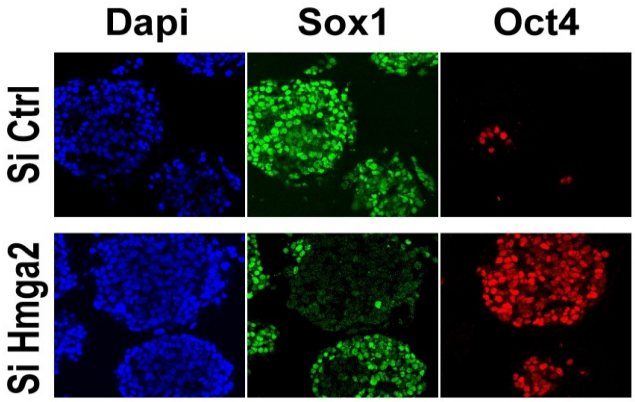
UNIVERSITA' DI NAPOLI FEDERICO II

**DOTTORATO DI RICERCA IN BIOCHIMICA
E BIOLOGIA CELLULARE E MOLECOLARE
XXVII CICLO**

Angelica Navarra

TRANSCRIPTIONAL MECHANISMS GOVERNING THE EXIT OF MOUSE EMBRYONIC STEM CELLS (mESCs) FROM THE PLURIPOTENCY GROUND STATE



Academic Year 2013/2014

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Riassunto

Le cellule staminali embrionali (ESCs) sono cellule non specializzate capaci di auto-rigenerasi e differenziare in tutti i tipi cellulari di un organismo, mimando gli eventi che avvengono in *vivo* durante le prime fasi dello sviluppo. Per queste caratteristiche, una profonda conoscenza dei meccanismi che governano il destino delle ESC è fondamentale sia per la ricerca di base che per la terapia cellulare. Negli ultimi anni sono stati fatti enormi progressi nella comprensione della pluripotenza delle ESC, mentre i meccanismi che governano il destino delle ESC ed, in particolare, i meccanismi che governano l'uscita di queste cellule dallo stato basale di pluripotenza e le prime fasi del differenziamento, non sono stati ancora ben capiti. In questo contesto il nostro gruppo di ricerca ha sviluppato un approccio sistematico basato sullo screening di una libreria di shRNA per identificare fattori che governano il destino delle ESC. Tra i geni identificati abbiamo trovato il gene HMGA2, una proteina non istonica associta alla cromatina, ampiamente espresso durante lo sviluppo e che svolge un ruolo importante nei processi di adesione/differenziamento cellulare. Abbiamo trovato che il knockdown di HMGA2 promuove il mantenimento di uno stato di pluripotenza. Infatti, in condizioni che promuovono il differenziamento, il knockdown di HMGA2 mantiene l'espressione dei marcatori di staminalità Oct3/4 e Nanog. Inoltre, abbiamo trovato che l'espressione dei marcatori di staminalità è accompagnata da una riduzione del marcatore del neuroectoderma Sox1. Un'analisi dettagliata del fenotipo indotto dalla soppressione di HMGA2, ha mostrato che il blocco del differenziamento avviene nelle prime fasi, ovvero nella transizione delle ESCs a cellule staminali dell'epiblasto (EpiSCs). Gli stessi effetti, accompagnati da un fenotipo molto più evidente, sono stati osservati in seguito all'induzione del differenziamento di cellule staminali pluripotenti indotte (iPS) knock out per HMGA2. Successivamente, abbiamo trovato che l'espressione di HMGA2 è regolata da un fattore trascrizionale (FT) chiave Otx2, che da solo o in combinazione con Oct4 guida l'attivazione di enhancer precoci durante l'uscita delle ESC dallo stato basale verso la

la formazione dell'epiblasto. Inoltre, abbiamo osservato che HMGA2 coopera con Otx2 nella formazione di enhanceosomi. In conclusione, i risulati ottenuti durante la mia tesi di dottorato, dimostrano che HMGA2 agisce prendendo parte ai meccanismi di regolazione che guidano l'uscita delle ESCs dallo stato basale di pluripotenza.

