenon was not surprising because Nodal/Activin and BMP4 belong to the TGF-β superfamily and share the common

mediator Smad4 which balances the transcriptional activity of Smad 2/3 (dependent on Nodal/Activin) and Smad 1/5/8 (dependent on BMPs). Thus, we hypothesized that the up-regulation of Nodal/Activin signaling was responsible for the effect of Dies1 suppression. To address this point, we treated Dies1 knock-down cells with the specific Nodal/Activin inhibitor SB-431542. The treatment with SB-431542 restored proper differentiation ability in the absence of LIF, leading to the rescue of Dies1 knock-down effect. Taken together our results suggest that the balance between BMP4 and Nodal/Activin exerts a crucial role in regulating ES cell pluripotency and differentiation.

In summary, we have identified Dies1 as novel component of BMP4 signaling pathway required for proper ES cell differentiation.

INTRODUCTION

1. Embryonic Stem cells: Past and future

Research on teratocarcinoma and Embryonal Carcinoma cells (EC) has been carried out over the past 50 years. Teratoma (benign) and teratocarcinoma (malignant) are tumours that are most commonly found in the gonads but also occasionally in extragonadal sites. Their name, which stems from the Greek word 'teratos', describes their appearance, as these tumours are composed of a mixture of adult tissues. In 1964 Kleinsmith and Pierce demonstrated that a single cell derived from a tumour injected intraperitoneally can produce all cell types that are encountered in a teratocarcinoma (Kleinsmith and Pierce 1964). This finding suggested that teratocarcinoma possess a unique type of stem cell, which has the capacity to grow indefinitely, ensuring the malignant nature of the tumour and at the same time being able to differentiate into multiple adult cell types. The existence of such pluripotent stem cells and the observation of specific structures in the tumours similar to early embryos indicated the embryonic origin of teratocarcinoma. Given the similarities between mouse EC cells and the cells of early embryos it was tempting to investigate whether EC cells retain their embryonic nature. Brinster in 1974 demonstrated that EC cells could contribute to the development of chimaeras following injection into the mouse blastocyst (Brinster 1974). In the early 1980s the attempt to derive cells similar to EC cells directly from the embryo resulted in the isolation of mouse embryonic stem (ES) (Evans and Kaufman 1981). ES cells are capable of complete differentiation that gives rise to all tissues found in an adult animal. This differentiation takes place *in vitro*, *in vivo,* in tumours derived from injected ES cells and in chimaeras. Moreover, an ES cell is able to give rise to germ cells in chimaeras and the germ cells in turn are able to develop into normal, fertile adults. Of great concern is that mouse ES cell differentiation follows the same hierarchical steps of embryo development, resulting in an excellent model for studying embryo development in vitro. Furthermore, the use of ES cells as a vehicle for introducing targeted genetic modifications into the germ line makes ES cells a favourite tool for gene function analysis (Solter 2006). Thus, the class of pluripotent cells includes EC cells, ES cells and primordial germ cells because of the extensive similarity in the expression of various markers between these cell types. These considerations, as well as the germ cell origin of spontaneous teratocarcinoma, led to attempts to derive ES-like cells from primordial germ cells. These attempts resulted in the isolation of embryonic germ cells (Matsui, Zsebo et al. 1992). In most aspects embryonic germ cells are identical to ES

cells, although their biology has not yet been studied extensively. It was therefore obvious to ask whether ES cells can also differentiate into germ cells. In the last few years, some reports have suggested that ES cells can differentiate into cells that are very similar to male and female germ cells (Toyooka, Tsunekawa et al. 2003). However, so far there is no indication that these cells are completely functional gametes that can support normal embryonic development. Recently, great promise for regenerative medicine is given by reprogramming of somatic cells into induced pluripotent stem cells (iPS). Takashasi and Yamanaka have demonstrated that Oct4, Sox2, Klf4 and c-myc (also known as Yamanaka factors) are able to "*reprogramming*" fibroblasts into pluripotent cells capable to colonize ICM and give raise to an high rate of chimaerism (Takahashi and Yamanaka 2006). iPS can be induced without c-Myc, but with even lower efficiency (Wernig, Meissner et al. 2008). Hong and collaborators reported a higher rate of "reprogramming" in fibroblasts lacking p53 into iPS cells even without the Myc retrovirus. The p53 deletion also promoted the induction of integration-free mouse iPS cells with plasmid transfection. Thus, the suppression of p53 increases the efficiency of mouse and human iPS cell generation (Hong, Takahashi et al. 2009). The links between pluripotency and tumorigenicity are exemplified by the fact that genes used to produce iPS cells are oncogenes such as c-myc and Klf4 or are linked to tumorigenesis such as Sox2, Nanog, and Oct3/4. Moreover the finding that p53 suppression promotes iPS generation demonstrates that the p53-p21 pathway serves as a barrier not only in tumorigenicity, but also in iPS cell generation (Kawamura, Suzuki et al. 2009). Of great concern is that nearly all iPS cells described in published works have been demonstrated to cause teratoma, proving pluripotency but also tumorigenicity, and that mice genetically derived to contain some tissues from iPS cells have a malignant tumor incidence of 20%. Thus, these studies reveal that differentiation is reversible, and provide strong foundation for the development of technologies that will enable the generation of patient-derived pluripotent cells. Immunologically matched pluripotent cells are an ideal source for the generation of cells for transplantation, and patient-derived pluripotent cells may also be useful tools for the study of disease states and drug therapies.

2. Pluripotency and differentiation

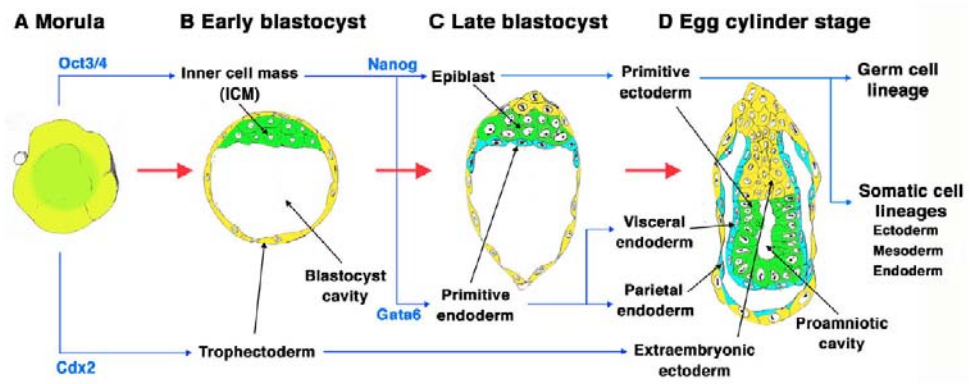
Mouse embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the pre-implantation embryo at stage of blastocyst (day 3.5 post coitum). As the embryo develops, the ICM gives rise to two distinct cell lineages: the extra-embryonic endoderm, which goes on to form the extra-embryonic tissues and the epiblast, which gives rise to the primitive ectoderm at the egg-cylinder stage of embryogenesis, from which the embryo proper arises (Fig. 1). The primitive ectoderm is the only cell lineage in which pluripotency is maintained at this stage of development, enabling it to give raise to all three embryonic germ layers and to primordial germ cells. However, as it lacks the ability to differentiate into primitive endoderm lineages, the primitive ectoderm is less pluripotent than the cells of the ICM. ES cells, in fact, are defined as pluripotent cells because they have the ability to give rise to all cells of embryo and adult but they give a limited contribution in extra-embryonic tissues (Bradley, Evans et al. 1984). In fact, through the ICM that they colonize, ES cells can contribute to extra-embryonic mesoderm and therefore to composite annexes such as the amnion, the allantois and the yolk sac, but they do not give rise to the trophoectoderm, which is consistent with the first cell lineage decision that separates ICM and trophectoderm in the blastocyst. However, Niwa and collaborators demonstrated that ES cells can differentiate *in vitro* into trophoectoderm in particular culture conditions such as Oct4 suppression by RNA interference (Niwa, Toyooka et al. 2005). Therefore, Niwa proposed a new definition of pluripotency for ES cells: the ability to generate all cell types, including the trophectoderm, without the self-organizing ability to generate a whole organism (Niwa 2007).

Mouse ES cell differentiation follows the same hierarchical steps of embryo development. Indeed, the same markers are expressed in both ES cell and embryo at same time during ES cell differentiation and embryo development. ES cells, in fact, express Oct4 and Nanog, which are expressed in ICM, whereas they do not express Cdx2 that marks throphoectoderm in the blastocyst. In addition, ES cells give rise to primitive endoderm, as ICM does *in vivo.* Primitive endoderm is recognized by the expression of Gata6 that is mutually exclusive with Nanog, which marks pluripotent cells that will give rise to primitive ectoderm and then to the three germ layers: ectoderm, endoderm and mesoderm. The formation of Embryoid Bodies from ES cells gives rise to the three germ layers. Early markers of differentiation of the three germ layers may be recognized *in vitro* as well as *in vivo*: Sox1 marks ectoderm, Brachyury (also named T) marks mesoderm, Gata4 and Hnf3β marks endoderm. Culture conditions influence differentiation process, promoting the

formation of a particular germ layer and then the differentiation into a particular cell type. To date, lots of protocols for efficient differentiation of ES cells towards the desired cell type are available.

Pluripotency is maintained in ES cell through the prevention of differentiation and the promotion of proliferation. Thus, ES cells can self-renew continuously for years if they are cultured under conditions preventing their differentiation. Pluripotency can also be re-established in a differentiated or non-pluripotent cell by exposure of the nucleus to external factors. The introduction of the nucleus of a somatic cell into an enucleated oocyte, termed Somatic Cell Nuclear Transfer (SCNT) results in a cell that is able to give rise to all cells of the mature organism, as demonstrated when this process was used to create Dolly the sheep and some others species of mammalian clones subsequently (Wilmut, Schnieke et al. 2007). More recently, the use of four specific transcription factors, c-myc, Sox2, Oct4 and Klf4, has been shown to induce pluripotency in both human and mouse somatic (Takahashi and Yamanaka 2006) (Fig. 2). Loss of pluripotency and commitment to specific cell lineages results in changes in gene expression that include the down regulation of the key pluripotency transcription factors and the up regulation of regulators of differentiation (Ivanova, Dimos et al. 2002) (Ramalho-Santos, Yoon et al. 2002) and is accompanied by a range of epigenetic alterations including DNA methylation (Carlone, Lee et al. 2005) (Yeo, Jeong et al. 2007) and chromatin modifications (Azuara et al., 2006; Mikkelsen et al., 2007). Use of ES cells in medicine is a promising scenario for next years. Understanding the molecular mechanism underlying ES cell pluripotency and differentiation is crucial to develop efficient therapeutic strategies.

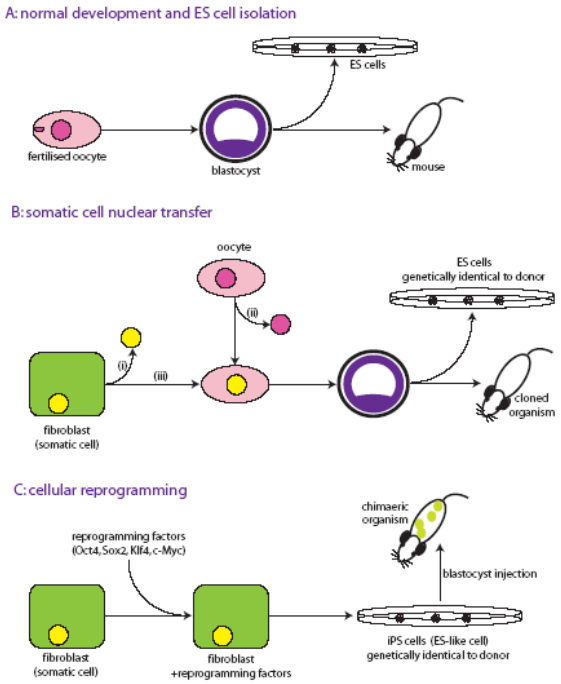
FIGURE 1: EMBRYO DEVELOPMENT



From: (Niwa 2007)

Pluripotent cells are stained in green. The inner part of the morula (A) forms the inner cell mass (ICM) of the blastocyst (B). The blastocyst is composed by two different cell types: the Inner cell mass (ICM) and the trophoectoderm. The ICM contains pluripotent cells which will give rise to all cells of the embryo. Thus, ES cells are isolated from ICM. In the late blastocyst (C) the ICM gives rise to primitive endoderm (Gata6 positive cells) and to the epiblast (Oct4 and Nanog positive cells). The primitive ectoderm origins from the epiblast. When gastrulation occurs (D), primitive ectoderm gives rise to three germ layers: ectoderm, mesoderm and endoderm.

FIGURE 2: THREE WAYS TO OBTAIN PLURIPOTENT CELLS



From: (Johnson, Shindo et al. 2008)

3.The transcriptional network regulating ES cell pluripotency

ES cells require extrinsic growth factors, such as LIF, for the maintenance of pluripotency in culture. These extrinsic factors are responsible for the maintenance of a network of key transcription factors within the cell that controls pluripotency. This network includes the homeodomain transcription factor Oct4 (Niwa, Miyazaki et al. 2000), the variant homeodomain transcription factor Nanog (Chambers, Colby et al. 2003) and the high mobility group (HMG)–box transcription factor Sox2 (Avilion, Nicolis et al. 2003) (Fig. 3).

Oct3/4 is a POU domain transcription factor that is expressed by all pluripotent cells during mouse embryogenesis, in mouse Embryonic Stem cell lines as well as in Embryonic Germ cell lines, even if its expression is weaker in germline stem cells. Oct3/4 deficient mouse embryos only develop to a stage that looks like a blastocyst, and although cells are allocated to the interior, these blastocysts are composed of trophectodermal cells. As these structures lack a genuine ICM, they cannot be used to produce ESC cell lines (Nichols, Zevnik et al. 1998). Oct4 has therefore been viewed as being involved in preventing trophoectoderm and perhaps somatic cell differentiation from the ICM, as well as being crucial for maintaining the pluripotent state during embryonic development. An enhancer containing Oct3/4 and Sox2 binding motifs, which is highly active in undifferentiated ES cells but not in differentiated cells regulates the expression of most pluripotency associated genes, including Fgf4, Utf1, Fbxo15, Lefty1 and Nanog. Oct3/4and Sox2, which bind independently to their respective binding motifs act synergistically to activate this enhancer (Yuan, Corbi et al. 1995).

Sox2 is a member of the HMG-domain DNA-binding-protein family that is implicated in the regulation of transcription and chromatin architecture. Sox2 is expressed in ES cells, but it is also expressed in neural stem cells. The Oct–Sox enhancers are important in promoting the expression of Oct3/4 and Sox2 themselves, suggesting that these two transcription factors are regulated by a positive-feedback loop (Chew, Loh et al. 2005). Chromatin immuno-precipitation studies in both human and mouse ES cells indicate that Oct3/4 and Sox2 cooperatively activate or repress a set of genes through their Oct–Sox enhancers. However, neither Oct3/4 nor Sox2 are able, even if over-expressed, to sustain ES cell self-renewal in the absence of LIF *in vitro*.

On the contrary, the homeodomain transcription factor Nanog was found to confer LIF-independence to ES cells. However, the physiological levels of Nanog in ES cells do not prevent their differentiation after LIF withdrawal. On the contrary, mouse ES cells over-

expressing Nanog are resistant to the spontaneous differentiation that occurs after LIF withdrawal (Chambers, Colby et al. 2003). The persistence of Nanog therefore seems to delay, rather than block, the differentiation of ES cells. On the other hand, Nanog–/– mouse ES cells differentiate slowly into extra-embryonic endoderm lineages, which is consistent with the absence of a primitive ectoderm in Nanog–/– embryos at E5.5 *in vivo* (Mitsui, Tokuzawa et al. 2003) (Silva, Nichols et al. 2009). However, although they are prone to differentiate, ES cells can self-renew indefinitely in the permanent absence of Nanog. Expanded Nanog null cells colonize embryonic germ layers and exhibit multilineage differentiation both in fetal and adult chimaeras. Although they are also recruited to the germ line, primordial germ cells lacking Nanog fail to mature on reaching the genital ridge. Thus, Nanog is dispensable for expression of somatic pluripotency *in vivo* but is specifically required for formation of germ cells (Chambers, Silva et al. 2007). Of great concern is that Nanog expression levels are not constant in ES cells. Thus, Chambers and collaborators proposed that Nanog regulates the efficiency of self-renewal in a manner analogous to a rheostat. In this paradigm, fluctuating Nanog levels in wild-type cultures confer a variable resistance to differentiation upon individual embryonic stem cells. Low levels of Nanog may constitute a 'window of opportunity' in which intrinsic or environmental perturbations can become consolidated into a lineage commitment decision, or may be reversed by re-expression of Nanog. Nanog can block primitive endoderm differentiation and neuronal differentiation (Ying, Nichols et al. 2003). Moreover, Nanog has been shown to interact with Smad1 to inhibit the expression of Brachyury (Suzuki, Raya et al. 2006), blocking mesodermal differentiation and with another transcription factor involved in maintenance of pluripotency, Sall4. Nanog and Sall4 form a positive regulatory loop for their expression (Wu, Chen et al. 2006). A feedback loop in which Oct3/4, Sox2 and Nanog function to maintain their expression promotes continuous ES cells self-renewal (Loh, Wu et al. 2006). (Ivanova, Dobrin et al. 2006). This loop determines the differentiation fate of ES cells by influencing the expression of transcription factors, such as Cdx2 (which promotes trophoectodermal differentiation) and Gata6 (which promotes primitive endoderm differentiation). However, during mouse development, Nanog transcription is down-regulated in the epiblast and in early primitive ectoderm (Hart, Hartley et al. 2004), where Oct3/4 and Sox2 continue to be expressed (Avilion, Nicolis et al. 2003) (Rosner, Vigano et al. 1990). Moreover Nanog expression levels in P19 embryonal carcinoma (EC) cells is much lower than that in ES cells, although both EC and ES cells express similar levels of Oct3/4 and Sox2 (Chambers, Colby et al. 2003). This suggests that the positive-feedback circuitry in the pluripotent transcription factor network

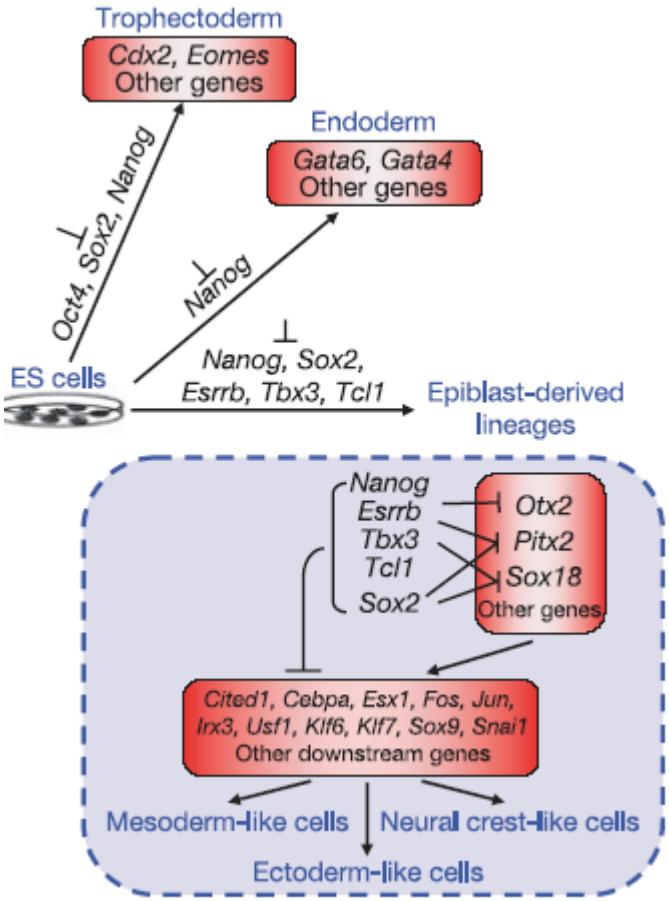
does not always require Nanog, and that the transcription factor network can establish a different stable circuit that maintains the levels of Oct3/4 and Sox2 expression with or without Nanog. The transition of the pluripotent transcription factor network to either the trophoectodermal or extra-embryonic/endodermal network is most likely to be regulated by the presence or absence of extracellular signals, such as the removal of LIF from mouse ES cells or the formation of EBs. However, the activation of Cdx2 or the repression of Oct3/4 might occur in mouse ES cells under particular culture conditions. This tallies with evidence that Oct3/4 and Cdx2 compete with each other to be expressed during blastocyst formation, and with evidence that Oct3/4 expression is dominant in the ICM. Therefore, the gatekeeper function of Nanog, which is an Oct3/4 target and prevents extra-embryonic endoderm differentiation, appears to be more important in mouse ES cells, as these cells are regulated by extra-cellular signals. Indeed, Nanog could be at the hub of these multiple signal transduction pathways. Nanog expression, in fact, is regulated by extrinsic factors, such as Wnts (Pereira, Yi et al. 2006) and LIF. Klf4 and Klf5, together with Klf2, were shown to form a Klf-based molecular circuitry that regulates ES cell self-renewal by activating Nanog expression (Jiang, Chan et al. 2008) (Hall, Guo et al. 2009). These members of the Kruppel-like transcription factors family, are direct targets of LIF (Bourillot, Aksoy et al. 2009), thus linking Nanog and LIF. Klf4 is one of the 4 Yamanaka factors, playing a very important role in reprogramming somatic cells into induced pluripotent stem cells (iPS) (Takahashi and Yamanaka 2006) and is able to maintain pluripotency in combination with Tbx3 in the absence of LIF (Niwa, Ogawa et al. 2009). However both Klf2 and Klf5 are able to work in "reprogramming" experiments, suggesting a functional redundancy of these three transcription factors (Nakagawa, Koyanagi et al. 2008). This is supported by the fact that they share common regulation of many genes and that the same DNA binding site has been identified for these 3 transcription factors. Klf5 play a crucial role in embryo development *in vivo*, as Klf5 knock-out mice die before gastrulation (Shindo, Manabe et al. 2002), showing aberrant blastocyst formation (Ema, Mori et al. 2008).