Summary

Embryonic stem cells (ESCs) are non-specialized cells able to self-renew and to differentiate *in vitro* giving rise to all cell types of an organism, mimicking the events that take place *in vivo* during the early stages of the development. For these characteristics, a deep knowledge of the mechanisms that govern ESC fate is fundamental for both basic research and cell replacement therapy. In the recent years, enormous progresses have been made in the understanding of ESC pluripotency, while the mechanisms governing the ESC differentiation and, in particular, the mechanisms governing the exit from the pluripotent state and the first steps of differentiation are still not definitively understood. In this context, our group has developed a systematic approach based on the screening of a shRNA library to identify factors governing ESC fate. Among the identified genes, we have found the high mobility group AT-hook 2 (HMGA2), a non-histone chromatin factor that is widely expressed during embryogenesis and is known to have important roles in development and cell adhesion/differentiation processes. We have found that the knockdown of HMGA2 promotes the maintenance of pluripotent state. Indeed, in conditions promoting differentiation, HMGA2 knock-down cells maintain the expression of stemness markers Oct3/4, Nanog. Interestingly, we have found that the expression of stemness markers is accompanied by a decrease of neuro-ectodermal marker Sox1. A more detailed analysis of the phenotype induced by HMGA2 suppression has shown that the block of differentiation occurs during first steps, i.e. the transition from ESCs to epiblast stem cells (EpiSCs). The same effects, accompanied by a more dramatic phenotype, were observed upon differentiation of induced pluripotent stem cells (iPS cells) knock-out for HMGA2. We have found that HMGA2 expression is regulated by a key transcription factor Otx2 that alone or in combination with Oct4 drives early enhancer activation during the exit from ground state of ESCs to EpiSCs. Indeed, we have shown that HMGA2 cooperates with Otx2 in enhanceosome formation. Finally, the results obtained during my

doctoral thesis have demonstrated that HMGA2 acts taking part to the regulatory mechanisms that guide the exit of ESCs from ground state.

Index

List of Figures

Pag.

1. Introduction

Embryonic Stem Cells (ESCs) are primitive cells that have the remarkable capacity to self-renew and differentiate *in vitro* in all specialized cell types of an organism, thus mimicking the events that take place *in vivo* during the early stages of development. They were first derived from the inner cell mass (ICM) of blastocyst-stage embryos in 1981 (Evans and Kaufman 1981). The blastocyst is a vesicular structure comprising two cell types: the inner cell mass (ICM), a cluster of approximately 20 cells, adhering to one side of the vescicle and of an epithelial outer layer, the trophectoderm (TE), enclosing a fluid filled space. After the embryo has implanted in the uterus, the trophectoderm overlying the ICM proliferates (Rossant and Cross 2001) and grows into a thick column of extraembryonic ectoderm (ExE), which extends into the blastocoel and carries a compact epiblast at its distal pole. The embryonic epiblast is composed of a single layer of pseudostratified epithelia called primitive ectoderm, which gives rise to all three embryonic germ layers (ectoderm, mesoderm and definitive endoderm) and primordial germ cells (Pfister, Steiner et al. 2007). All these steps of embryogenesis can be recapitulated by ESCs because they express different sets of genes that, changing their expression level, drive the events of embryogenesis. The ESCs exhibit two remarkable features in culture: i) under appropriate conditions, they can be propagated indefinitely as a stable self-renewing population by retaining stem-cell identity; this characteristic allows ESCs to be cultured over extended periods; ii) ESCs under appropriate conditions and after removal of factors that maintain ESCs in undifferentiated state, can generate progeny consisting of derivatives of the three embryonic germ layers: ectoderm, endoderm and mesoderm, (Keller 1995) (Figure1).