In our laboratory we have demonstrated a unique role for Klf5 in ES cell self-renewal (Parisi, Passaro et al. 2008). Klf5 is strongly expressed in ES cell and in blastocyst (E 3.5), whereas it is down-regulated when differentiation occurs, being undetectable 48 hours after the induction of ES cell differentiation *in vitro* and in the epiblast of gastrulating embryos at E 6.5. I contributed to the characterization of Klf5 function in ES cells, analyzing the effect of Klf5 suppression by RNA interference. Klf5 knock-down causes a strong down-regulation of Oct4, Sox2 and Nanog expression. Thus, ES cells were Klf5 is suppressed

(Klf5 KD cells) show aberrant expression of mesodermal and trophoectodermal markers which are undetectable in then controls. Indeed, about 12% of Klf5 KD cells express Brachyury (mesodermal marker) and 11% express Cdx2 (trophoectodermal marker). The aberrant expression of these differentiation markers is coherent with the down-regulation of Oct4, Sox2 and Nanog. This effect is specific of Klf5, as Klf4 KD cells do not show a significant down-regulation of Oct4, Sox2 and Nanog in our culture conditions.

In this work we demonstrate that Klf5 directly controls Oct4 and Nanog with ChIP analysis and luciferase assays. The involvement of Klf5 in the transcriptional circuitry which controls ES cell self-renewal and proliferation is also supported by the fact that Klf5 over-expression alone is able to maintain undifferentiated markers such as Alkaline Phosphatase, Oct4 and Nanog when cells are grown in the absence of LIF.

FIGURE 3: THE TRANSCRIPTIONAL NETWORK REGULATING ES CELL SELF-RENEWAL AND PLURIPOTENCY



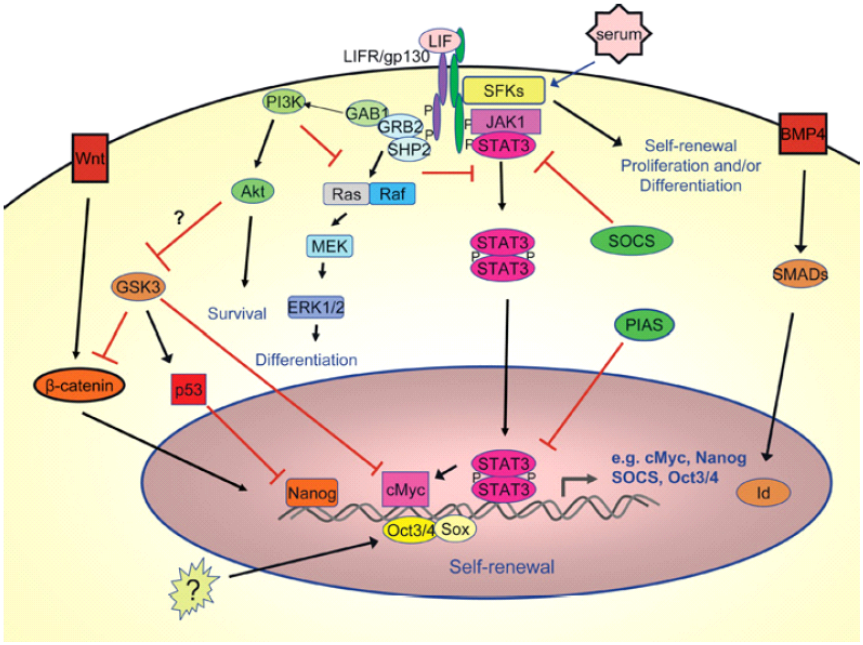
Modified from: (Ivanova, Dobrin et al. 2006)

Many transcription factors such as Oct4, Sox2 and Nanog form a core transcriptional network that regulates ES cell pluripotency. This network blocks differentiation and promotes proliferation. Moreover, Oct4, Sox2 and Nanog are involved in the regulation of their own expression each other, indicating that a positive feedback loop sustains the network.

4. Extrinsic factors are necessary for maintaining ES cell pluripotency in vitro

The maintenance of pluripotency within ES cells requires extrinsic factors, either added to the growth medium or provided on a feeder layer of differentiated cells (Smith, Heath et al. 1988) thereby creating an appropriate external signalling environment (Fig. 4).

FIGURE 4: PATHWAYS REGULATING ES CELL SELF-RENEWAL AND PLURIPOTENCY



A transcriptional network including Oct4, Sox2 and Nanog, maintains ES cell undifferentiated. This transcriptional network is positively regulated by feed-back loop and is sustained by signals present in the serum. Leukemia inhibitory factor (LIF) is necessary, even if not sufficient, for sustaining the core transcriptional network and thus for maintaining ES cells undifferentiated. The binding of LIF to its receptor promotes the phosphorylation and, in turn the activation of the transcription factor STAT3. STAT3 is involved in control of proliferation, promoting the transcription of c-myc, and sustains the core transcriptional network regulating the transcription of other transcription factors, such as Kruppel-like factors (Klfs). Moreover, STAT3 works in combination with Nanog in regulating some genes and in combination to BMPs-dependent Smads. BMPs-dependent Smads regulate the transcription of Id genes which block neural commitment. Thus, BMP4 is required for maintaining ES cells undifferentiated. BMP4 exerts its effect also blocking ERK phosphorylation which is a pro-differentiative signal. Surprisingly, LIF promotes ERK phosphorylation, suggesting that LIF regulates a fine balance between self-renewal and differentiation. Wnts are also involved in the control of ES cell self-renewal, promoting the transcription of STAT3 and balancing signals sustaining self-renewal and promting differentiation. The involvement of Wnts is suggested also by the inhibitory effect of GSK3 kinase on this process. PI3 kinase activity, sustained by LIF, blocks GSK3 kinase, promoting survival and proliferation.

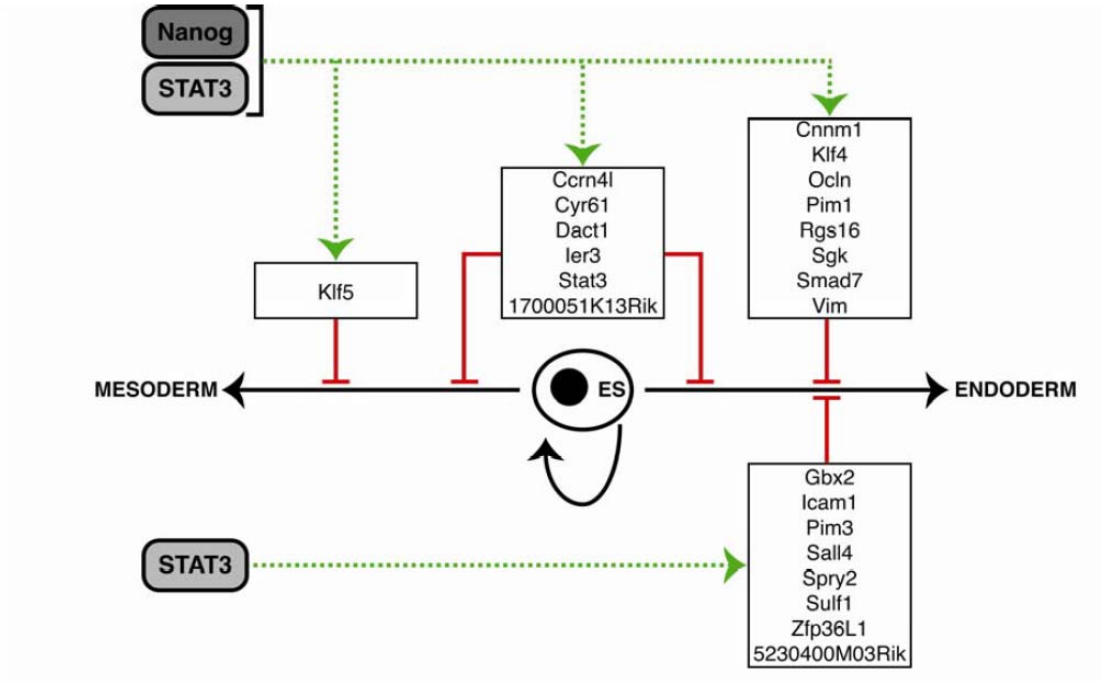
4.1. LIF

The factor required to sustain self-renewal of mouse ES cells is the leukaemia inhibitory factor (LIF) (Smith, Heath et al. 1988). LIF is not required for pluripotency of the ICM *in vivo* (Nichols, Chambers et al. 2001) and is unable to maintain pluripotency in human ES cells suggesting that alternative mechanisms function in the maintenance of pluripotency within these contexts. LIF exerts its effect by binding to the LIF receptor (LIFR)–gp130 heterodimer receptor on the cell membrane and activating the signal transducer and activator of transcription-3 (STAT3). In the presence of LIF in the culture medium, STAT3 binds to phosphor-tyrosine residues on activated LIFR–gp130 heterodimer receptors (Niwa, Burdon et al. 1998) and undergoes phosphorylation and homodimerization. Phosphorylated STAT3 dimers then translocate to the nucleus, where they function as transcription factors. A conditionally active form of STAT3 that is induced by tamoxifen (a STAT3–oestrogen-receptor (ER) fusion protein) (Matsuda, Nakamura et al. 1999) can be used to maintain the pluripotent phenotype when LIF is removed from the culture media.

In addition to the pathway leading to STAT3 nuclear translocation, the intracellular domains of the LIFR–gp130 heterodimer can, upon binding to LIF, recruit the non-receptor tyrosine kinase Janus (JAK) and the anti-phospho-tyrosine immunoreactive kinase (TIK) and activate other pathways. The treatment of ES cells with LIF also induces the phosphorylation of extracellular signal-regulated protein kinases, ERK1 and ERK2 and increases mitogen-activated protein kinase (MAPK) activity (Matsuda, Nakamura et al. 1999) which are pro-differentiative signals. This occurs through activation of a bridging factor between the cytokine and the MAPKs the widely expressed tyrosine phosphatase Src homology Sh domain 2 containing protein tyrosine phosphatase. Functional inactivation of SHP-2 facilitates self-renewal (Burdon, Stracey et al. 1999) suggesting that LIF stimulates self-renewal by co-activating positive and negative signaling pathways in a coordinated manner. The most known STAT3 direct target is the proto-oncogene c- Myc. The forced expression of a mutated form of c-Myc temporarily inhibits the differentiation of mouse ES cells induced by withdrawal of LIF (Cartwright, McLean et al. 2005). Recently Bourillot and collaborators identified 22 STAT3 target genes whose knock-down induces spontaneous differentiation (Bourillot, Aksoy et al. 2009). The 22 identified genes encode transcription factors, various types of proteins involved in protein and nucleic acid modification (serine/threonine kinases, sulfatase, and deadenylase), adhesion molecules, extra-cellular matrix and cytoskeleton associated proteins, and a stress-response factor. They include Kruppel like factors Klf4 and Klf5 which are known to play a pivotal role in the maintenance of the undifferentiated state (Jiang, Chan et al. 2008), as mentioned

before, Smad7 which is a regulator of TGF-β superfamily signaling and Sgk. Sgk encodes the protein kinase B (PKB)-related protein serum and glucocorticoid-inducible kinase. SGK is a downstream effector of the PI3K/3-phosphoinositide-dependent protein kinases (PDK) signaling pathway, which contributes to the maintenance of the undifferentiated state (Paling, Wheadon et al. 2004). The LIF/STAT3 and LIF/PI3K pathways may act synergistically to activate SGK and promote self-renewal. SGK-like PKB inactivates glycogen synthase kinase (GSK)-3 *in vitro.* Inhibition of GSK-3 activity is known to facilitate self-renewal of mouse ES cells providing a possible explanatory mechanism for SGK action. The observation that both Klf4 and Klf5 are regulated by STAT3 activity functionally links the LIF/STAT3 pathway to the regulation of Nanog expression. In addition, Bourillot and collaborators observed that Sall4 is also regulated by STAT3. This suggests that LIF/STAT3 signaling contributes to the regulation of Nanog transcriptional activity, and indirectly feeds the Nanog/Oct4/Sox2 core regulatory network, through activation of Klf4, Klf5 and Sall4. Moreover Bourillot and collaborators found that STAT3 and Nanog co-regulate the expression of 14 genes involved in pluripotency control, including Klf4 and Klf5. Of these 14 genes, 12 contain both STAT3 and Nanog binding sites identified by ChIP-Seq (Chen, Xu et al. 2008) indicating that they are all direct targets of both transcription factors. The identification of pluripotency genes coregulated by STAT3 and Nanog helps explain how Nanog overcomes differentiation induced by LIF starvation when over-expressed (Fig. 5).

FIGURE 5: STAT3 AND NANOG COOPERATE



From: (Bourillot, Aksoy et al. 2009)

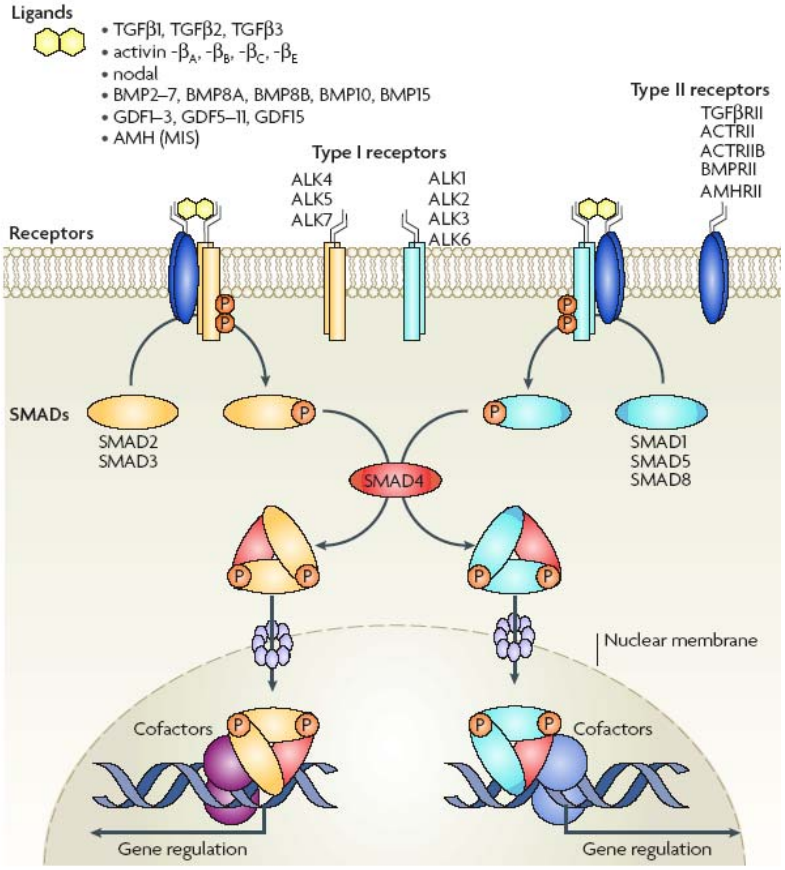
LIF exerts its function through the activation of STAT3. STAT3 promotes proliferation regulating the transcription of c-myc and blocks differentiation repressing some genes involved in. Moreover, in combination with Nanog, STAT3 regulates its own transcription and the expression of some other genes such as Kruppel-like factors (Klfs). Klfs promote the expression of Nanog functionally linking LIF with the core transcriptional network that works within the cells. The common regulation of many genes exerted by Nanog and STAT3 may explain why the over-expression of Nanog is able to delay differentiation in the absence of LIF.

4.2. TGF-β superfamily

LIF is necessary but not sufficient for maintaining ES cell undifferentiated in culture. Serum is also required for mouse ES cells. In serum free culture, in fact, LIF is not able to block neural differentiation. Bone morphogenetic protein 4 (BMP4) was found to be necessary for maintaining ES cell undifferentiated in combination with LIF (Ying, Nichols et al. 2003). Bone morphogenetic proteins (BMPs) belong to the Transforming growth factor (TGF-β) superfamily that includes also TGF-βs, Activin, Nodal and other related proteins. These proteins play an important role in the establishment of body plan and tissue differentiation through their effect on cell proliferation, differentiation and migration. The model of induction of signalling responses by TGF-β related factors is a linear signalling pathway from the type II to the type I receptor kinase to Smad activation, resulting in ligand-induced transcription (Derynck and Zhang 2003). In mammals, only 5 type II receptors and 7 type I receptors have been identified. Ligand binding to type I receptors induces dimerization with type II receptors. The activation of the type I receptor kinase and consequent signalling, requires phosphorylation of its GS domain by the type II receptor in the heteromeric complex. Phosphorylation of GS-domain enables the recruitment of receptor-regulated SMAD proteins (R-SMADs). The type I receptor phosphorylates the R-SMADs which allows them to form complexes with the common mediator SMAD4. SMAD 1,5 and 8 are activated by type I receptors Alk 1,2,3 and 6 whereas SMAD 2,3 are activated by type I receptors Alk 4,5 and 7. In mouse ES cells the most expressed receptors are Alk3, which binds to BMP4 and Alk 4 and 7 which bind to Nodal and Activin. SMAD complexes accumulate in the nucleus, where they are directly involved in regulating the transcription of target genes, both positively and negatively (Schmierer and Hill 2007) (Fig. 6).

TGF-β family signalling has been reported to be involved in the maintenance of ES cell identity. Mouse embryos deficient in Smad4 display defective epiblast proliferation and delayed outgrowth of the inner cell mass (Sirard, de la Pompa et al. 1998).

FIGURE 6: TGF-β SUPERFAMILY SIGNALING



From: (Schmierer and Hill 2007)

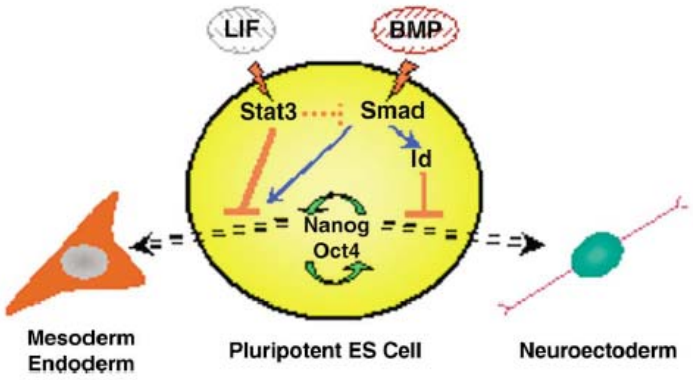
Ligand binding to the type I receptors induces dimerization of type I receptors and type II receptors. Type II receptors phosphorylate type I receptors that, in turn, recruit SMADs and activate them through phosphorylation. TGF-β1, Activin and Nodal exert their function through type I receptors Alk 4,5 and 7 that induce SMAD 2-3 phosphorylation, whereas BMPs bind to Alk 1,2,3 and 6 that induce SMAD 1-5-8 phosphorylation. SMAD 2-3 and SMAD 1-5-8 share the common mediator SMAD4 which is necessary for their nuclear translocation and recruitment of cofactors to activate or repress transcription.

4.2.1 BMP4

The only BMP dependent type I receptor expressed in the inner cell mass and in ES cells in Alk3 (Mishina, Suzuki et al. 1995). Mice lacking Alk-3 (also named BMP type IA receptor) exhibit reduced cell proliferation in the epiblast (Mishina, Hanks et al. 2002), indicating that BMP signaling plays important roles in the maintenance of mouse ES cell identity. Since BMPs are potent inhibitors of neural differentiation in vertebrate embryos (Wilson and Hemmati-Brivanlou 1995) the growth stimulatory activities of BMPs should be mainly mediated by their inhibitory effects on neural differentiation of mouse ES cells. Ying and collaborators (Ying, Nichols et al. 2003) reported that BMP-4 sustains self renewal of mouse ES cells in concert with LIF, and that the critical contribution of BMP-4 to self renewal is mediated by the induction of the helix-loop-helix protein Id (inhibitor of differentiation). Id genes encode repressive basic helix-loop-helix (bHLH) factors and have been shown to be induced by BMP/Smad in neuroepithelial cells (Nakashima, Takizawa et al. 2001) and in C2C12 myoblasts (Lopez-Rovira, Chalaux et al. 2002). The induction of Id proteins by BMP has also been reported in differentiating ES cell cultures (Hollnagel, Oehlmann et al. 1999) and is dependent by BMP but not by LIF. Moreover, neither activin nor TGF-β induces the expression of Id genes indicating that this response is specific to SMADs downstream of the BMP receptor. The neurogenic bHLH transcription factors are known to be antagonized by direct binding of Id proteins in the developing CNS (Lyden, Young et al. 1999). Thus, Id expression may be necessary to prevent continuous neural differentiation of ES cells triggered by precocious expression of pro-neural bHLH factors.
BMP4 regulation of Ids is only part of the story. Qi and co-workers (Qi, Li et al. 2004) reported that BMP-4 is necessary for the maintenance of ES cell self-renewal, and that effect of BMP-4 is accomplished by inhibition of both extra-cellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways. Inhibition of the p38 MAPK pathway was shown to overcome the block in deriving ES cells from blastocysts lacking Alk3. In addition, Lee and collaborators demonstrated that BMP4 is involved in ES cell proliferation (Lee, Lim et al. 2009). BMP4, in fact, increases thymidine incorporation and induces cyclin D1 expression in a SMAD-dependent manner. Wnt1 seems to be mediate BMP4 involvement in control of proliferation because its knock-down blocks BMP4 effect on thymidine incorporation and cyclin D1 induction.
A mechanism of cooperative transcriptional regulation between Smad1 and STAT3 has been characterized in neuroepithelial cells. This involves formation of a ternary complex particular bridged by the ubiquitous transcriptional coactivator p300 and results in

synergistic activation of glial-specific promoters. Ying and collaborators investigated whether a complex containing STAT3 and Smads may be formed in ES cells stimulated with LIF plus BMP. Immunoprecipation following transfection with 3XFLAG-tagged Smad1 indicated that activated STAT3 and Smad1 may colocalize. This conclusion was corroborated by co-immunoprecipitation of endogenous phosphorylated Smad1 and STAT3 following LIF plus BMP stimulation. These data support the hypothesis that BMP4/Smad1 sustains LIF/STAT3 function in mouse ES cells. In parallel, the capacity of BMP4 to induce mesodermal and endodermal differentiation is constrained by STAT3, probably involving direct as well as indirect mechanisms. Withdrawal of LIF therefore, results in a switch in BMP action from supporting self-renewal to promote lineage commitment. Taken together this results indicate a tight cross-talk between BMP4 and LIF.

FIGURE 7: LIF AND BMP4 COOPERATE TO MAINTAIN ES CELL UNDIFFERENTIATED



From: (Ying, Nichols et al. 2003)

LIF redirects the pro-differentiative action of BMP4 to the sustain of pluripotency. In presence of LIF, BMP4 induces the expression of Id genes blocking neuroectodermal commitment and blocks pro-differentiative signals such as ERK phosphorylation. Otherwise, in the absence of LIF, BMP4 promotes differentiation. In presence of serum, in fact, BMP4 promotes differentiation into mesoderm and endoderm. On the other hand, in serum-free conditions, BMP4 promotes non-neural ectodermal fate, giving rise to epithelioid cells committed toward epidermal fate.

4.2.2 Nodal/Activin

Nodal involvement in maintenance of ES cell properties is demonstrated by the finding that Nodal-deficient mouse embryos exhibit a thick epiblast with very low levels of Oct3/4 expression (Mesnard, Guzman-Ayala et al. 2006). Nodal itself and its co-receptor Cripto as well as Nodal antagonists Lefty1/2 and Bambi (Bmp and Activin membrane bound inhibitor) are controlled by the Nodal/Activin pathway through the activation of SMAD 2/3 complex in mouse ES cells, indicating that auto-regulatory loops regulate the signaling (Guzman-Ayala, Lee et al. 2009). The treatment with Nodal/Activin inhibitor SB-431542 (Inman, Nicolas et al. 2002) dramatically decreases the proliferation of mouse ES cells, suggesting that Activin/Nodal signaling is indispensable for proliferation of mouse ES cells (Ogawa, Saito et al. 2007). Recently, Activin and Nodal were shown to be sufficient to maintain undifferentiated ES cell where E-cadherin is suppressed even in the absence of LIF suggesting a role also in maintenance of pluripotency (Soncin, Mohamet et al. 2009). Moreover, Activin/Nodal are necessary to maintain the pluripotent status of human ES cells (James, Levine et al. 2005). Inhibition of Activin/Nodal signalling by follistatin and by over-expression of Lefty or by the Activin receptor inhibitor SB-431542, accelerates human ES cells differentiation. Nevertheless, neither Nodal nor Activin is sufficient to sustain long-term human ES cells growth in a chemically defined medium without serum. Long-term maintenance of pluripotency can be achieved with a combination of Activin or Nodal plus FGF2 in the absence of serum. However, this effect of FGF on human ES cells pluripotency depends on Activin/Nodal signalling, because it is blocked by SB-431542 (Vallier, Alexander et al. 2005). Activin/Nodal exert their effect directly controlling the expression of Nanog in human embryonic stem cells (hESCs) and in mouse epiblast stem cells (EpiSCs) (Vallier, Mendjan et al. 2009). Nanog in turn prevents neuroectodermal differentiation induced by FGF signalling and limits the transcriptional activity of the Smad2/3 cascade, blocking progression along the endoderm lineage.

4.2.3 Regulation of TGF-β superfamily signalling

TGF-β superfamily signaling is finely regulated. Many regulatory proteins exerts their function at different levels.

a) Many accessory proteins enhance ligand-binding specificity to receptors. For example, efficient binding of Nodal to its type I receptor Alk4 and consequent signalling require association of fucosylated Cripto with Alk4 (Bianco, Adkins et al. 2002) (Strizzi, Bianco et

al. 2005). In parallel, betaglycan and endoglin provide high affinity TGF-β presentation to the TGF-β receptor complex.

b) R-Smads (Smad 1-5-8 and Smad 2-3) and Smad4 contain a conserved MH1 domain and C-terminal MH2 domain, flanking a divergent middle linker sequence. Inhibitory Smads (Smad 6-7) lack a recognizable MH1 domain, but have a MH2 domain. Both MH1 and MH2 domains can interact with selected sequence-specific transcription factors, whereas the C-terminus of the R-Smads interacts with and recruits the related coactivators CREB-binding protein (CBP) or p300. With the exception of Smad2, the MH1 domains of Smads can bind to DNA, whereas the MH2 domains mediate Smad oligomerization and Smad receptor interaction. Although differentially controlled during development, R-Smads and Smad4 are expressed in most, if not all, cell types. In contrast to R-Smad expression, expression of inhibitory Smad6 and Smad7 is highly regulated by extra-cellular signals. Furthermore, induction of Smad6 and Smad7 by BMP and TGF-β represents an auto-inhibitory feedback mechanism for ligand induced signalling (Massague 2000). Smad6 and Smad7 regulate activation of R-Smads, binding with their MH2 domains to type I receptor, preventing recruitment and phosphorylation of effector Smads. Ubiquitin-proteasome mediated degradation also controls the levels of Smads post-translationally. The HECT (homologous to the E6-AP carboxy terminus) E3 ubiquitin ligases, Smurf1 (Smad-ubiquitination-regulatory factor 1) and Smurf2 antagonize TGF-β family signalling by interacting with R-Smads and targeting them for degradation. Smurf1 interacts with Smad1 and 5, thereby affecting BMP responses, whereas Smurf2 interacts more broadly with different R-Smads, allowing interference with BMP and TGF-β/Nodal/Activin signalling (Zhu, Kavsak et al. 1999) (Zhang, Chang et al. 2001). In addition, recruitment of a complex of Smad7 with Smurf1 or Smurf2 to type I receptors, results in degradation of receptors. Proteasomal degradation also regulates R-Smad levels after translocation into the nucleus. Thus, C-terminally phosphorylated Smad2 can undergo ubiquitination, and inhibition of proteasomal degradation enhances its nuclear accumulation (Lo and Massague 1999). In contrast to R-Smads, Smad4 is not subjected to ubiquitin mediated degradation. However, some tumor-associated mutations allow ubiquitination and/or decrease Smad4 stability (Wan, Cao et al. 2002).

c) TGF-β ligands induce receptor internalization in endosomes, which may be required for efficient TGF-β signalling through Smads. Thus, receptor associated proteins may have a role in vescicular trafficking as well as facilitating internalization and Smads recruitment to receptors. For example, SARA and Dab2, which are enriched in endosomes and clathrin

coated vescicles, bind to both receptors and Smads, promoting Smad phosphorylation (Tsukazaki, Chiang et al. 1998).

d) Some kinases further regulate Smad signalling. The linker region between MH1 and MH2 domains contains several serine and threonine residues that can be phosphorylated by kinases. Mitogen-activated protein kinases (MAPKs), which are activated by growth factors and cytokines, modulate Smad signaling (Funaba, Zimmerman et al. 2002) (Kretzschmar, Doody et al. 1999). In the BMP signaling pathway, Erk phosphorylates the linker region of Smad1 to inhibit Smad1 nuclear accumulation and transcriptional activity. In the TGF-β signaling pathway, activated MAPKs inhibit TGF-β signaling by inducing the cytoplasmic retention of Smad2 and/or Smad3 via phosphorylation of the linker region Conversely, Kamaraju and Roberts reported that phosphorylation of the Smad3 linker region by p38 MAPK was required for maximal transcriptional activation and growth inhibition by TGF-β (Kamaraju and Roberts 2005).

4.3. Wnts

Wnt signalling acts synergistically with LIF, regulating STAT3 transcription (Hao, Li et al. 2006) and with BMP4, mediating cyclin D1 induction (Lee, Lim et al. 2009). Wnt pathway activation by 6-bromoindirubin-3'-oxime, a specific pharmacological inhibitor of GSK-3, maintains the undifferentiated phenotype in human and mouse ES cells and sustains expression of the pluripotent state-specific transcription factors Oct3/4, Rex1 and Nanog (Sato, Meijer et al. 2004). Stimulation of the canonical Wnt signaling pathway causes the transcriptional coactivator β-catenin to translocate to the nucleus, where it interacts with Tcf/Lef proteins to activate target gene. The most important effector of Wnts is T-cell factor 3 (Tcf3) which represses Nanog expression, thus demonstrating that a fine regulation of the stemness master genes occurs in ES cell (Pereira, Yi et al. 2006). Tcf3 co-occupies promoters of many genes involved in self-renewal in association with Oct4 and Nanog. Thus, Tcf3 also is an integral component of the core regulatory circuitry of ES cells. Both Tcf3 depletion and Wnt pathway activation cause increased expression of Oct4, Nanog, and other pluripotency factors and produce ES cells that are refractory to differentiation (Cole, Johnstone et al. 2008). However, Wnts acts balancing signals sustaining pluripotency or promoting differentiation, as shown *in vivo*. Wnt3-deficient embryos, in fact, express high levels of Oct4 (Liu, Wakamiya et al. 1999). The entire Wnt3-deficient embryo remains bilayered at gastrulation and continues to express Oct4, thus providing Wnt3 requirement in primary axis formation in mice.

5. Are extrinsic factor dispensable for maintaining ES cell pluripotency?

Ying and co-workers proposed that the LIF and serum/BMP signals mainly exert their action blocking ES cell differentiation. In this view, LIF and BMP4 should act downstream of phospho-ERK to block commitment (Ying, Wray et al. 2008). To test this hypothesis they used selective small-molecule inhibitors SU5402 and PD184352 to inhibit FGF receptor tyrosine kinases and the ERK cascade, respectively. They found that, in combination with LIF, either inhibitor replaces the requirement for serum/BMP and supports robust long-term ES cell propagation. Lineage commitment does not occur despite a reduced expression of Ids proteins due to the absence of BMP4 in growth medium. This result, observed with several independent ES cell lines, suggests that the minimal requirements for ES-cell self-renewal may be to deflect commitment signals emanating from FGF receptor and ERK signalling. However, apoptosis is relatively high in cells treated with these two inhibitors (2i) and cells survive poorly at clonal density. To overcome these problems they added to the culture medium a glycogen synthase kinase-3 inhibitor, CHIR99021. ES cell propagation, in fact, was previously reported to be enhanced by GSK3 inhibitors. Alone, CHIR99021 enhances survival at low cell density but also induces non-neural differentiation. However the combination of all three inhibitors (3i) results in a highly efficient expansion of undifferentiated colonies, even at a low cell density. These results were confirmed in STAT3 null background confirming that 3i dependent ES cell propagation does not involve STAT3 signaling cascade. As consequence of results obtained *in vitro*, Ying and collaborators observed that the derivation of ES cells from embryos cultured with the 2 previous described inhibitors SU5402 and PD184352 (2i) from the 8-cell stage was very efficient. Nichols and collaborators (Nichols, Silva et al. 2009) used this system to address the ability of the individual epiblast cells to form ES cells. They used immunosurgery to isolate inner cell mass (ICM) from 129 embryos cultured for 3 days in 2i from the 8-cell stage. They dissociated the ICM and deposited single cells into individual wells of 96-well plates in 2i or 2i plus LIF. Of individual cells plated in 2i alone, 2 out of 33 (6%) gave rise to undifferentiated ES cell colonies. By contrast, 25 out of 46 cells (54%) plated in 2i plus LIF yielded ES cell colonies. Addition of LIF to 3i has previously been shown to enhance clonogenicity of ES cells by activation of the Stat3 pathway. The colonies produced in 2i alone were morphologically identical to those derived in 2i plus LIF. These findings indicate that self-renewal is shared between pre-implantation epiblast and ES cells. The intrinsic properties exhibited by mouse ES cells seems thus not to be an adaptation to culture, but directly represents autonomous

expansion of early epiblast. In the unperturbed embryo, self-renewal is short-lived due to the inductive action of Fgf4 and other extrinsic Erk stimuli, but this stimulus can be arrested in diapause, during which the epiblast can remain in a naïve state for weeks. ES cell lines can most readily be derived from diapause blastocysts (Ying, Wray et al. 2008). Upon explant culture, the epiblast will rapidly lose pluripotency and differentiate under the influence of Erk signalling (Buehr and Smith 2003). Disrupting this signal with 3i/2i prevents this progression and releases ES cells.

6. Aim of the project

In our laboratory we performed the screening of a shRNA library to identify genes involved in ES cell self-renewal and pluripotency. The knowledge of the molecular machinery involved in ES cell biology will improve their use in regenerative medicine and the knowledge of embryo development. By using the screening approach we found 50 genes. First of all, we focused on the transcription factor Klf5 (Kruppel-like factor 5) and on its specific function in ES cell (Parisi, Passaro et al. 2008). More in detail, I analysed the effect of Klf5 suppression in ES cell and the role of this transcription factor within the network that controls ES cell self-renewal. In the last two years, I focused on the unknown gene riken cDNA 4632428N05, we named Dies1 which stands for Differentiation of ES cell 1. The investigation of Dies1 function is the object of this thesis. Dies1 was characterized as a new player required for proper ES cell differentiation. Indeed, Dies1 was identified as novel component of BMP4 signaling pathway, shedding light on the crucial role of the balance of TGF-β superfamily signaling in the control of ES cell pluripotency. A part of the work described here was published on "The Journal of Biological Chemistry" in December 2009 (Aloia, Parisi et al. 2009).

MATERIAL AND METHODS

Cell culture

E14Tg2a (BayGenomics) mouse ES cells were maintained on feeder-free, gelatine coated plates in the following medium (ES cell medium): GMEM (Sigma) supplemented with 2mM glutamine (Invitrogen), 100U/ml penicillin/streptomycin (Invitrogen), 1mM sodium pyruvate (Invitrogen), 1x non essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 10% FBS (Hyclone), 2 mM glutamine (Invitrogen), and 10^3 U/ml leukemia inhibitory factor (LIF) (Chemicon).

For neural differentiation cells were seeded at the density of $3x10^3$ cells for cm^2 . Cells were cultured in neural differentiation medium (NDM): Knockout Dulbecco's minimal essential medium supplemented with 10% Knockout Serum Replacement (both from Invitrogen),0.1mM β-mercaptoethanol, 2 mM glutamine, 100 U/mL penicillin-streptomycin.

To perform the screening, the promoter of β-actin gene was inserted in pEGFP N1 vector (pCBA-GFP vector). A clone stably transfected with pCBA-GFP was selected for futher experiments and renamed α1 tubulin-GFP ES cell line.

α1-tubulin-EGFP cell line was plated in gelatine-coated 96-wells and transfected after 12h with shRNAs using ArrestIn (both from Open Biosystems). Recombinant clones were selected with puromycin (2μg/ml, Sigma) for three days. The selected clones were trypsinised into a single cells suspension and plated in ES cell medium (see above) at low confluence ($1-5x10^3$ cells/cm^2) for four-five hours to allow them to attach to the plate. Then, ES cell medium were replaced with NDM (see above) that was changed on alternated days. Within 4-5 days of differentiation, EGFP-positive cells appear. Starting from day 7 of differentiation onward, a population of cells showing a strong EGFP expression (neurons) was observed which produce a complex network surrounding the clones. We analyzed the presence/absence of EGFP by observation at fluorescence inverted microscope (Leica Microsystems) at different time points (4, 7, 10 days of differentiation).

HEK 293 were cultured in DMEM (GIBCO) supplemented with 10%FBS (HighClone), 2 mM glutamine and100 U/mL penicillin/streptomycin.

ES cells were transfected with Lipofectamine 2000, following manufacturer's instructions.

HEK 293 cells were transfected using calcium phosphate transfection method.

RNA interference

Open Biosystems Mouse pSM2 retroviral shRNA mir was used as source of shRNAs. Sequences of shRNAs used are listed below:

Dies1 shRNA1:

TGCTGTTGACAGTGAGCGAGGAACCCTGCTCCTTGCTATTTAGTGAAGCCACA
GATGTAAATAGCAAGGAGCAGGGTTCCCTGCCTACTGCCTCGGA

Oct3/4 shRNA:

TGCTGTTGACAGTGAGCGCGCAGAAGGAGCTAGAACAGTTTAGTGAAGCCACA
GATGTAAACTGTTCTAGCTCCTTCTGCATGCCTACTGCCTCGGA

Id1 shRNA:

TGCTGTTGACAGTGAGCGCGCAGCATGTAATCGACTACATTAGTGAAGCCACA
GATGTAATGTAGTCGATTACATGCTGCATGCCTACTGCCTCGGA

Id3 shRNA:

TGCTGTTGACAGTGAGCGACCTGATTATGAACTCTATAATTAGTGAAGCCACA
GATGTAATTATAGAGTTCATAATCAGGGTGCCTACTGCCTCGGA

Dies1 shRNA2 (targeting Dies1 3'UTR) was generated using Open Biosystems protocols available on line. The sequence is:

TGCTGTTGACAGTGAGCGCGGAACCACTGTGAGCTCATTATAGTGAAGCCACA
GATGTATAATGAGCTCACAGTGGTTCCTTGCCTACTGCCTCGGA

Non silencing siRNA, Dies1 siRNA (riken 4632428N05), Nanog siRNA, Sox2 siRNA and Alk3 siRNA are available in Dharmacon Smart Pool library.

Plasmids

The vector used for all the constructs is pCBA-GFP. This vector was derived from p-EGFPN1, with β-actin promoter instead of CMV. Dies1 cDNA was obtained from NIH Mammalian Gene Collection, Invitrogen clone IMAGE: 3488482. Dies1 cDNA was inserted using BamH1-NotI restriction sites, using the following oligonucleotides:

5'BamH1 Dies1 full-length for:

GGATCCGCCGCCATGGGTGTCCCCGCGGTCCCAGAGGCCAGC

3'NotI/HindIII Dies1 full-length rev:

GCGGCCGCAAGCTTTTAGATGGCTTCAGAGTTAGGGGAGTCAG

Then, using the inserted site HindIII and Not I, 3xFLAG was added at 3'end of Dies1 sequence with *in vitro* annealing:

HindIII 3xflag:

AGCTTGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTAC
AAGGATGACGATGACAAGTAAGC

NotI rev 3xflag:

GGCCGCTTACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATC
ACCGTCATGGTCTTTGTAGTCA

Thus, Dies1 full-length 3xFLAG was generated.

By this vector, all the others constructs were generated.

Dies1-ECD 3xFLAG was obtained by replacing Dies1 full-length, with Dies1 extra-cellular domain (ECD) (aa 1-189), with the following oligonucleotides:

5'BamH1 Dies1-ECD for:

GGATCCGCCGCCATGGGTGTCCCCGCGGTCCCAGAGGCCAGC

3'Hind III Dies1-ECD rev:

AAGCTTTTACGTGATGCTGTCACTGTCCTGCTCATTAGACG

Both Dies1 full-length and Dies1-ECD were tagged with HA and with Strep-tag replacing 3xFLAG (HindIII/NotI) with the following oligonucleotides:

HindIII streptag for

CTTCTCAAATTGTGGGTGGCTCCAGCCGCCGCC TAAGC

NotI streptag rev

TTAGGCGGCGGCTGGAGCCACCCACAATTTGAGAAGA

Thus Dies1 full-length/ECD- Streptag was generated.

HindIII HA for

TACCCATACGATGTTCCAGATTACGCTTAAGC

NotI HA rev

TTAAGCGTAATCTGGAACATCGTATGGGTAA

Thus Dies1 full-length/ECD- HA was generated

RNA isolation, reverse transcription and real-time PCR analysis

Total RNAs were extracted using Trizol ultra pure reagent (Invitrogen) according to the manufacturer's instructions and subsequently incubated with DNAse RNAse-free (Ambion) to minimize genomic DNA contamination. was used for reverse transcription. Total RNA (2 μg RNA/reaction) was reverse transcribed with SuperScript Vilo transcriptase (Invitrogen) using random hexamers (Amersham). Real Time RT-PCR was

carried out on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using Power SYBR Green PCR Master mix (Applied Biosystems). Gene specific primers used for amplification are listed in the table below:

Gene	Forward primer	Reverse primer
Gapdh	GTATGACTCCACTCACGGCAAA	TTCCCATTCTCGGCCTTG
Dies1	AGGCAGGCAAAGGCTCG	CTGTCCTGCTCATTAGACGCC
Oct3/4	AACCTTCAGGAGATATGCAAATCG	TTCTCAATGCTAGTTCGCTTTCTCT
Nanog	TCAGAAGGGCTCAGCACCA	GCGTTCACCAGATAGCCCTG
Sox2	CTGCAGTACAACTCCATGACCAG	GGACTTGACCACAGAGCCCAT
Stat3	TTCCCATTCTCGGCCTTG	TCTTGATGACTAAGGGCCGGT
Klf2	CCAACTGCGGCAAGACCTAC	CAATGATAAGGCTTCTCACCTGTGT
Klf4	ACTCACACAGGCGAGAAACCTTAC	TCAGTTCATCGGAGCGGG
Klf5	GGTCCAGACAAGATGTGAAATGG	TTTATGCTCTGAAATTATCGGAACTG
Id1	GAGCAGCAGGTGAACGTCCT	TCCTTGAGGCGTGAGTAGCA
Id2	TTCCCATTCTCGGCCTTG	TGATGCAGGCTGACGATAGTG
Id3	GTAAGAGCCCGTCGACCGA	GCAGTGGTTCATGTCGTCCA
Nodal	CCTCCAGGCGCAAGATGT	ACCAGATCCTCTTCTTGGCTCA
Lefty1	CTCGGGTCACCATTGAATGG	TGGACACGAGCCTAGAATCGA
Lefty2	GTCACCATTGAATGGCTGAGAG	GTGGATGGACACGAGCCTAGAG
Rex1	TCATCTTCTTGCTCTTCTTCAGAGTC	TAGTCCATTTCTCTAATGCCCACAG
Cripto	ATCCAGTGTGGTTTTGCTTGTG	TCTCTGATGGCAAGGTCTCTCC
Alk3	CTCATGTTCAAGGGCAGAATCTAG	TTTCTGGCTTCTTCTGGTCCAA

Generation of Dies1 antibody

Dies1 was an uncharacterized and unpublished gene. To further explore Dies1 expression we generated polyclonal antibodies using the N-terminal part of the protein as it was predicted to be strongly immunogenic in rabbit. Firstly, we purified Dies1 extra-cellular domain (aa 39-168) fused to GST domain from bacteria (Escherichia Coli BL21). This protein was injected into 2 rabbits by PRIMM and then the serum (named serum 1 and 2) was collected after one month. We tested both serum by Western Blot but they didn't get satisfying results. Thus, we decided to generate others antibodies using a peptide as immunogen. The peptide ELKNHHPEQRFYGSME (aa 149-164) was synthesized by PickCell Laboratories, injected into 2 rabbits and after one month serum (named 11375 and 11376) was collected. We tested both serum and selected the serum 11375 as it gave better results in western-blot and immuno-staining analysis.