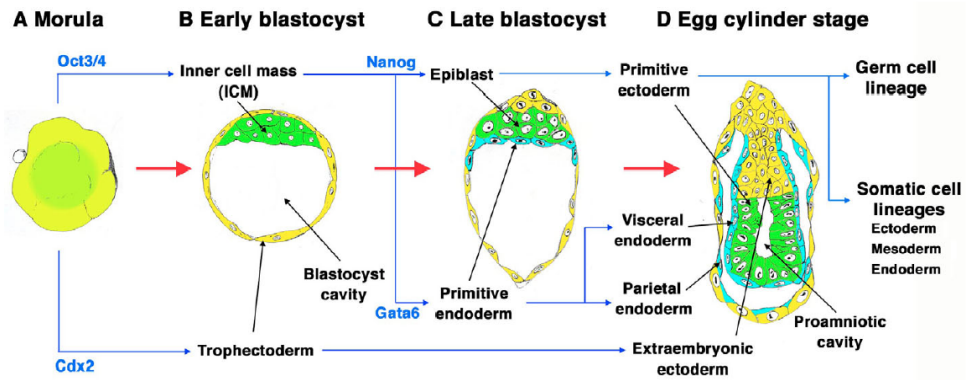


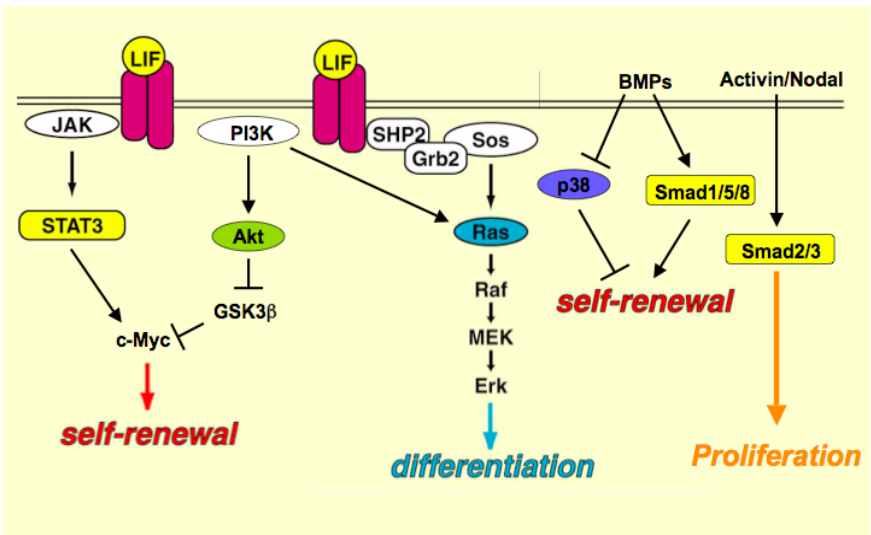
Figure1: Embryo development: Pluripotent cells are stained in green. The inner part of the morula (A) forms the inner cell mass (ICM) of the blastocyst (B). The blastocyst is composed by two different cell types: the Inner cell mass (ICM) and the trophoectoderm. The ICM contains pluripotent cells which 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 From: (Niwa 2007)

It seems that there is no intrinsic limitation to the ability of ESCs to differentiate in vitro and ESC progeny can express appropriate markers and functional characteristic of specific cell sub-types, such as neurons or cardiomyocytes (Maltsev, Rohwedel et al. 1993; Strubing, Ahnert-Hilger et al. 1995; Lee, Hart et al. 2004). For these characteristics, nowadays, the ESCs are widely used for different purposes including gene targeting, cell therapy, tissue repair, organ generation and so on. They represent a major advance in biology and experimental medicine, so the understanding of the mechanisms underlying ESC differentiation is necessary to provide new ESC-based developmental models and for clinical applications of cell therapy. The ESC stemness maintenance is supported by transcriptional and post-transcriptional mechanisms. The transcriptional mechanisms include both the responsiveness to external stimuli that activate the main pathways involved in stemness maintenance (Xiao et al., 2006; Ying et al., 2003), and the regulation of the expression levels of the "stemness transcription factors" such as Oct3/4, Sox2, Nanog, Stat3 and Klfs (Yuan et al., 1995; Nichols et al., 1998). On the other hand, post transcriptional regulation of gene expression by microRNA is also important in the maintenance of stemness.

1.1 LIF pathway is the main signal that modulate ESCs state

During self-renewal, ESC pluripotency is maintained through the balance between prevention of differentiation and promotion of proliferation. LIF (Leukemia inhibitory factor), a member of the IL-6 family of cytokines, is a key factor that sustains pluripotency and prevents differentiation (Smith et al. 1988). LIF stimulates ESCs through the receptor gp130, which works as heterodimer together with LIF-Receptor. This activated complex signals the induction of Janus-associated tyrosine kinase (JAK) and the signal transducer and activation of transcription (STAT). Activated STAT3 translocates into the nucleus and induces the expression of important genes involved in the stemness and pluripotency, such as the Kruppel like factors (Klfs),