Protein extraction and western blotting

To obtain protein extracts, cells were lysated in RIPA buffer: 20 mM Tris-HCl (pH 7.5) 150 mM NaCl, 1 mM EDTA, 1% Triton, 1% sodium deoxycholate, 2,5 mM sodium pyrophosphate,1 mM b-glycerophosphate, 1 mM Na3VO4. The lysates were separated by SDS-PAGE (Biorad Laboratories) and transferred to PVDF membrane (Millipore). The

membrane was probed with the following antibodies: Anti-Oct3/4 (Santa Cruz), anti-GAPDH (Santa Cruz), anti FLAG M2 (Sigma), anti HA (Upstate), anti pTyr (Santa Cruz), anti Nanog (Calbiochem), anti Smad1 (Epitomics), anti p-Smad 1/5/8 (Cell signaling), anti Smad2 (Cell Signaling), anti p-Smad 2/3 (Cell Signaling). Antibody-protein complexes were detected by HRP-conjugated antibodies (Santa Cruz and Amersham Biosciences) and ECL (Amersham Biosciences).

Immunoflurescence of cells and embryos

ES cells, HEK 293 and blastocysts were fixed in 4% paraformaldehyde and permeabilized with 0.2% TX-100 in 10% normal goat serum (Dako Cytomation, Glostrup, Denmark, http://dakocytomation.com)/1% BSA in 1X PBS for 15 min at room temperature. Thus the samples were incubated with primary antibodies at the following working dilutions: anti-βIII-tubulin (1:400; Sigma-Aldrich), anti-Oct3/4 (1:100; Santa Cruz), anti-Nanog (1:100, Calbiochem), anti-Flag (1:250; Sigma), anti-E cadherin (1:100 Sigma), anti Dies1 (1:250 serum 11375). Following primary antibodies incubation, either cells or blastocyst were incubated with appropriate secondary antibodies detecting mouse, rabbit, goat and rat IgG conjugated with Alexa Fluor 594 or 488 (Molecular Probes). Images were captured with an inverted microscope (DMI4000, Leica Microsystems) or with a confocal microscope (LSM 510 META, Zeiss).

Alkaline Phosphatase assay

ES cells were seeded at low density ($1x10^3$ for 10 cm plates) and then cultured for 6 days in medium (see above) not supplemented by LIF. Then they were fixed with formaldehyde and stained with Red Violet (Sigma).

FACS analysis

$5x10^6$ cells were resuspended in 1 ml of SALINA GM+EDTA. Then the cells were fixed adding ethanol at kept overnight at 4°C. The pellet obtained after centrifugation at 1000 rpm was resuspended in 2 ml of iodure propidium (20 μg/ml) and then analysed with a FACScanto (BD Bioscence).

Teratoma Formation

Nude mice were injected subcutaneously with $2x10_6$ ESC cells transfected with Dies-1 shRNA (left side) or NS shRNA (right side). Four weeks after the injection, tumors were surgically dissected from the mice. Samples were fixed for 24 hours in 4%

paraformaldehyde, and embedded in paraffin. Sections were stained with heamatoxylin and eosin.

Luciferase reporter assays

Firefly and Renilla luciferase activities were measured with the dual-luciferase reporter system (Promega) by Sirius Luminometer (Berthold Detection Sistems). Cells were transfected with Id BRE luc or with CAGA luc (kindly provided by Caterina Missero) and with NS siRNA and Dies1 siRNA. 16 hours post-transfection BMP4 30 ng/ml (R&D), Activin (50 ng/ml), Nodal (4 μg/ml) were added to the following medium: GMEM supplemented with KSR and LIF (see above). After 24 hours lysates were harvested. The data generated were expressed as relative to mock transfected cells or to non-silencing siRNA control transfection, after normalization to Renilla luciferase reading. All transfection experiments were repeated in triplicate.

Elisa assay

ELISA was performed by using Delfia EU labeling kit (Perkin Elmer) following the manufacturer instruction. Briefly, 96 well plates Strep-MAB Immunocoated (IBA) were incubated at 4°C for multiple rounds with the conditioned medium of HEK293 cells secreting Strep-tagged Dies1 extracellular domain (ECD). Then, 100 ng/ml of BMP4 (R&D), marked with Europium (Perkin Elmer), was added to the wells and incubated for 2h at RT. For competition assay some wells were pre-treated with unlabelled BMP4 at reported concentration. Fluorescence signal was detected with Envision 2102 Multilabel Reader (Perkin Elmer).

RESULTS

Identification of genes involved in mouse ES cell differentiation

We developed a systematic approach based on the screening of a shRNA library (Open Biosystems) covering all known mouse transcriptome (Silva, Li et al. 2005). Our goal was to identify genes involved in ES cell self-renewal and/or differentiation. To this aim, we generated an ES cell line expressing the gene reporter Enhanced Green Fluorescent Protein (EGFP) under the control of the promoter of α1 tubulin gene, which is a specific marker of neuronal precursors and young neurons (Schmandt, Meents et al. 2005). α1 tubulin-GFP cell line showed proper differentiation ability and retained all the properties specific of wild-type E14 Tg2A ES cells. To obtain neural differentiation, α1 tubulin-GFP cells were cultured in monolayer with neural differentiation medium (NDM), which contains knock-out medium supplemented with knock-out serum, a serum-free defined cocktail of factors. In these culture conditions, α1 tubulin and, in turn, EGFP started to be expressed at day 4 of differentiation and strongly increased day by day during differentiation until day 13. In fact, 10-13 days were necessary to gain complete differentiation, and thus to obtain cells positive to Glial Fibrillar Acidic Protein (GFAP), a marker of astrocytes and to β3 tubulin, that is specifically expressed in mature neurons.

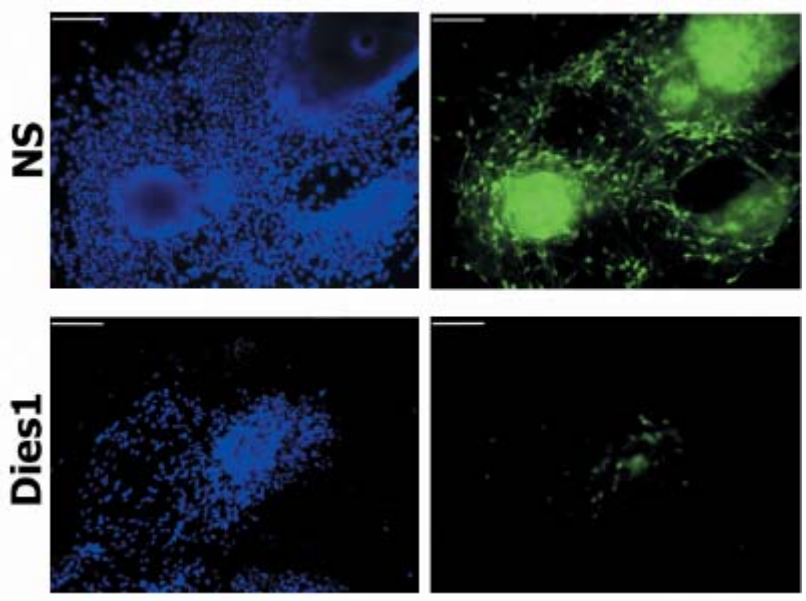
α1 tubulin-GFP cells were transfected with a single shRNA and, after selection with puromycine, pool of clones were seeded in monolayer in NDM. The readout of the screening was the detection by eye at microscope of EGFP expression that was monitored at day 4, 7 and 10 of differentiation. The differentiation conditions used are very powerful to study the impact of a single gene avoiding extrinsic influences and not reproducible effects due to serum. We speculated that a gene whose knock-down by RNA interference affects the generation of neurons may be involved not only in differentiation but also in maintenance of self-renewal and pluripotent status. Thus, we focused our attention on genes whose knock-down impaired EGFP expression and, in turn, neuronal differentiation. Indeed, we selected genes with a strong effect on EGFP signal and then we confirmed these results in three rounds of transfection. To date, we have screened about 10500 genes and more than 30000 shRNA constructs. As a result of the first part of the screening we found 50 genes whose knock-down affects ES cell differentiation. In 2008 we published a paper where we characterized one of these 50 genes, the transcription factor Klf5, as a key factor involved in ES cell self-renewal through the regulation of Oct3/4 and Nanog expression (Parisi, Passaro et al. 2008). Among the 50 genes selected, we found 6

uncharacterized and unpublished genes (table1). Among these 6 genes we selected for further studies the riken cDNA 4632428N05, that we named Dies1 (Differentiation of ES cell 1), given its effect on ES cell differentiation (Fig. 8)

TABLE 1: LIST OF UNCHARACTERIZED GENES WHOSE KNOCK-DOWN AFFECTS NEURONAL DIFFERENTIATION

Riken cDNA 4632428N05	Riken cDNA E130310K16
Riken cDNA 4632404H12	Riken cDNA 4921522P10
Riken cDNA 4933412D19	Riken cDNA 4833418A01

FIGURE 8: RIKEN cDNA 4632428N05 (NAMED DIES1) SUPPRESSION BY RNA INTERFERENCE AFFECTS ES CELL DIFFERENTIATION INTO NEURONS

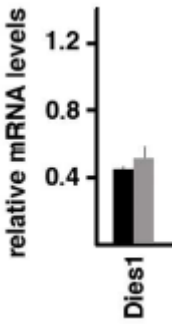


α1-tub GFP cells, transfected with NS shRNA or with Dies1 shRNA, were cultured 13 days in neural differentiation medium. EGFP expression is driven by the neuronal specific promoter α1 tubulin.

Dies1 suppression impairs ES cell differentiation into neurons

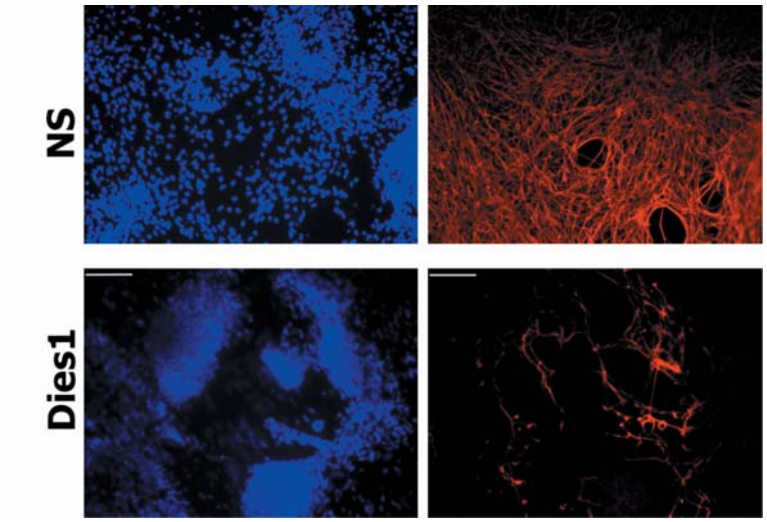
First of all, we attempted to confirm results obtained in α1 tubulin-GFP cells. To this aim, we transfected mouse embryonic stem cell line, E14 tg2A, with the shRNA targeting Dies1 available in the library (sh1). This shRNA is able to reduce Dies1 mRNA level to 40-50% (Fig. 9). Pools of clones stably expressing Dies1 KD1 (Dies1 KD1 cells) were cultured in neural differentiation medium (NDM). At day 13 of differentiation we stained β3 tubulin as marker of mature neurons. As shown in Fig. 10, the number of neurons is strongly reduced in Dies1 KD1 cells compared to cells transfected with non-silencing shRNA construct (NS KD). This result confirmed what we observed in α1 tubulin-GFP cells. To avoid off target effects, we designed others 3 shRNA targeting Dies1 mRNA. One of them, targeting 3'UTR of Dies1 mRNA (sh2), is efficient in silencing Dies1 mRNA as well as Dies1 KD1 (Fig. 9). We performed the same experiment described above with cells stably expressing Dies1 shRNA2. As expected, the staining with β3 tubulin antibody in Dies1 KD2 cells at day 13 of differentiation displayed the same phenotype obtained in Dies sh1 cells (not shown). Indeed, about 60% of NS KD cells are positive to β3 tubulin staining at day 13 of differentiation compared to less than 30% of Dies1 KD cells (Fig. 11). We noted also a reduction in the number of glial cells, suggesting that Dies1 suppression was involved not only in neuronal differentiation, but also in glial differentiation. In fact, about 12% of NS KD cells were stained with the antibody of the astrocyte marker GFAP, compared to less than 4% of Dies1 KD cells (Fig. 11). No signs of non-ectodermal differentiation were detected in our culture conditions. In fact, only few cells (about 1-3%) were stained with the antibody of the endodermal marker HNF3β and no cells were stained with antibodies of markers of early mesoderm as Brachyury or of late mesoderm such as α-myosin heavy chain (α-MHC) (not shown). Thus, our results indicated that Dies1 suppression affects ES cell differentiation in both neurons and astrocytes.

FIGURE 9: DIES1 TARGETING SHRNA1 (BLACK) AND SHRNA2 (GREY) REDUCE DIES1 mRNA EXPRESSION LEVEL



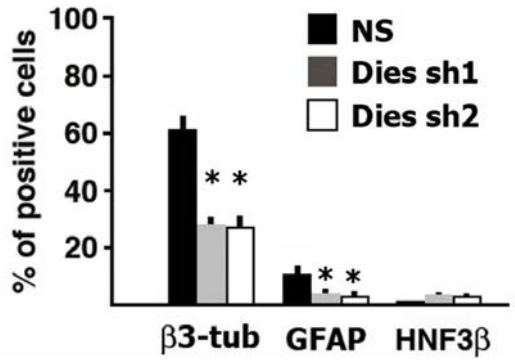
ES cells were transfected with NS shRNA or with shRNA 1 (black) or with shRNA2 (grey) targeting Dies1. Real Time PCR analysis was performed to detect Dies1 mRNA levels. Values are reported as fold-changes compared to Dies1 mRNA levels in cells transfected with NS shRNA

FIGURE 10: DIES1 KNOCK-DOWN AFFECTS NEURONAL DIFFERENTIATION, REDUCING THE NUMBER OF β3 TUBULIN POSITIVE CELLS



ES cells, transfected with NS shRNA or Dies1 shRNA, were cultured in neural differentiation medium. These cells were stained with antibody β3 tubulin at day 13 of differentiation (red). Nuclei were stained in blue with Dapi.

FIGURE 11: DIES1 KNOCK-DOWN AFFECTS DIFFERENTIATION INTO NEURONS AND ASTROCYTES

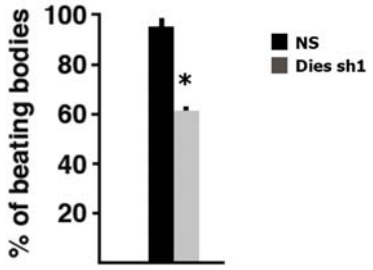


ES cells, transfected with NS shRNA or with Dies1 shRNAs were cultured in neural differentiation medium. These cells were stained at day 13 of differentiation with the following antibodies: β3 tubulin as marker of neurons, GFAP as marker of astrocytes and HNF3β as marker of the endodermal layer. The number of cells positive to these markers was counted in 7 different fields and reported in graph as percentage of total cells, which were stained with Dapi. This experiment was performed in triplicate.
**, $p<0,001$

Dies1 suppression impairs ES cell differentiation into cardiomyocytes

Given its effect on differentiation into neurons and astrocytes, we asked whether Dies1 suppression by RNA interference affected specifically neural differentiation. To answer this question we cultured Dies1 KD cells as Embryoid Bodies (EBs) with hanging drop differentiation method. When ES cells are differentiated as EBs with hanging drop differentiation, they form all three germ layers and every EB contains one or more beating hearts if cultured in cardiomyocytes differentiation medium (CDM). CDM contains GMEM supplemented with an empirically selected FBS serum. Every EB contains one or more beating hearts after 7-9 days in CDM, whereas 12 days are required to obtain completely differentiated cardiomyocytes. As shown in Fig. 13, Dies1 KD cells cultured as Embryoid Bodies in CDM gave rise to a lower number of EB containing beating hearts at day 8 of differentiation (about 60% compared to 100% of EBs derived from NS shRNA cells) (Fig. 12). This result suggested that Dies1 was involved also in differentiation into cardiomyocytes, impairing proper differentiation when it is suppressed by RNA interference.

FIGURE 12: DIES1 KNOCK-DOWN AFFECTS PROPER DIFFERENTIATION INTO CARDIOMYOCYTE**S**

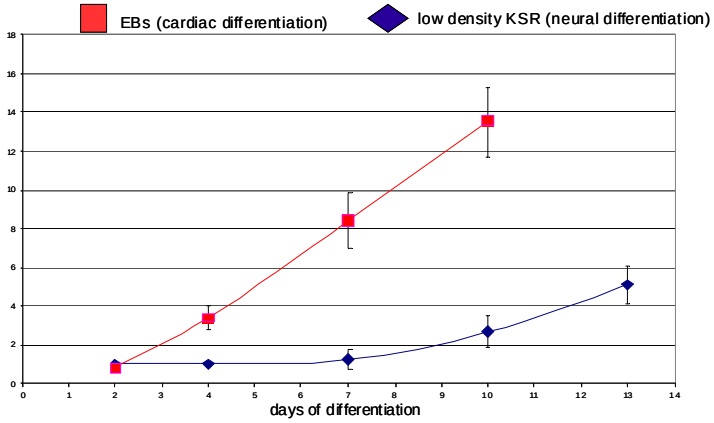


ES cells, transfected with NS shRNA (black) or with Dies1 shRNA1 (grey), were cultured as Embryoid Bodies (EBs) with hanging drop differentiation method. At day 8 of differentiation the EBs containing beating hearts were counted (total number= 48). The experiment was done in triplicate.
*,p<0,01

Induction of Dies1 expression is an early event of differentiation

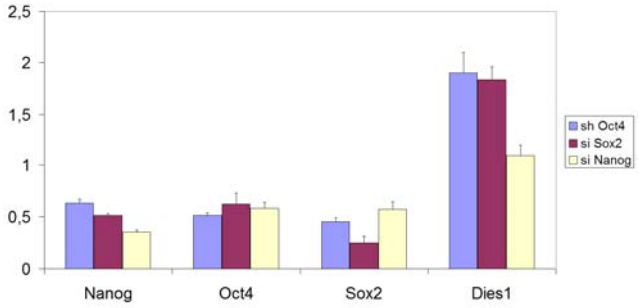
Dies1 suppression impaired ES cell differentiation into neurons, astrocytes and cardiomyocytes. This data were supported by the increase of Dies1 mRNA expression levels when cells were cultured in conditions promoting differentiation (Fig. 13). In addition, Dies1 mRNA expression levels increased in cells where Oct4 and Sox2 were suppressed by RNA interference, suggesting that the increase of Dies1 expression level is an early event of differentiation (Fig. 14). However, the suppression of Nanog did not exert any effect on Dies1 expression at early time points.

FIGURE 13: DIES1 EXPRESSION INCREASES DURING DIFFERENTIATION



ES cells were cultured in monolayer in neural differentiation medium or as Embryoid Bodies (EBs) with hanging drop differentiation method. RT Real Time PCR analysis was performed to detect Dies1 mRNA levels at different time points during differentiation. The Dies1 mRNA levels are expressed as fold-changes compared to undifferentiated ES cells.

FIGURE 14: DIES1 EXPRESSION INCREASES IN CONSEQUENCE OF Oct4 AND Sox2 SUPPRESSION



ES cells were transfected with siRNA targeting Sox2 or Nanog, or with shRNA targeting Oct4. RT-Real Time PCR analysis was performed to detect the mRNA expression of Nanog, Sox2, Oct4 and Dies1 48 hours after transfection. The values are reported as fold-changes compared to control (non silencing si/sh RNA)

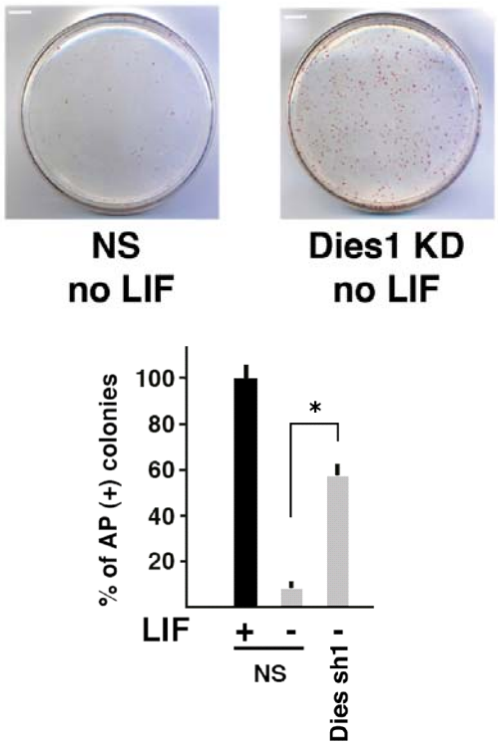
Dies1 knock-down maintains Alkaline Phosphatase expression in the absence of LIF

The results showed before suggested that Dies1 was involved in early steps of ES cell differentiation. Dies1 suppression, in fact, impaired ES cell differentiation and exerted its effect in different culture conditions. We hypothesized two different fates for ES cells where Dies1 is suppressed (Dies1 KD cells):

1. Dies1 KD cells were unable to differentiate, thus they remained completely or partially undifferentiated.
2. Dies1 KD cells underwent an alternative differentiation fate, becoming a specific lineage.

To test the first hypothesis, we cultured Dies1 KD cells in the absence of LIF for 6 days and then we stained the marker of undifferentiated state Alkaline Phosphatase (AP) with a colorimetric assay (AP assay). As mentioned before, LIF is necessary for maintaining ES cell undifferentiated in culture. Thus, the absence of LIF leads to a weak and uncontrolled differentiation. ES cells grown without LIF, progressively loose the expression of markers of undifferentiated state, such as the transcription factors Oct4 and Nanog and the membrane protein AP. The AP assay evaluates the ability of cells to remain undifferentiated in the absence of LIF, giving rise to colonies stained in red, if the cells are positive to AP staining and thus, still undifferentiated. We speculated that if Dies1 was involved in pluripotency or in early steps of differentiation, Dies1 KD cells should maintain the expression of undifferentiated even in the absence of LIF. As expected, less than 10% of NS KD colonies are positive to AP staining when they are cultured for 6 days in the absence of LIF. This result indicated that they underwent proper differentiation, loosing the expression of undifferentiated markers in the absence of LIF. On the contrary about 60% of Dies1 KD colonies were positive to AP staining, indicating that they maintained the expression of undifferentiated markers when they are cultured without LIF (Fig. 15). Thus, Dies1 KD cells were, at least in part, not dependent on LIF for maintaining undifferentiated state.

FIGURE 15: DIES1 KNOCK-DOWN MAINTAINS ALKALINE PHOSPATASE EXPRESSION IN THE ABSENCE OF LIF



ES cells, transfected with NS shRNA and Dies1 shRNA1, were cultured in the absence of LIF. At day 6, Alkaline Phosphatase (AP) was used as marker of the undifferentiated state. The number of colonies (red) positive to AP present in a 10 cm dish was counted and reported as percentage compared to colonies positive to AP when cells are cultured in presence of LIF. The experiment was done in triplicate.

*, $p<0,01$

Dies1 suppression maintains the expression of undifferentiated markers in conditions promoting differentiation

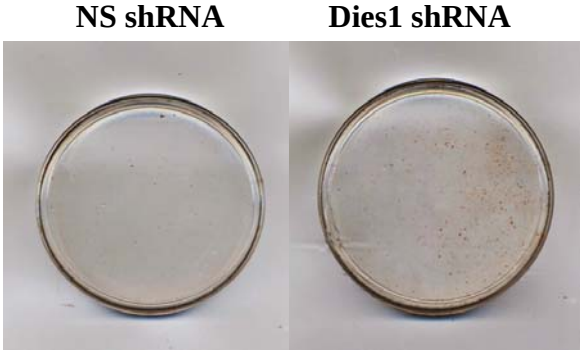
Given that Dies1 KD cells maintained, at least in part, Alkaline Phosphatase (AP) expression when the cells were grown in the absence of LIF we investigated if AP expression was maintained also when Dies1 KD cells were grown in neural differentiation medium (NDM). To this aim, we stained AP in Dies1 KD cells cultured 7 days with NDM. In this case, it was not easy to count the number of cells positive to AP, because they are not seeded at clonal density. However, the results obtained (Fig. 16) encouraged us to explore further in detail the expression of undifferentiated markers in Dies1 KD cells grown in conditions promoting differentiation. To this aim, we stained Oct4 and Nanog as marker of undifferentiated state at day 13 in NDM. Indeed, in NS KD cells Oct4 and Nanog were undetectable at day 13 of neural differentiation whereas Dies1 KD cells formed many clusters of cells positive to Oct4 and Nanog staining, retaining ES-cell like morphology (Fig. 17 A). To confirm this result, we measured Oct4 and Nanog mRNA expression levels (Fig. 17 B). No changes in Oct4 and Nanog expression levels between NS KD and Dies1 KD cells were detected in undifferentiated cells. On the contrary, in NS KD cells Oct4 and Nanog expression strongly decreased during differentiation becoming almost undetectable, whereas in Dies1 KD cells their expression was significantly higher than in controls at day 7 and increased at day 13, suggesting that the fraction of cells still expressing Oct4 and Nanog was still proliferating. In addition, Oct4/Nanog positive cells expressed also other markers of undifferentiated state, as E-cadherin (Fig. 17 C).

The persistence of undifferentiated markers was further explored in Dies1 KD cells grown in conditions promoting differentiation into cardiomyocytes. To this aim, Oct4 and Nanog were stained in EBs derived from Dies1 KD cells grown in cardiomyocytes differentiation medium for 8 days. Indeed, in EBs derived from NS KD cells Oct4 and Nanog expression was restricted to a small area whereas they were widely expressed in EBs derived from Dies1 KD cells (Fig. 17 D).

Taken together, these results demonstrated that Dies1 KD cells were unable to differentiate retaining undifferentiated features such as Oct4, Nanog and AP expression when they were cultured in conditions promoting differentiation.

FIGURE 16: DIES1 KNOCK-DOWN MAINTAINS ALKALINE PHOSPHATASE EXPRESSION IN CONDITIONS PROMOTING NEURAL DIFFERENTIATION

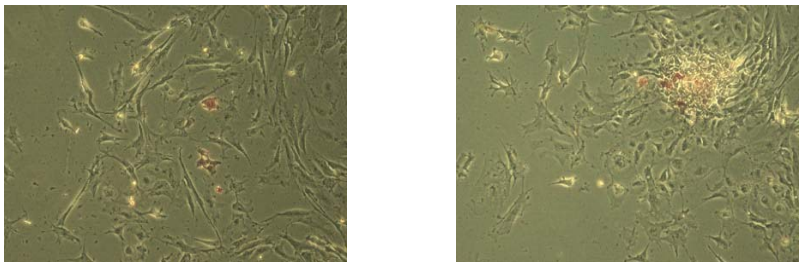
A) AP staining in 10 cm dish



ES cells, transfected with NS shRNA or Dies1 shRNA were cultured in conditions promoting neural differentiation. At day 6, Alkaline Phosphatase was stained in red.

B) Detail of colonies stained with Alkaline Phosphatase assay

NS shRNA



Dies1 shRNA

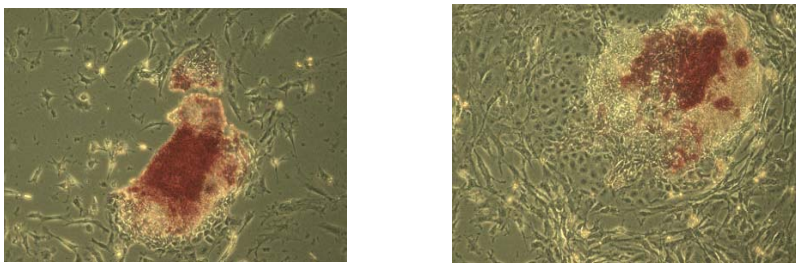
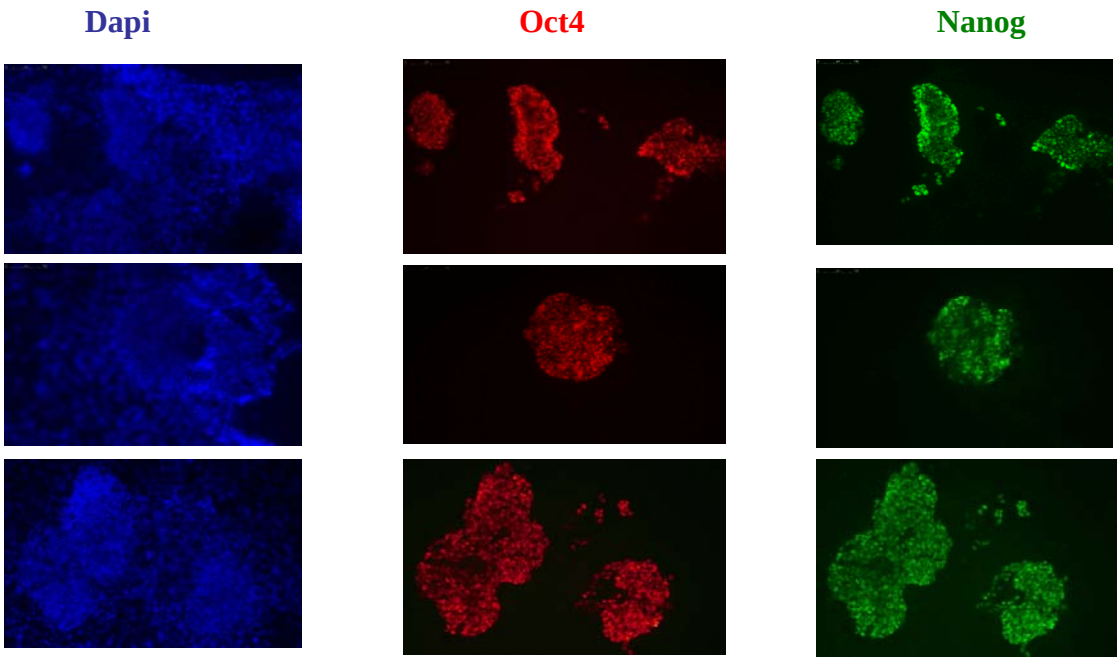


FIGURE 17: DIES1 KNOCK-DOWN MAINTAINS THE EXPRESSION OF UNDIFFERENTIATED MARKERS IN CONDITIONS PROMOTING DIFFERENTIATION

A) Dies1 knock-down (KD) cells form many colonies positive to Oct4 and Nanog staining in conditions promoting neural differentiation

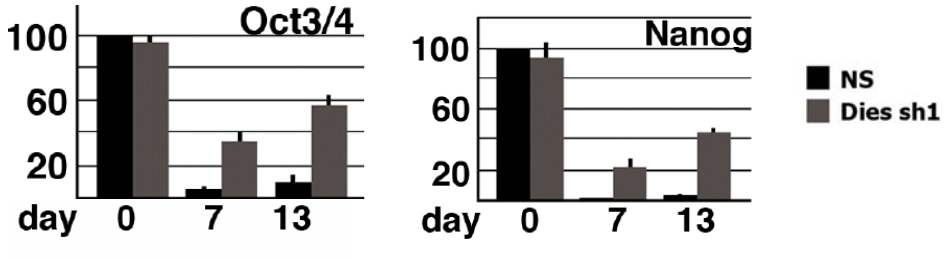
NS KD

Dies1 KD



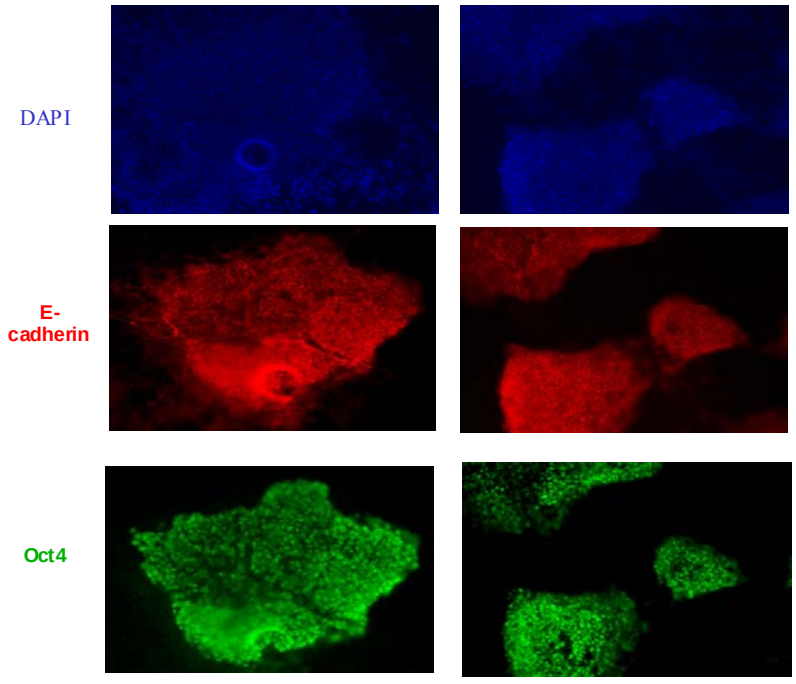
ES cells, transfected with NS shRNA or Dies1 shRNA, were cultured in conditions promoting neural differentiation. At day 13 of differentiation Oct4 (red) and Nanog were stained. Nuclei were stained with Dapi (blue).

B) Dies1 KD cells maintain higher Oct4 and Nanog mRNA expression levels than controls in conditions promoting neural differentiation



ES cells, transfected with NS shRNA or Dies1 shRNAs, were cultured in conditions promoting neural differentiation. Real Time PCR analysis was performed to detect Oct4 and Nanog mRNA expression in undifferentiated conditions (day 0) and at day 7 and 13 of differentiation. The values are reported as percentage relative to Oct4 and Nanog expression in undifferentiated cells transfected with NS shRNA.

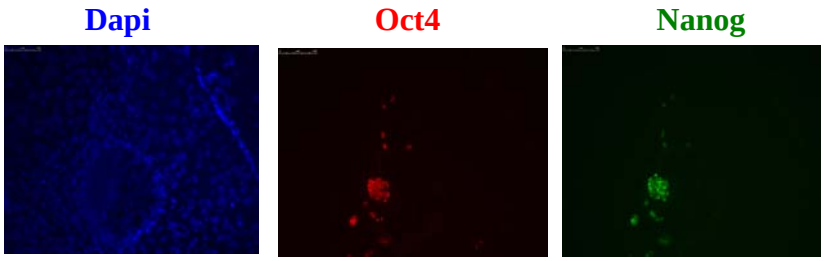
C) Dies1 KD cells, which are still positive to Oct4 in conditions promoting neural differentiation, co-express the undifferentiated marker E-cadherin



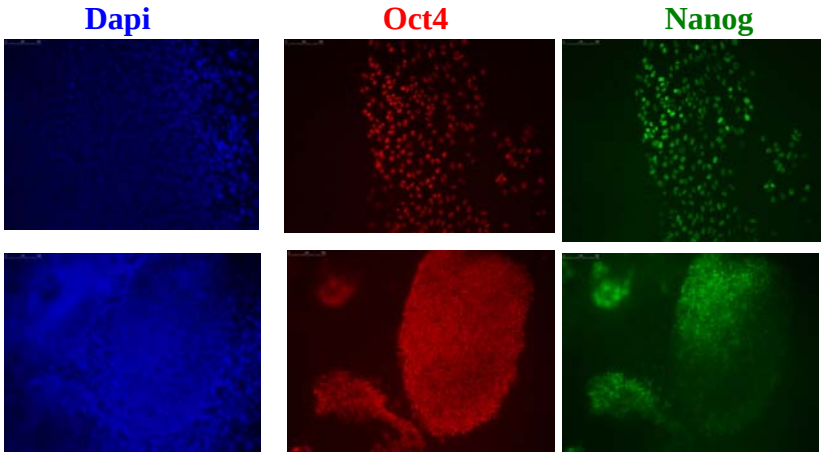
ES cells transfected with Dies1 shRNA, were cultured in conditions promoting neural differentiation. At day 13 of differentiation these cells were stained with Oct4 and E-cadherin antibodies. Nuclei were stained in blue with DAPI.

D) Dies1 KD Embryoid Bodies maintain wide Oct4 and Nanog expression

NS KD Embryoid Bodies



DIES1 KD Embryoid Bodies



ES cells, transfected with NS shRNA or Dies1 shRNA, were cultured as Embryoid Bodies with hanging drop differentiation method in cardiomyocytes medium. At day 8 of differentiation Oct4 (red) and Nanog (green) were stained. Nuclei were stained in blue with Dapi.

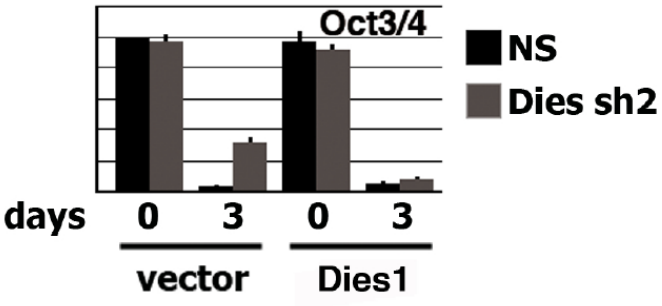
Dies1 re-expression rescues Dies1 knock-down effect

To be sure that Dies1 was responsible for Dies1 knock-down effect on ES cell differentiation, we performed a "rescue" experiment. We transfected Dies1 coding cDNA in ES cells stably expressing Dies1 shRNA2 (Dies1 KD2 cells). The shRNA2 targets Dies1 3'UTR, thus not affecting the expression of Dies1 coding cDNA expression. We tested the ability of exogenous Dies1 to rescue the effect of the suppression of endogenous Dies1. As mentioned before, Dies1 suppression led to maintenance of the expression of the undifferentiated markers in conditions promoting differentiation. This effect indicated that Dies1 KD cells were not able to properly differentiate. To explore the ability of Dies1 re-expression to rescue Dies1 knock-down we measured Oct4 expression in "*rescued*" cells. As shown in figure 18 Dies1 over-expression did not modify Oct4 expression levels in NS KD cells at day 0 (when they are undifferentiated) compared to NS KD cells transfected with an empty vector (NS KD mock). In parallel, Dies1 transfection in Dies1 KD2 cells had no effect on Oct4 expression at day 0, compared to the transfection with an empty vector (Dies1 KD2 mock). Thus, when cells were grown in undifferentiated condition no difference in Oct4 expression occurred .

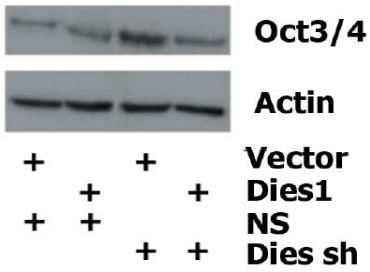
As mentioned before, Dies1 KD cells were unable to undergo proper differentiation, maintaining the expression of undifferentiated markers. As expected, at day 3 of differentiation, Dies1 KD2 mock transfected cells exhibited higher Oct4 expression than NS KD mock transfected cells. However, at day 3 of differentiation Dies1 transfection did not affect Oct4 expression in NS KD cells compared to NS KD mock transfected cells. On the contrary, Oct4 expression levels were strongly reduced in Dies1 KD2 cells transfected with Dies1 compared to Dies1 KD2 mock transfected cells. This phenomenon resulted in restoring Oct4 expression to levels very similar, if not identical, to controls. As a consequence of Oct4 proper expression in the "*rescued*" cells, we may conclude that Dies1 re-expression was able to restore proper differentiation ability in cells where endogenous Dies1 is suppressed by RNA interference.

FIGURE 18: DIES1 RE-EXPRESSION "RESCUES" DIES1 KNOCK-DOWN EFFECT ON NEURAL DIFFERENTIATION, RESTORING PROPER OCT4 EXPRESSION

A) Real Time RT PCR analysis of Oct4 mRNA levels



B) Western Blot analysis of Oct4 protein levels



Real Time PCR (A) or Western Blot (B) analysis was performed to detect Oct4 expression levels. In the panel A Oct4 expression was analysed in undifferentiated conditions and at day 3 of differentiation, whereas the panel B Oct4 protein expression level was analysed at day 3 of differentiation.

Dies1 knock-down affects ES cell developmental potential *in vivo*

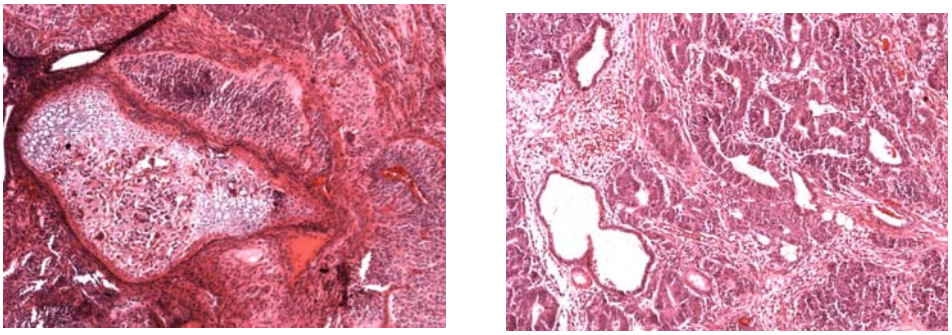
To investigate the effect of Dies1 suppression *in vivo*, we tested the ability of Dies1 knock-down cells to form teratoma. As mentioned before, ES cells are able to give rise to teratoma when they are injected subcutaneously into nude mice. Stemness and tumorigenesis, in fact, are strictly linked each other. We transfected ES cells with NS shRNA (NS KD cells) or with Dies1 shRNA (Dies1 KD cells) and then cultured the cells with puromycin for 6 days. A pool of stable clones where Dies1 was strongly down regulated was selected and then injected subcutaneously into 3 SCID mice (NS KD cells on the right flank and Dies1 KD cells on the left flank). After 3 weeks we explanted tumors from animals. As shown in the figure 19A, the tumors formed by Dies1 KD cells were smaller than the controls. This result suggested that a consistent fraction of Dies1 KD cells lost proper differentiation ability, giving rise to a small tumor, confirming the result obtained *in vitro*. Indeed, more than 50% of cells were shown to be unable to differentiate *in vitro,* thus explaining the reduced size of the tumors *in vivo*. However, the tumors derived by Dies1 KD cells were formed many different cell types, as typically occurs in teratoma. This result suggested that the fraction of cells still able to differentiate as it gave rise to teratoma without any clear histological difference even if the size was decreased (Fig. 19 B). Thus, Dies1 suppression affected ES cell pluripotency as *in vivo* as in *vitro*.

FIGURE 19: DIES1 KNOCK-DOWN AFFECTS ES CELL DEVELOPMENTAL POTENTIAL IN VIVO

A) Dies1 KD decreases the size of the teratomas

NS KD and Dies1 KD cells were injected into 3 SCID mice. Tumors were explanted after 3 weeks. The figure represents an example of the explanted teratomas.

B) Histological analysis of teratomas



NS KD Dies1 KD

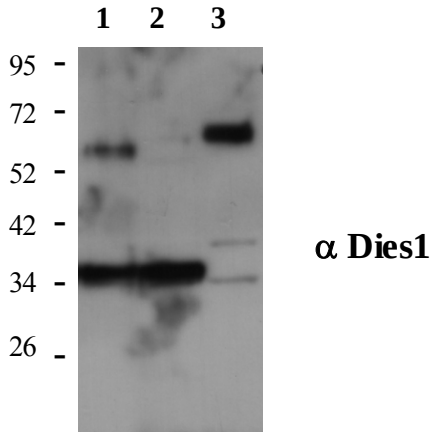
Teratomas deriving from NS KD and Dies1 KD cells were fixed in PFA and embedded into paraffin. Then they were coloured with haematoxylin and eosine

Dies1 is expressed in blastocyst, in ES cells and in some adult organs

Western blot analysis with Dies1 antibody 11375 (see material and method) in mouse ES cells identified a specific band whose molecular weight is about 60 kilodalton, suggesting strong post-translational modifications as the expected size of Dies1 protein is 35 KD. An aspecific band, whose molecular weight is about 30 KD, was present in mouse ES cells total extract and was recognized by pre-immune serum also (not shown). The band of 60 kilodalton disappears in ES cells total extracts after the transfection of the shRNA targeting Dies1 (Fig. 20 A). As further control, we also transfected Dies1 cDNA in HEK 293 and detected a band of a bit higher molecular weight because of the presence of the 3xFLAG tag. To confirm the expression of Dies1 *in vivo* we stained mouse blastocysts with antibody 11375 (Fig. 20 B). The staining with serum 11375 showed signals on the surface of some cells in the blastocyst. Then we asked if Dies1 is expressed in mouse adult. To this aim we performed Real Time PCR analysis. This analysis indicated that Dies1 is strongly expressed in lung, spleen and bone and is detectable in others organs such as brain, testis heart and liver even if at lower level (Fig. 21 A), as suggested also by data available in databases of ESTs (Unigene). The strong expression in lung and spleen was confirmed by Western Blot analysis with Dies1 antibody (Fig. 21 B). The band of higher molecular weight detected in brain and testis could be the result of different post-translational modifications and needs to be confirmed as a specific Dies1 signal**.**

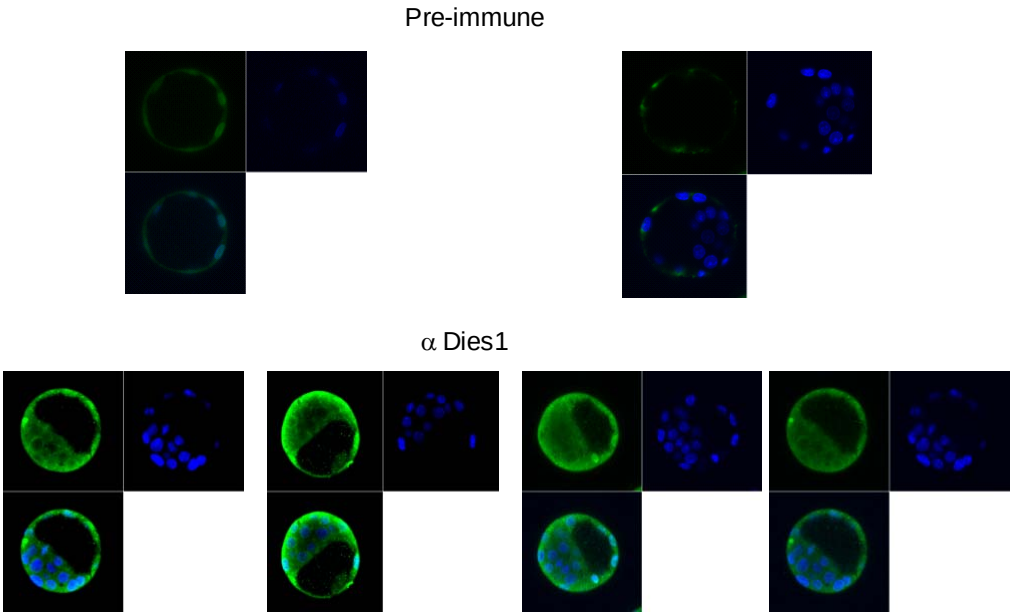
FIGURE 20: DIES1 IS EXPRESSED IN MOUSE ES CELL AND BLASTOCYST

A) Western Blot in mouse ES cells



ES cells were transfected with NS shRNA (lane 1) or with Dies1 shRNA (lane 2). HEK 293 cells were transfected with Dies1 cDNA (lane 3) as control. Western Blot analysis with Dies1 antibody 11375 was performed to detect Dies1 protein expression

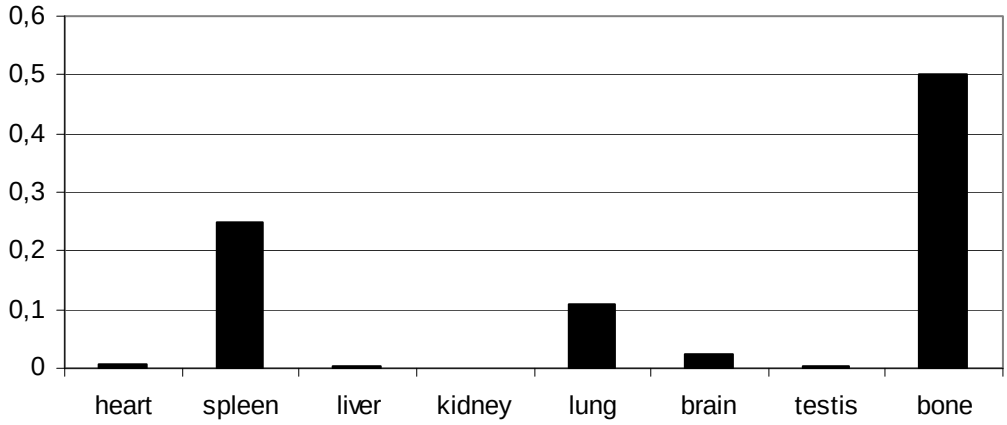
B) Confocal microscopy images of blastocyst



Mouse blastocysts (E 3,5) were fixed and incubated with pre-immune serum (green) or with Dies1 antibody 11375 (green). Nuclei were stained with Dapi (blue).

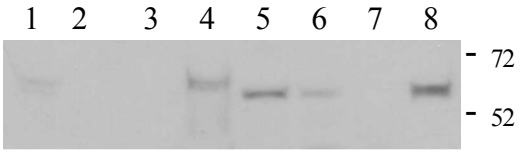
FIGURE 21: DIES1 EXPRESSION IN ADULT MOUSE ORGANS

A) Dies1 mRNA expression levels



Real Time PCR analysis was performed to detect Dies1 mRNA expression levels in mouse adult organs. Values are reported as fold-changes relative to GAPDH expression level in each organ.

B) Dies1 protein expression level



Western blot analysis was performed to detect Dies1 protein expression in mouse adult testis (lane 1), kidney (lane 2), heart (lane 3), brain (lane 4), spleen (lane 5), lung (lane 6) and liver (lane 7). HEK 293 cells were transfected with Dies1 cDNA HA tagged as control (lane 8).

Dies1 is a surface protein

To investigate Dies1 function we performed an *in silico* analysis of Dies1 sequence that is available on Ensembl database. As shown Fig. 22A, Dies1 protein consists of 314 aminoacids (aa). The sequence of aa 1-32 is a typical signal peptide and the aa 192-213 consists of a putative trans-membrane tract. This observation suggested a possible localization in membrane. Of great concern is the presence of an Ig-like domain in the N-terminal part of the protein (the putative extra-cellular/extra luminal part). Ig-like domains are involved in protein-protein interaction and can be commonly found in proteins whose function is adhesion, as cadherins and receptor activity such as EGFR, PDGFR and other related proteins. To confirm bioinformatics prediction we performed an immuno-staining in mouse ES cells and in HEK 293 over-expressing Dies1 full-length protein fused with 3xFLAG tag at C-terminus of the protein. The cells were fixed, permeabilyzed and incubated with FLAG antibody. Confocal images showed that Dies1 was expressed on cell surface, supporting bioinformatics prediction (Fig. 22B). Moreover, the staining resulted also in signals present in intracellular vessels suggesting trafficking from endoplasmic reticulum to surface and vice versa.

As many proteins expressed on cells surface, Dies1 contains an Ig-like domain. As Ig-like domains are commonly glycosylated, we tested if Dies1 was glycosylated in ES cells. Dies1, in fact, contains at least 3 putative sites of N-glycosylation at the N-terminal part of the protein. To this aim, we treated ES cells over-expressing Dies1 fused to 3xFLAG tag with tunicamycin, an N-glycosylation inhibitor, at different concentrations for 24 hours. Western blot analysis with FLAG antibody resulted in a strong change of Dies1 electrophoretic mobility. This is dose-dependent and at high concentrations of tunicamycin the electrophorectic mobility become very close to the expected size of the protein, 35 kilodalton (Fig. 22 C). This result indicated that Dies1 was N-glycosylated and this post-translational modifications could be responsible for its electrophorectic migration around 60 kilodalton.

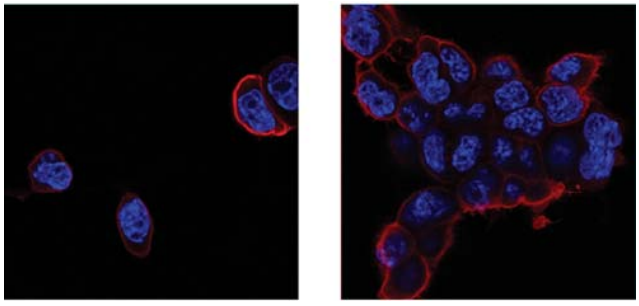
FIGURE 22: DIES1 IS A SURFACE PROTEIN

A) Dies1 sequence

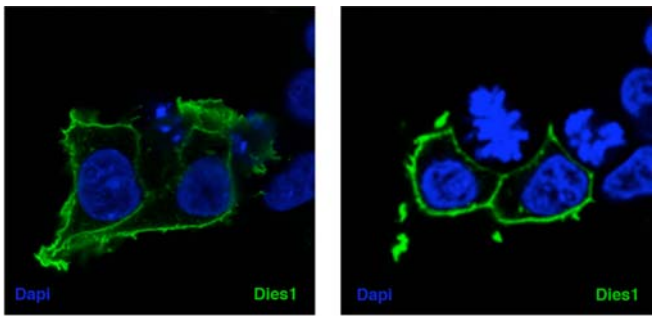
*MGVPAVPEASSPRWGTLLLAIFLAA*SRGLVAAFKVTTPYSLYVCPEGQ**N**A
TLTCRILGPVSKGHDVTIYKTWYLSSRGEVQMCKEHRPIR**N**FTLQHLQHH
GSHLKA**N**ASHDQPQKHGLELASDHHGITAAALATGACIVGILCLPLILLL
VYKQRQVASHRRAQELVRMDSSNTQGIENPGFETTPPFQGMPEAKTRPPL
SYVAQRQPSESGRYLLSDPSTPLSPPGPGDVFFPSLDPVPDSPNSEAI

In italic the signal peptide (aa 1-32). Underlined the transmembrane domain (192-213). In bold putative sites for N-glycosylation.

B) Dies1-3XFLAG (red) is expressed on ES cell surface

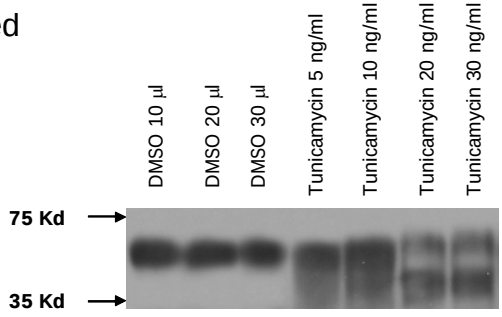


C) Dies1-3XFLAG (green) is expressed on HEK 293 cell surface



Confocal images of ES cells (40X magnification) (B) and HEK 293 (63X magnification) (C), transfected with Dies1 fused to 3xFLAG were stained with FLAG antibody

C) Dies1 is N-glycosylated



WB α FLAG

ES cells transfected with Dies1-3xFLAG were incubated for 24 hours with DMSO or with the inhibitor of N-glycosylation tunicamycin at different concentrations. Western Blot analysis was performed with FLAG antibody to analyse Dies1 electrophoretic mobility.

Dies1 extra-cellular domain plays a crucial role for Dies1 function

As Dies1 knock-down has a strong impact on ES cell differentiation we investigated the effect of Dies1 over-expression. Dies1 full-length (f.l.) over-expression in undifferentiated cells did not affect the expression of markers of undifferentiated state such as Oct4, Sox2 and Nanog and proliferation (not shown). Moreover, Dies f.l. cells were able to efficiently differentiate in neural differentiation medium and did not show any difference in Alkaline Phosphatase (AP) staining compared to mock transfected cells (control) if grown with or without LIF for 6 days. Thus, Dies1 f.l. over-expression did not display any clear and relevant effect on ES cells.

As Dies1 is a surface protein, its extra-cellular domain (ECD) should play an important role. Moreover, Dies1-ECD contains an Ig-like domain which is usually required for binding to interactors. Thus, Dies1-ECD should be crucial for Dies1 proper function. To evaluate Dies1-ECD role, we expressed only Dies1-ECD (aa 39-168) fused to 3xFLAG tag in HEK 293 and in ES cells. First of all we evaluated Dies1-ECD expression in ES cells staining Dies1-ECD cells with FLAG antibody (Fig. 23 A). Then, Western Blot analysis of HEK 293 lysates and conditioned medium confirmed that Dies1-ECD was properly expressed and secreted in the conditioned medium (Fig. 23 B). We speculated that the expression of Dies1-ECD alone may work as a Dies1 dominant negative form: it may compete with endogenous full-length Dies1 for binding to interactors inside and outside the cell. To test this hypothesis, we transfected Dies1-ECD in mouse ES cells and tested its effect on self-renewal and differentiation. In undifferentiated cells, Dies ECD has no effect on proliferation and on the expression of undifferentiated state markers such as Oct4, Nanog and Sox2, as well as Dies1 f.l. over-expression (not shown). On the contrary, Dies1-ECD exerted a clear effect when cells are cultured in the absence of LIF. Indeed, Dies1-ECD led to the maintenance of AP expression in about 40% of colonies when cells are cultured 6 days without LIF (Fig. 24 A). This result has to be compared to the percentage of colonies that maintain AP expression in the controls (less than 10%) and after Dies1 f.l. over-expression (about 15%). This confirmed that Dies1-ECD worked as Dies1 dominant negative form, resembling Dies1 KD effect. As mentioned before, also Dies1 suppression by RNA interference led to maintenance of AP expression, even if its effect is stronger than Dies1-ECD resulting in 60% of colonies positive to AP staining. The effect of Dies1-ECD in the maintenance of undifferentiated markers was confirmed by the increase of Oct4 expression levels in Dies1-ECD cells at day 6 without LIF **(**Fig. 24 B/C). No differences were seen in Nanog expression that continues to be very low in both cases.

Further in detail, we analysed Dies1-ECD colonies when the cells were grown in the absence of LIF. Dies1-ECD cells formed bigger colonies and showed stronger Oct4 expression than controls, confirming previous results (Fig. 24 D).
On the other hand, Dies1-ECD cells were able to differentiate as well as controls in neural differentiation conditions, suggesting that others mechanisms may overcome Dies1-ECD dominant negative effect in particular contests.
Given the fact that Dies1-ECD cells maintained, at least in part, AP expression and markers of undifferentiated state as Oct4 when ES cells were cultured in the absence of LIF we speculated that Dies1-ECD may be used as soluble protein instead of LIF to supplement ES cell growth medium. To test this hypothesis we tried two different approaches:

1. We purified Dies1-ECD fused to 3xFLAG from HEK 293 conditioned medium, using anti-FLAG resine and added this protein to growth medium (not supplemented by LIF);
2. We supplemented normal ES cell medium (without LIF) with conditioned medium of mouse ES clones stably expressing Dies1-ECD fused to 3xFLAG

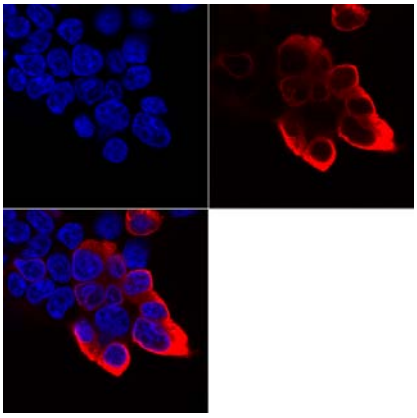
We cultured ES cells for 6 days in the absence of LIF to perform AP assay. In both cases, there was no difference in AP staining compared to controls. This result could be explained in different ways:

1. There were some technical problems in protein purification from 293 medium and in concentration of conditioned medium of ES stable clones;
2. The amount of protein added to growth medium is too low to exert any effect;
3. Dies1-ECD binds to Dies1 intracellular interactors, exerting its effect inside the cells. Thus, Dies1-ECD is not able to work as soluble protein.

If the last hypothesis is correct, Dies1-ECD should impair the formation of a complex whose proper assembly is essential for the function of Dies1 and its partners. Thus, it should work as a dominant negative form inside the cells, for example in vescicles, impairing the proper assembly of a receptor complex.
Taken together these results supported that Dies1-ECD plays a very important role and shed light on possible Dies1 function as surface receptor.

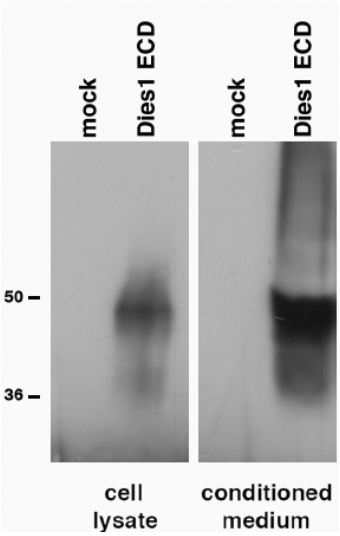
FIGURE 23: DIES1 EXTRA-CELLULAR DOMAIN (ECD) EXPRESSION

A) Dies1-ECD expression in ES cells



ES cells were transfected with Dies1-ECD fused to 3XFLAG. After 48 hours cells were stained with FLAG antibody (red) and then analyzed at confocal microscope.

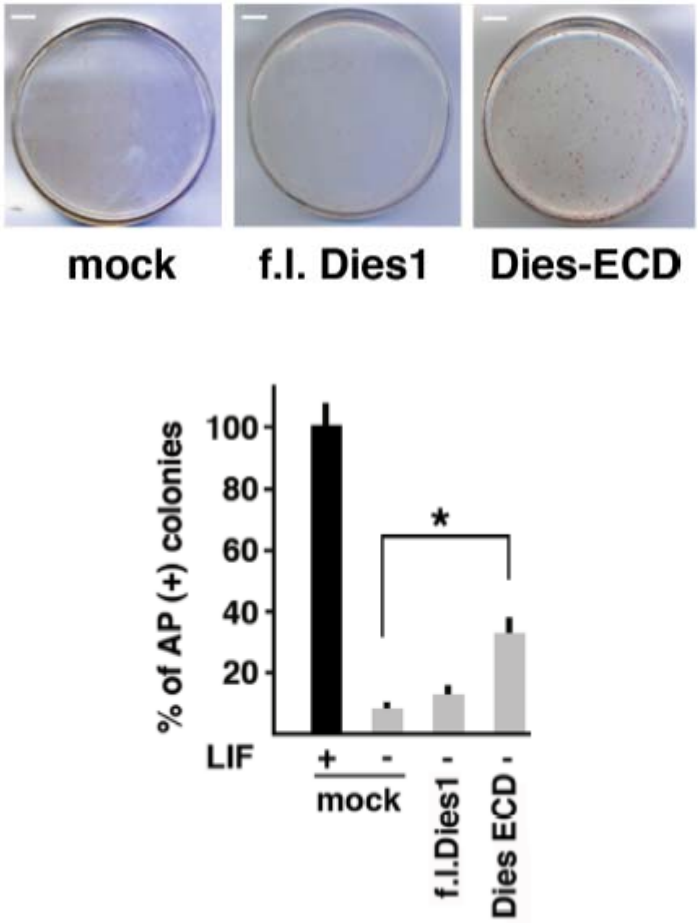
B) Dies1-ECD is properly secreted in culture medium when transfected alone



HEK 293 cells were transfected with Dies1 extra-cellular domain (ECD) fused to 3XFLAG. Western Blot analysis with FLAG antibody was performed on cell lysate and on conditioned medium.

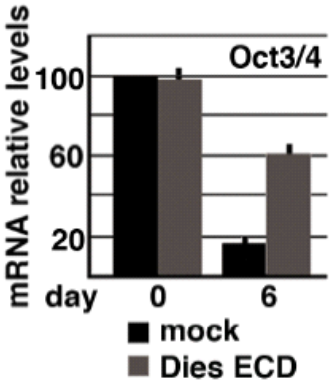
FIGURE 24: Dies1-ECD MAINTAINS OCT4 AND ALKALINE PHOSPHATASE (AP) EXPRESSION IN THE ABSENCE OF LIF

A) Dies1-ECD maintains AP expression in the absence of LIF



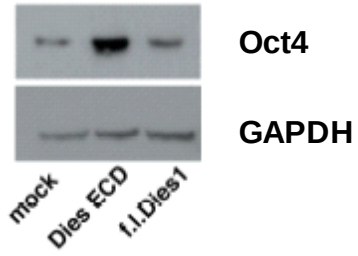
ES cells, transfected with Dies1 full-length or Dies1 extra cellular domain (ECD) were cultured in the absence of LIF. Dies1-ECD maintains AP expression at day 6 in the absence of LIF in more than 30% of colonies. Dies1 full-length has no significant effect compared to control (mock transfected cells)
*, $p<0,01$

B) Oct4 mRNA expression level in the absence of LIF



ES cells, transfected with an empty vector (mock) or with Dies1-ECD, were cultured in the absence of LIF. RT Real Time PCR analysis was performed to detect Oct4 mRNA expression in undifferentiated conditions (day 0) and at day 6 in the absence of LIF. The values are reported as percentage of Oct3/4 expression compared to cells transfected with empty vector (mock) in undifferentiated conditions (time 0).

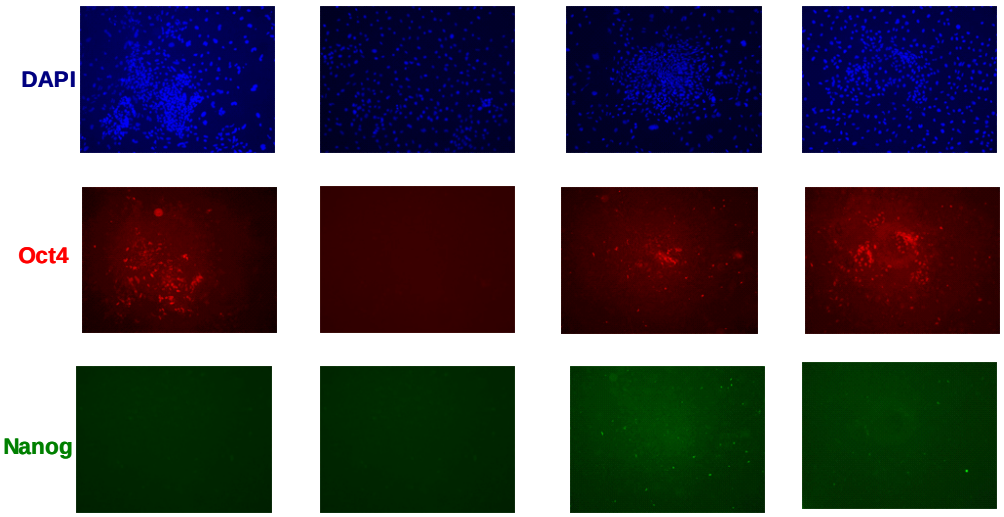
C) Oct4 protein expressionlevel in the absence of LIF



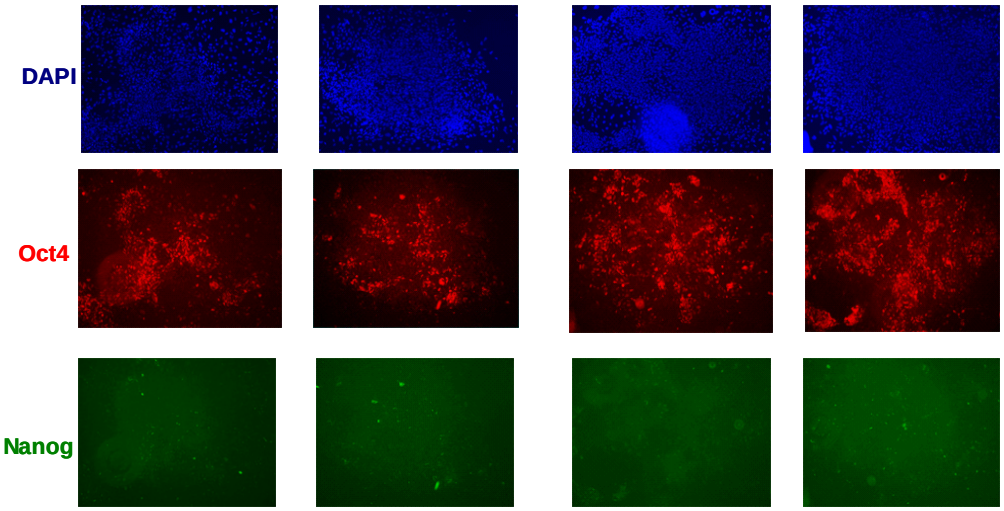
ES cells, transfected with an empty vector (mock), with Dies1-ECD or with Dies1 full-length were cultured in the absence of LIF. Western Blot analysis was performed to detect Oct4 expression at day 6 in the absence of LIF.

D) Staining of Dies1 ECD colonies in the absence of LIF

Mock transfected colonies



Dies1-ECD colonies

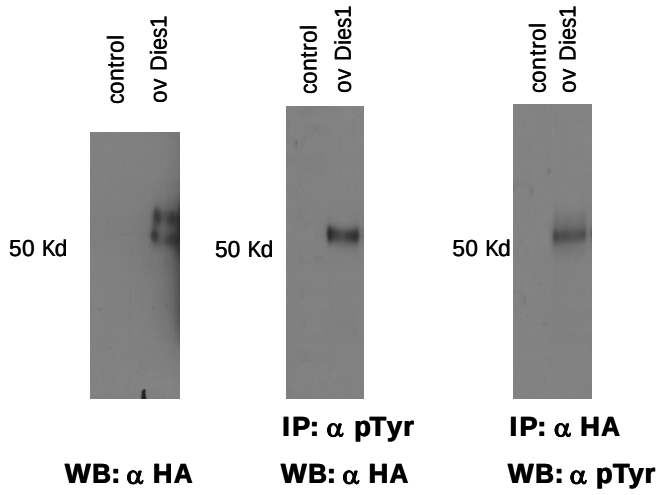


ES cells, transfected with an empty vector (D) or with Dies1-ECD (E) were cultured without LIF. At day 6 Oct4 (red) and Nanog (green) were stained. Nuclei were stained in blue with Dapi.

Dies1 knock-down affects BMP4 signaling pathway

As many receptors are tyrosine-phosphorylated, we tested whether Dies1 also was phosphorylated in tyrosine. Dies1, in fact, contains two tyrosine residues in the C-terminal of the protein. To this aim we transfected HEK 293 with Dies1 cDNA fused to HA tag. Western Blot analysis of immunoprecipitation experiments with HA antibody and with p-Tyr antibody demonstrated that Dies1 was phosphorylated (Fig. 25).

FIGURE 25: DIES1 IS PHOSPHORYLATED IN TYROSINE

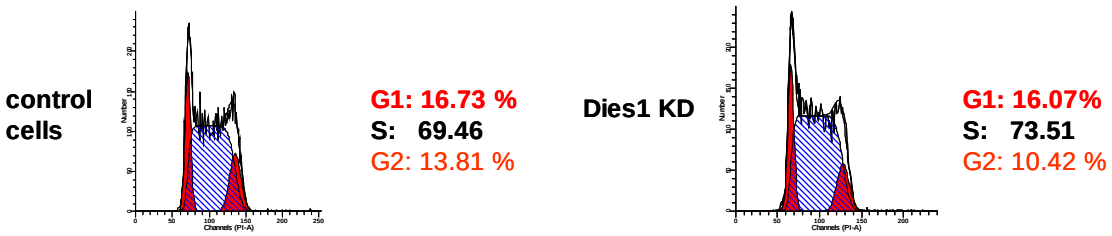


HEK 293 cells were transfected with Dies1 full-length fused to HA tag or with an empty vector (control). In this first panel Western Blot (WB) analysis with HA antibody was performed. In the second panel, immunoprecipitation (IP) with pTyr antibody and then WB with HA antibody were performed. In the third panel, IP with HA antibody and WB with pTyr antibody were performed.

Given the possible role for Dies1 as a receptor we speculated that Dies1 knock-down (KD) or the expression of the extra-cellular domain (ECD) may affect some signalling pathway required for proper ES cell developmental potential. Dies1 KD cells, in fact, were unable to differentiate in different culture conditions and were at least in part independent by LIF. As mentioned before, mouse ES cells are sensitive to extrinsic signals in culture and proper signal transduction machinery is required for maintaining ES cell pluripotency. Thus, we hypothesized that the alteration of some signalling pathway due to Dies1 knock-down resulted in the impaired ability of ES cells to properly differentiate. To address this point, we focused on LIF and BMP4 pathways, that are the most important pathways involved in the maintenance of self-renewal and pluripotency (Ying, Nichols et al. 2003). To this aim, we analysed the expression of genes directly controlled by LIF and BMP4, comparing their mRNA expression levels in non-silencing knock-down cells (NS KD) or Dies1 KD cells, cultured in undifferentiated conditions. Dies1 suppression did not affect proliferation (Fig. 26) or the expression of markers of undifferentiated state such as Oct4, Nanog, Sox2 and Rex1 when cells were cultured in undifferentiated conditions (Fig. 27A). Moreover, Dies1 KD cells may be cultured as wild type cells for long periods and did not show any clear morphological difference. In addition, LIF targets such as STAT3 (also target of Wnts) and Kruppel-like factors such as Klf2, Klf4 and Klf5 were not affected by Dies1 suppression. On the contrary Id1, Id2 and Id3 expression was affected by Dies1 suppression (Fig. 27B). Ids 1-3 are direct transcriptional targets of SMAD1/5/8-SMAD4 complex that is activated by BMP4. To confirm that reduction of Id1-3 expression levels was direct consequence of impairment in transducing BMP4 signal, we performed a luciferase assay. To this aim, we transfected mouse ES cells with a vector encoding for luciferase under the control of the BMP responsive element of Id1 promoter (Id1 BRE luc) (ten Dijke, Korchynskyi et al. 2003). Mouse ES cells were transiently transfected with Id1 BRE luc and with siRNA non silencing or with siRNA targeting Dies1. To minimize effects due to serum, ES cells were cultured in GMEM supplemented with Knock-Out serum supplemented by LIF. As shown in Fig. 27C, BMP4 treatment (20 ng/ml) for 24 hours, resulted in an increases up to 25 times of luciferase activity in NS siRNA cells compared to an increase up to 16 times in Dies1 siRNA cells. This supported that BMP4 signaling was directly affected by Dies1 suppression. To investigate whether the reduction of Id 1-3 expression levels was dependent on a decreased activity of SMAD 1/5/8 complex, we analyzed the amount of phosphorylated and, thus, active, SMAD 1/5/8. As previously described, SMAD 1/5/8 proteins are phosphorylated and thus, activated, by type I receptors upon BMP4 binding. The active, phosphorylated form of SMADs translocates to the nucleus in association with

SMAD4, activating or repressing their target genes. As shown in Fig. 27D in Dies1 KD cells grown in undifferentiated conditions, the amount of SMAD1-5-8 phosphorylated was decreased, whereas total SMAD1 protein level was identical to controls. These results indicated that Dies1 KD impaired BMP4 signaling cascade, down-regulating Id1-3 in a SMAD dependent manner.

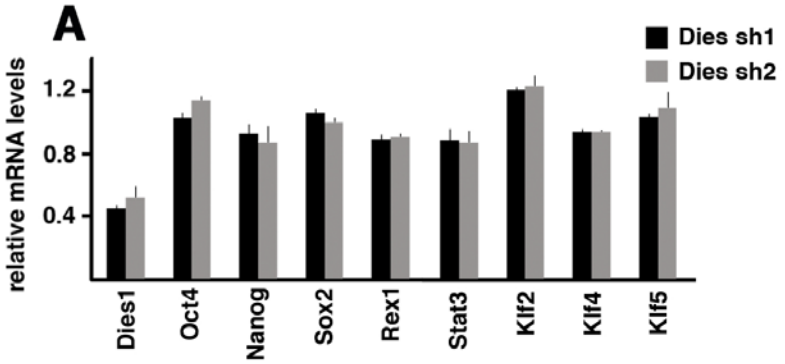
FIGURE 26: DIES1 KNOCK-DOWN DOES NOT AFFECT PROLIFERATION IN UNDIFFERENTIATED CONDITIONS



ES cells were transfected with NS shRNA (control) or with Dies1 shRNA. Then FACS analysis was performed to determine the number of cells in the different phases of cell cycle.

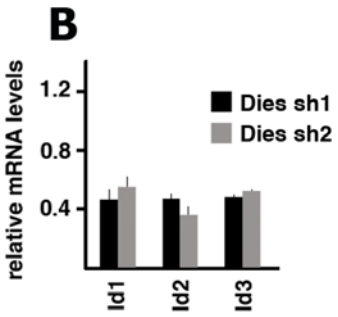
FIGURE 27: DIES1 KNOCK-DOWN DOES NOT AFFECT THE EXPRESSION OF STEMNESS MARKERS IN UNDIFFERENTIATED CONDITION, WHEREAS IT IMPAIRS PROPER BMP4 SIGNALING

A) Undifferentiated markers and LIF targets are not affected by Dies1 knock-down



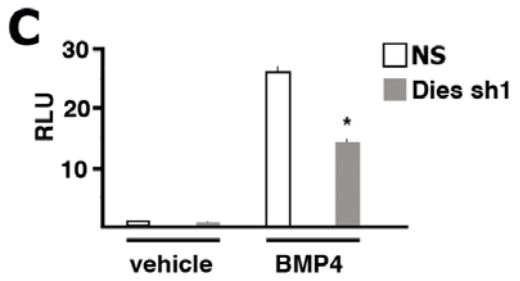
ES cells were transfected with NS shRNA or Dies1 shRNAs. Real Time PCR analysis was performed to detect the expression of undifferentiated markers and LIF targets. The values are reported as fold-changes compared to cells transfected with NS shRNA.

B) BMP4 targets are down-regulated in Dies1 knock-down cells



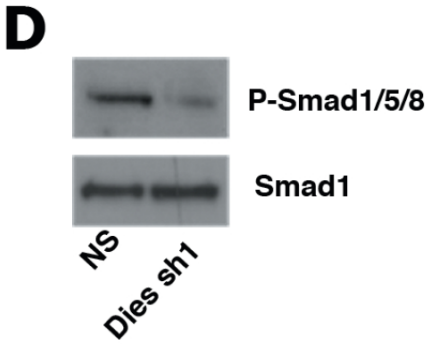
ES cells were transfected with NS shRNA or Dies1 shRNAs. Real Time PCR analysis was performed to detect the expression of BMP direct targets such as Id 1/2/3. The values are reported as fold-changes compared to cells transfected with NS shRNA.

C) Dies1 knock-down reduces luciferase activity in response to BMP4 treatment



ES cells were transfected with a vector in which luciferase gene is under the control of the BMP responsive element of Id1 gene promoter (Id1 BRE LUC). These cells were transfected also with siRNA non silencing or with siRNA targeting Dies1 and then grown in knock-out serum supplemented by LIF. 16 hours after transfection, the growth medium was supplemented with BMP4 (20 ng/ml) for 24 hours. The values are reported as fold-changes compared to luciferase activity (normalized with Renilla) compared to cells transfected with siRNA non silencing cultured in the absence of BMP4.

D) SMAD 1/5/8 phosphorylation is reduced in Dies1 knock-down cells

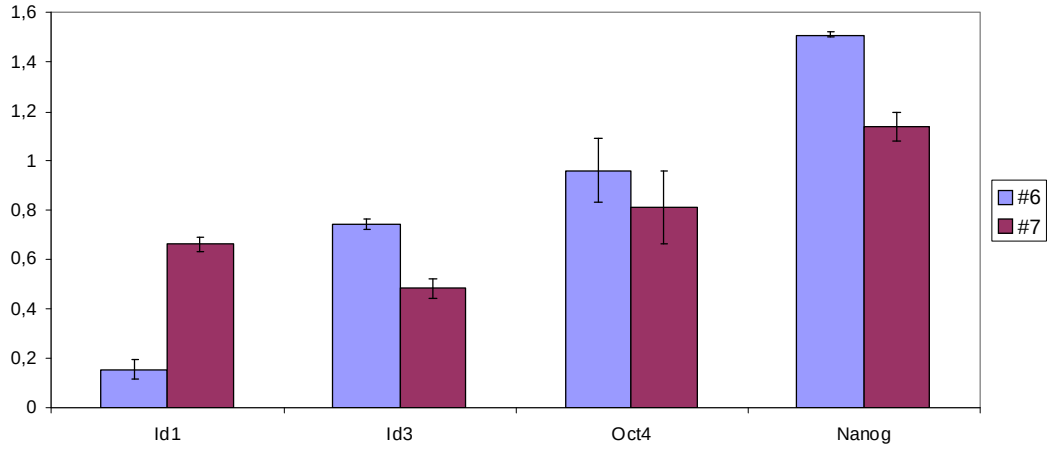


ES cells were transfected with NS shRNA or with Dies1 shRNA. Western Blot analysis was performed to detect the phosphorylated and thus active form of Smad 1/5/8. Total amount of Smad1 protein was used to normalize the samples

Down-regulation of Id genes does not affect ES cell undifferentiated state

Dies1 suppression affects BMP4 signaling, down-regulating Id 1-3 mRNA levels in a SMAD dependent manner. As mentioned before, Id 1-3 regulation seems to be crucial for sustaining ES cell self-renewal pluripotency. Id 1-3 impairs the expression of pro-neural factors, thus maintaining ES cell undifferentiated when the growth medium is supplemented only with LIF and BMP4. In addition, Ying and collaborators demonstrated that Id over-expression may overcome the absence of BMP4 in the growth medium (Ying, Nichols et al. 2003). In our culture conditions, Id expression levels are reduced to 40-50% in consequence of Dies1 suppression by RNA interference. Thus, Id expression is decreased but not abolished. Of great concern is that Id down-regulation seems to be in contrast with the maintenance of undifferentiated markers in conditions promoting differentiation due to Dies1 knock-down. One explanation could be that the reduced levels of Ids does not affect Ids proper function, thus having no impact on ES cells, in contrast to what observed by Ying and Nichols in 2003 when Id 1-3 expression is completely off. This hypothesis is supported by the data obtained by Ying and collaborators in 2008 (Ying, Wray et al. 2008). They observed that a strong reduction of Id1 protein due to the absence of BMP4 in culture does not lead to differentiation when ES cells are cultured just with LIF plus two ERK/FGF4 inhibitors. To prove that Id 1-3 down-regulation did not affect ES cell self-renewal and pluripotency, we transfected both Id1 and Id3 targeting shRNA in ES cells. 2 out of 48 clones screened displayed decreased mRNA levels of both Id1 and Id3. These clones did not show any significant difference in the expression of undifferentiated markers such as Oct4, Nanog and Sox2 (Fig. 28) and were maintained in culture as well as controls at least for 2 weeks. These results strongly supported the hypothesis that Id down-regulation did not affect ES cell ground state.

FIGURE 28: ID1/ID3 DOWN-REGULATION DOES NOT AFFECT THE EXPRESSION OF UNDIFFERENTIATED MARKERS



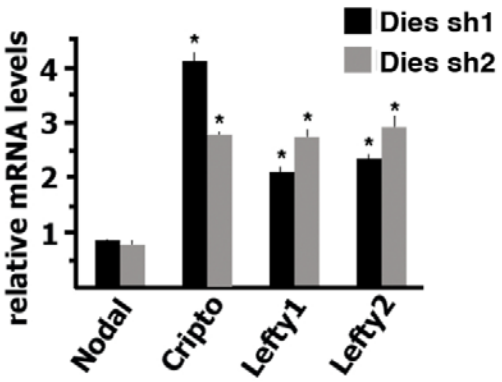
ES cells were transfected with a shRNA targeting Id1 and a shRNA targeting Id3. 24 clones were screened and two of them (#6 and # 7) showed reduced mRNA expression of Id1 and Id3 as shown by Real Time PCR analysis. In these clones mRNA expression levels of Oct4 and Nanog were analyzed. The values are reported as fold-changes compared to cells transfected with NS shRNA.

Dies1 knock-down induces the up-regulation of Nodal/Activin targets

Dies1 knock-down affects BMP4 signaling in ES cells, reducing the amount of phosphorylated SMAD 1/5/8 and their transcriptional targets such as Inhibitor of Differentiation (Ids) 1-3. As mentioned before, BMP4 belongs to TGF-β superfamily which includes also Nodal and Activin. Nodal and Activin bind to type I receptors Alk4 and 7 which phosphorylate and in turn, activate SMAD 2-3. Active SMAD 2-3 form complexes with SMAD4 and translocate to the nucleus. Nodal/Activin and BMP4 share the common effector SMAD4 that is essential for SMAD 1/5/8 and SMAD 2/3 transcriptional activity. Moreover, inhibitory SMADs (SMAD 6-7) are able to block SMADs signalling cascade dependent on both BMP4 and Nodal/Activin. Thus, BMP4 and Nodal/Activin pathways are tightly related each other. Given Dies1 knock-down effect on BMP4 signalling, we investigated Dies1 involvement in Nodal/Activin signalling. As shown in Fig. 29, Dies1 knock-down increased the mRNA levels of the Nodal/Activin targets such as Cripto, and Lefty 1-2. (Fig. 29) On the contrary, Nodal expression, that is regulated itself by Nodal, is not affected by Dies1 knock-down. An interesting hypothesis was that the alteration of Nodal and Activin pathway could be responsible for the effect of Dies1 knock-down on ES cell differentiation. As mentioned before, Nodal/Activin pathway controls Nanog expression in human ES cells and maintains mouse ES cell undifferentiated when E-cadherin is suppressed by RNA interference (Vallier, Mendjan et al. 2009) (Soncin, Mohamet et al. 2009). Thus, increasing evidence suggests that Nodal and Activin are important players in maintenance of pluripotency. To investigate whether Nodal/Activin are involved in the maintenance of the undifferentiated markers due to Dies1 suppression, we inhibited Nodal/Activin activity with the chemical compound SB-431542. SB-431542 specifically inhibits type I receptor activity dependent on Nodal and Activin. We hypothesized that inhibition of Nodal/Activin pathway may rescue Dies1 effect, restoring proper Alkaline Phosphatase (AP) expression when cells were grown in the absence of LIF. The SB-431542 inhibitor was added to normal ES cell growth medium for 16 hours and then to the medium without LIF for 2 days. The cells were cultured without LIF for 6 days and then analysed for AP expression. As shown in Fig. 30, the treatment with SB-431542 did not significantly affect the AP staining in NS KD cells. On the other hand, the treatment with SB431542 strongly decreased the number of Dies1 KD colonies positive to AP, thus restoring proper differentiation in the absence of LIF. To confirm this result we investigated the expression of markers of undifferentiated state in NS KD cells and in Dies1 KD cells treated with SB-431542. The treatment with SB-

431542 did not modify Oct4 and Nanog expression in NS KD cells, whereas it strongly decreased their expression levels in Dies1 KD cells at day 6 without LIF. As expected, when Dies1 KD cells were treated only with DMSO, the expression level of Oct4 and Nanog is much stronger than in controls, whereas the inhibition of Nodal and Activin restored proper expression of undifferentiated markers. This result was in agreement with that of AP staining. Taken together, these results demonstrated that Nodal and Activin activity were required for the maintenance of undifferentiated markers due to Dies1 knock-down. In fact, the inhibition of Nodal/Activin pathway rescued Dies1 knock-down effect, restoring differentiation in the absence of LIF. Thus, our data shed light on the fine regulation of Nodal/Activin and BMP4 activity in ES cells that appears to be crucial for the maintenance of pluripotency.

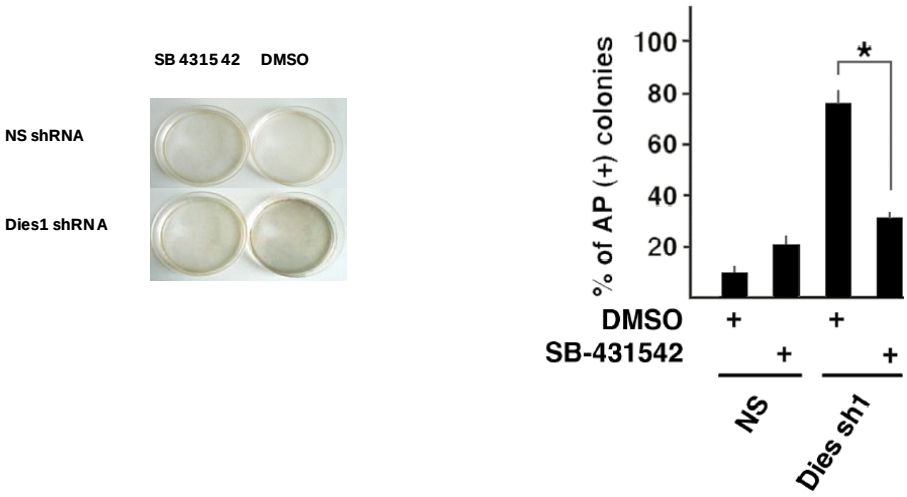
FIGURE 29: DIES1 SUPPRESSION INDUCES THE UP-REGULATION OF NODAL/ACTIVIN TARGETS



ES cells were transfected with NS shRNA or Dies1 shRNAs. Real Time PCR analysis was performed to detect mRNA expression levels of Nodal, Cripto, Lefty1 and Lefty2. Values are reported as fold-changes compared to cells transfected with NS shRNA.
*, $p<0,01$

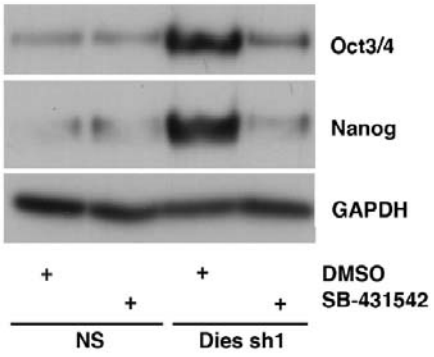
FIGURE 30: THE TREATMENT WITH NODAL/ACTIVIN PATHWAY INHIBITOR SB-431542 RESCUES DIES1 KNOCK-DOWN EFFECT

A) Treatment with SB-431542 restores proper Alkaline Phosphatase expression in Dies1 knock-down cultured in the absence of LIF



ES cells transfected with NS shRNA or Dies1shRNA were cultured in the absence of LIF with DMSO or Nodal/Activin inhibitor SB431542. At day 6 Alkaline Phosphatase (AP) was stained and the number of AP positive colonies were counted.
*, p<0,01

B) Treatment with SB-431542 restores proper Oct4 and Nanog expression in Dies1 knock-down cultured in the absence of LIF



ES cells transfected with NS shRNA or Dies1 shRNA were cultured in the absence of LIF with DMSO or Nodal/Activin inhibitor SB431542. Western blot analysis was performed to detect Oct4 and Nanog expression at day 6 in the absence of LIF.

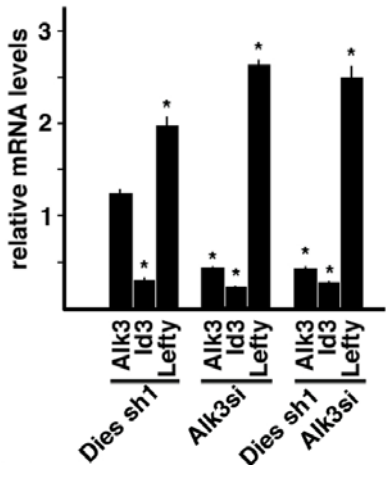
Dies1 is a component of the BMP4 signaling pathway

Dies1 suppression down-regulates BMP4 signaling and up-regulates Nodal/Activin pathway. This result is not surprising, given the balance occurring between BMP4 and Nodal/Activin. Dies1 knock-down cells displayed reduced levels of the BMP4 targets Id 1-3, in a SMAD dependent manner. In addition the response to BMP4 treatment was impaired in Dies1 knock-down cells, suggesting a direct involvement of Dies1 in BMP4 signaling.

To confirm a direct involvement of Dies1 in BMP4 signaling pathway, we tested Dies1 effect when BMP4 signaling was affected. To this aim we down-regulated the only BMP-type I receptor expressed in ES cells, Alk3 (Mishina, Suzuki et al. 1995).

We used siRNA to down-regulate Alk3 mRNA levels in ES cells (Alk3 KD cells). The effect of Alk3 knock-down is very similar to Dies1 knock-down effect. In fact Alk3 KD cells showed reduced levels of Id1 and 3 mRNA levels and increased mRNA levels of the Nodal/Activin target as Lefty1. Dies1 suppression in Alk3 KD cells did not exert any significant effect compared to Dies1 and Alk3 knock-down alone, thus suggesting that Dies1 and Alk3 are component of the same BMP4-dependent signalling pathway (Fig. 31)

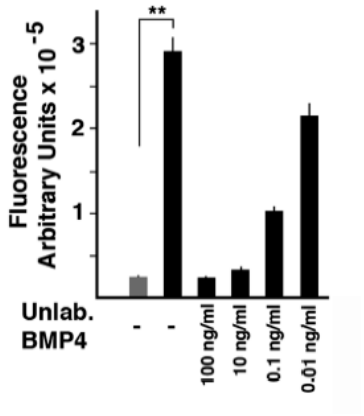
FIGURE 31: DIES1 AND ALK3 KNOCK-DOWN RESULTS IN THE SAME EFFECT ON THE REGULATION OF TGF-β SUPERFAMILY SIGNALING



ES cells were transfected with siRNA targeting Alk3 and siRNA control. Real time PCR was performed to detect mRNA expression levels of Id1, Id3 and Lefty 1. The same experiment was performed in Dies1 knock-down cells. Dies1 knock-down cells were transfected with siRNA control or with siRNA targeting Alk3. All the values are reported as fold-changes compared to cells transfected with siRNA control.
*, $p<0,01$

To address direct interaction between BMP4 and Dies1 we performed an ELISA. We incubated 96 well plates coated with StrepM antibody with medium harvested by HEK 293 transfected with Strep-tagged Dies1 extra cellular domain (ECD). As shown before, in fact, Dies1-ECD is secreted in the medium and could be harvested in the conditioned medium. We refreshed 4 times with new medium and then added BMP4 fluorescently marked with europium (Eu-BMP4) As control, we pre incubated some wells with different concentrations of non fluorescent BMP4 to perform a competition assay. As shown in Fig. 32, BMP4 binds to Dies1-ECD, increasing fluorescence signal up to 6 fold compared to control (wells incubated with medium harvested by mock transfected HEK 293). The pre-incubation with BMP4 significantly decreased fluorescent signal in a dose-dependent manner.

FIGURE 32: BMP4 BINDS TO DIES1 IN VITRO

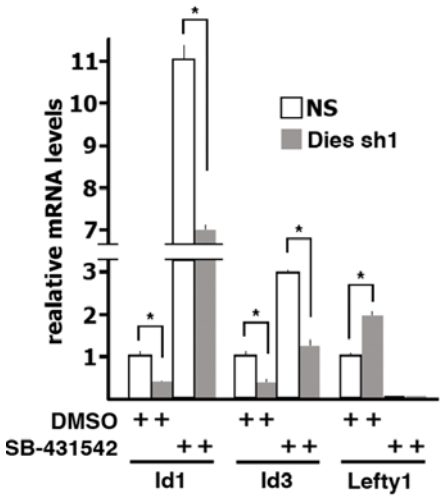


Conditioned medium harvested from HEK 293 mock-transfected (grey) or transfected with Strep-tagged Dies1-ECD (black) was incubated on 96 well plate coated with StrepM antibody. Some wells were pre-incubated with BMP4 .at reported concentrations Fluorescence was detected with Envision Elisa Reader.
**, p<0,001

Dies1 effect on BMP4 is independent on Nodal/Activin

Our results shed light on a direct involvement of Dies1 in BMP4 signaling. However, we could not exclude that the effect on BMP4 signaling was also a consequence of the up-regulation of Nodal/Activin signalling. To test this hypothesis, we treated mouse ES cells with Nodal/Activin inhibitor, SB-431543. This inhibitor specifically blocks the activity of type I receptors dependent on TGF-β1, Nodal and Activin but not dependent on BMP4. As expected, the treatment of mouse ES cells with SB431542 resulted in the block of SMAD 2/3 activity. In fact, it resulted in decreasing the expression of Nodal/Actvin targets as Lefty1, to more than 95% in NS KD cells as well as in Dies1 KD cells. In addition, SB-431542 treatment induced a strong up-regulation of BMP4 targets such as Id1 and Id3 (Fig. 33), confirming the hypothesis of a strong balance between BMP4 and Nodal/Activin. Of great concern is that Id1 and Id3 were increased upon SB-431542 treatment in NS KD cell and in Dies1 KD cells but the increase of Ids expression in response to Nodal/Activin inhibitor is less in Dies1 KD than in NS KD cells. This indicated that BMP4 signalling is affected by Dies1 knock-down even when Nodal/Activin signalling is off, demonstrating that Dies1 effect on BMP4 signaling was independent on Nodal/Activin activity.

FIGURE 33: DIES1 KNOCK-DOWN EFFECT ON BMP4 SIGNALING IS INDEPENDENT ON NODAL/ACTIVIN

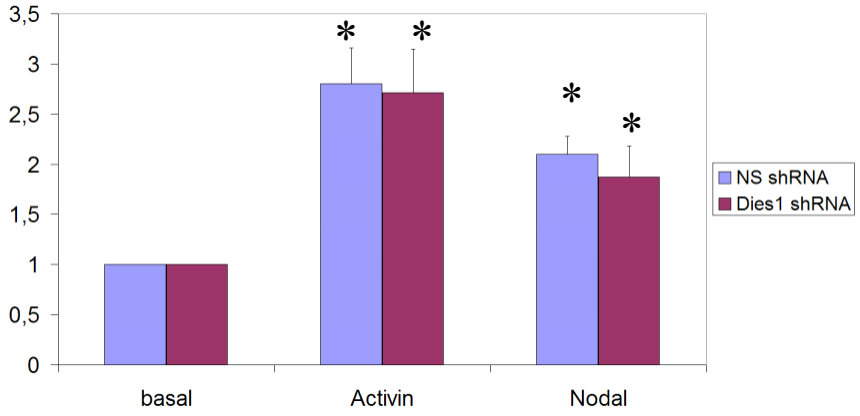


ES cells, transfected with NS shRNA or Dies1 shRNA were treated with DMSO or with Nodal/Activin pathway inhibitor SB-431542. Real Time PCR was performed to detect the mRNA levels of Id1, Id3 (BMP4 targets) and Lefty1 (Nodal/Activin target). The values are reported as fold-changes compared to levels in NS shRNA cells treated with DMSO.
*, $p<0,01$

Dies1 effect on Nodal and Activin is indirect

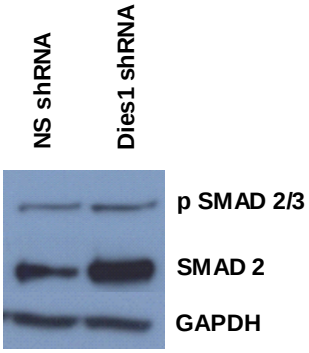
Dies1 knock-down up-regulates Nodal/Activin targets in ES cells. This could be due to a direct effect of Dies1 on Nodal/Activin or could be consequence of the effect on BMP4 signaling. To further explore Dies1 knock-down effect on Nodal/Activin signaling we transfected ES cells with a construct (CAGA-luc) containing the sequence encoding for luciferase driven by the sequence (CAGA) recognized by SMAD 2/3. The treatment with Nodal or Activin increased luciferase activity to 2-3 folds in NS KD cells. The suppression of Dies1 by RNA interference did not result in any significant difference in the response to Nodal and Activin compared to NS KD cells (Fig. 34) In addition, Dies1 KD cells showed just a weak, if significant, increase in phosphorylation of SMAD 2/3 even if the amount of total SMAD 2 is much higher than in controls (Fig. 35). The up-regulation of SMAD 2/3 total levels in Dies1 KD cells has to be further explored. Taken together, these results suggested that Dies1 effect on Nodal/Activin signalling is indirect. The up-regulation of Nodal/Activin targets in ES cells due to Dies1 knock-down could be consequence of the impairment of BMP4. One hypothesis is that the decreased amount of phosphorylation SMAD 1/5/8, could make SMAD 4 more available to SMAD 2/3, promoting SMAD 2/3 transcriptional activity.

FIGURE 34: Dies1 knock-down does not exert any effect on Nodal/Activin response in luciferase assay



ES cells were transfected with a vector which contains luciferase reporter gene. Its expression is driven by the SMAD2/3 responsive sequence. These cells were transfected also with NS siRNA or Dies1 siRNA. 24 hours after transfection, the growth medium was supplemented with Nodal or Activin. The values are reported as relative luciferase activity (normalized with Renilla) compared to untreated NS KD cells.
*, $p<0,01$

FIGURE 35: Dies1 knock-down does not affect SMAD 2/3 phosphorylation even if the amount of total SMAD2 is increased



ES were transfected with NS shRNA or with Dies1 shRNA. Western blot analysis was performed to detect the expression levels of total Smad2 and phosphorylated Smad 2/3. Gapdh was used to normalize the amount of protein in the extracts.

DISCUSSION

Molecular players involved in mouse ES cell self-renewal and differentiation are still largely unknown. The identification of the molecular machinery which controls these processes will contribute to better understand ES cell biology and to improve their use for regenerative medicine. Here we report the function of a previously uncharacterized gene, we named as Dies1 (Differentiation of Embryonic Stem cell 1), given its effect on ES cell differentiation. In our laboratory, the screening of a large collection of shRNA constructs led to the identification of 50 genes whose knock-down strongly affects ES cell differentiation. Among these ones, we focused on Dies1. We confirmed the results of the screening with different approaches, demonstrating that ES cells where Dies1 is suppressed by RNA interference are unable to properly differentiate into neurons, astrocytes and cardiomyocytes. In conditions promoting differentiation, in fact, Dies1 knock-down cells maintain the expression of undifferentiated markers such as Oct4, Nanog and Alkaline Phosphatase. In addition, Dies1 knock-down cells are, at least in part, independent from LIF, maintaining undifferentiated markers when they are cultured in the absence of LIF. Dies1 suppression exerts its effect on ES cell differentiation as *in vivo* as *in vitro*, giving rise to teratomas smaller than controls when Dies1 knock-down cells are injected into nude mice. Thus, Dies1 seems to be required for proper ES cell developmental ability. Dies1 is also expressed *in vivo* as shown by the staining of mouse blastocyst with Dies1 antibody, supporting the hypothesis that Dies1 plays an important role in ES cells. Immunostaining in blastocyst and in ES cell indicates that Dies1 is expressed on cell surface, confirming bioinformatics prediction based on the sequence. These *in silico* analysis suggest that Dies1 contains a trans-membrane domain and an Ig-like domain in the putative extra-cellular part of the protein. Ig-like domains are involved in protein-protein interaction and can be found in proteins which play a role in adhesion such as cadherins. Recently, E-cadherin suppression was shown to maintain ES cell undifferentiated in the absence of LIF, exerting the same effect of Dies1 suppression (Soncin, Mohamet et al. 2009)7. However, Ig-like domains can be found also in many receptors such as EGFR and PDGFR. As many others receptors, Dies1 is glycosylated in N-asparagine residues and is phosphorylated in tyrosine. The function of Dies1 as membrane receptor is further supported by the observation that Dies1 extra-cellular domain (ECD) works as Dies1 dominant negative form, resembling Dies1 suppression effect. ES cells transfected with Dies1-ECD alone maintain the expression of undifferentiated markers such as Oct4 and Alkaline Phosphatase when they are grown in the absence of LIF. Thus, the suppression of Dies1 or the expression of its extra-cellular domain alone, confers LIF independence to ES cells.

However, the effect of Dies1-ECD is weaker than Dies1 suppression. Indeed ES cells expressing Dies1-ECD are able to properly differentiate into neurons, astrocytes and cardiomyocytes. Thus, other mechanisms may overcome the dominant negative effect of Dies1 ECD in particular contests. Of great concern is that Dies1 ECD is not able to work as soluble protein instead of LIF, suggesting that Dies1 ECD works inside the cell. One explanation could be that the expression of Dies1-ECD impairs the formation of a complex, whose assembly is necessary for Dies1 proper function. All these results shed light on a possible involvement of Dies1 in the signalling pathways controlling ES cell pluripotency. ES cells in fact, are strictly dependent on proper transduction of extrinsic signals for maintaining self-renewal and pluripotency. Thus, we explored the effect of Dies1 suppression on the main signalling pathways governing ES cell biology: LIF and BMP4. LIF pathway seems to be unaffected by Dies1 knock-down, whereas the transcriptional targets of BMP4 such as Inhibitor of Differentiation (Id) genes are down-regulated in Dies1 knock-down cells. This down-regulation is Smad-dependent as the amount of phosphorylated and thus, active Smad 1/5/8 complex is decreased in Dies1 knock-down cells. The effect on BMP4 signaling pathway is consequence of a decreased response to the BMP4, as suggested by luciferase assays. Among the four BMPs-type I receptors which have been identified, Alk3 is the only one expressed in mouse ES cells and in the blastocyst. Alk3 knock-out mice exhibit reduced cell proliferation in the epiblast, suggesting that Alk3 plays a crucial role in ES cell. One hypothesis to explain Dies1 knock-down effect on BMP4 signaling is that Dies1 works as Alk3 co-receptor. Some co-receptors acting within the TGFβ/activin/nodal signal transduction pathways have been identified so far, whereas only the member of repulsive guidance molecule (RGM) Dragon has been identified as interactor of BMPs-type I receptors. In addition, Dragon is able to bind to BMP proteins (Babitt, Zhang et al. 2005). Given these data, it was logical to ask whether Dies1 sustains BMP4 signaling, directly interacting with BMP4. ELISA suggests that BMP4 binds to Dies1 *in vitro,* supporting the hypothesis that Dies1 may function as Alk3 co-receptors. In addition, it will be interesting to investigate the functional interaction between BMPs and Dies1 in other biological contexts. For example, BMPs play an important role in the bone, where Dies1 is strongly expressed. However, the identification of Dies1 as component of BMP4 signaling pathway, does not explain why Dies1 knock-down cells are LIF independent and maintain the expression of undifferentiated markers in conditions promoting differentiation. BMP4 in fact, exerts its action in combination with LIF in maintaining ES cell undifferentiated. Thus, we focused our attention on the balance of the signaling within the members of TGF-β superfamily, whose main components are

BMPs and TGF-βs/Nodal/Activin. These two members of the TGF-β superfamily share a common mediator, SMAD4 that is necessary for signal transduction. Both SMAD 1/5/8 which are activated by BMPs and SMAD 2/3 which are activated by TGF-βs, Nodal and Activin require association with SMAD4 to translocate into the nucleus and to exert their transcriptional activity. In addition, BMPs and TGF-βs/Nodal/Activin share common mechanisms of regulation, such as the inhibitory Smad 6 and Smad 7, which block both signalling pathways. We found that in Dies1 knock-down cells Nodal/Activin targets such as Cripto, Lefty1 and Lefty2 are up-regualated. This was not surprisingly, given the tight regulation between Nodal/Activin and BMP4. Given recent result supporting the crucial role for Nodal and Activin in the control of pluripotency (Soncin, Mohamet et al. 2009; Vallier, Mendjan et al. 2009), we hypothesized that Nodal/Activin signalling is crucial for Dies1 effect. This hypothesis was confirmed by treating Dies1 knock-down cells with the chemical compound SB-431542 which specifically inhibits Nodal/Activin pathway. The treatment with SB-431542 restores proper differentiation ability of Dies1 knock-down cells in the absence of LIF. Thus, the inhibition of Nodal/Activin pathway is able to rescue Dies1 knock-down effect. However, the effect of Dies1 suppression on Nodal and Activin signalling pathway seems to be indirect, as the level of phosphorylated and thus active Smad2/3 in Dies1 knock-down cells is identical to controls. In addition, Dies1 suppression does not exert any effect on Nodal/Activin response in luciferase assays. Our hypothesis is that the up-regulation of Nodal/Activin targets could be the consequence of the down-regulation of BMP4 signaling pathway. One possibility is that the decreased amount of active SMAD 1/5/8 in Dies1 knock-down makes SMAD4 more available for SMAD 2/3 complex. Such a regulation between Nodal/Activin and BMP4 is supported also by the observation that the down-regulation of BMP type I receptor Alk3 exerts the same effect of Dies1 knock-down on the transcription of the Nodal/Activin targets. Taken together our results suggest that the balance between BMP4 and Nodal/Activin exerts a crucial role in regulating ES cell pluripotency and differentiation. Thus, the identification of players regulating these signalling cascades will be very useful for the use of ES cell in therapy.

In conclusion, we have identified Dies1 as novel component of BMP4 signaling pathway which is involved in ES cell pluripotency and differentiation. The generation of animal models, as knock-out mice, will be crucial to elucidate the role of Dies1 *in vivo* in embryo development. Moreover, these models will be useful to further investigate the functional interaction between Dies1 and BMP4 in developmental processes and in adult organs as well as to shed light on uncovered Dies1 functions.

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