Myc and Sall4 (Cartwright et al., 2005; Niwa et al., 2009). Therefore, STAT3 activation represents a crucial point in the maintenance of ESC stemness and its activation is sufficient to prevent ESC differentiation in presence of serum (Niwa et al., 1998). In addition to STAT3 activation, LIF also induces the phosphorylation of extracellular signal-regulated protein kinases, ERK1 and ERK2 and increases the mitogen-activated protein kinase (MAPK), which promotes differentiation (Matsuda, Nakamura et al. 1999). Proliferation, survival and maintenance of pluripotency in ESCs, are sustained also by the Phosphoinositide 3-kinase (PI3K)/Akt pathway (Takahashi, Murakami et al. 2005). LIF/PI3K pathways may act by activating a protein kinase B/glucocorticoid-inducible kinase SGK which inactivates glycogen synthase kinase (GSK)-3 *in vitro.* Inhibition of GSK-3 activity is known to facilitate self-renewal of mouse ES cells. Recent paper showed that the inhibition of GSK3 and MEK (mitogen-activated protein kinase/ERK kinase), through small-molecules inhibitors (2i), is critical to establish and sustain ES cells (Wray et al., 2011) (Figure 1.1). Similarly, the Nodal/Activin pathway is crucial in ESCs. These proteins are present in various tissues and have a broad range of activities including the proliferation and differentiation of ESCs (Kunihiro Tsuchida et al.,2009) but, in this process, their role is still not clear. Some papers suggested a possible function of Nodal in the maintenance of ESC phenotype on the basis of the finding that Nodal-deficient mice show embryonic lethality due to a defective maturation of the ICM into epiblast with very low levels of Oct3/4 expression (Mesnard et al., 2006). Indeed, it has been demonstrated that Nodal/Activin signalling directly controls the Oct4 expression in ESCs through Smad2 (Lee et al., 2011).



Figue 1.1: Pathways required for maintaining pluripotency of ESCs. LIF signaling activates JAK-STAT3 to induce the expression of target genes crucial for pluripotency, such as c-myc. LIF also induces MAP kinase activation, that promotes differentiation. Activin/Nodal has been shown to contribute to ESC proliferation but not to pluripotency (Taken from Ohtsuka 2008 and modified).

1.2 Transcription factors

Self-renewal and pluripotency are maintained by perfectly balancing the expression of many transcription factors that act in a complex network (Niwa, et al .,2005). This network includes, in addition to the already mentioned STAT3, the homeodomain transcription factor Oct4 (Niwa et al., 2000), the variant homeodomain transcription factor Nanog (Chambers et al., 2003), the high mobility group (HMG)–box transcription factor Sox2 (Avilion et al., 2003) and the Kruppel-like factors (Klfs) (Jiang et al., 2008; Parisi et al. 2008) that are expressed specifically in pluripotent cells.

Oct3/4: Oct3/4 is highly expressed in ESCs and its expression quickly decreases when differentiation occurs. Oct3/4 is a POU domain-containing transcription factor that binds to an octamer sequence and acts by preventing ESC differentiation (Nichols et al., 1998). Oct3/4 has been reported to directly prevent differentiation towards trophectoderm by interacting with Cdx2 (a prominent gene for trophectoderm differentiation), to form a repressor complex (Niwa, Toyooka et al. 2005). Indeed, Oct3/4 suppression in undifferentiated ESC leads to an unappropriated differentiation into trophectoderm. On the other hand, Oct3/4 overexpression induces the differentiation into primitive endoderm (Niwa et al., 1998; Niwa et al., 2000), thus indicating that a tight control of this gene is crucial to maintain ESC undifferentiated state.

Nanog: Nanog is a homeobox-containing transcription factor with an essential role in maintaining the pluripotent cells of the ICM in vivo and ESCs in vitro. It is highly expressed in pluripotent cells and absent in differentiated cells (Chambers et al., 2003). Chambers and co-authors showed that Nanog KO ESCs can survive and proliferate by maintaining at least in part their undifferentiated state but with a marked tendency to differentiate into primitive endoderm (Chambers et al. 2007). It has been proposed that Nanog regulates pluripotency working as a transcriptional repressor of differentiation genes such as GATA4 and GATA6 (Chambers et al., 2007). However, Nanog can also activate genes necessary for self-renewal such as Rex1 and Oct3/4 (Pan, Li et al. 2006). The function of Nanog might not be

restricted to prevent the differentation of ESCs into primitive endoderm but it can also block neuronal differentiation induced by the removal of LIF and BMPs from serum-free culture (Ying, Nichols et al. 2003). In addition, Nanog can also control mesoderm specification by repressing Brachyury, which encodes the mesoderm-specific T-box transcription factor T (Suzuki, Raya et al. 2006).

Sox2: Sox2 is a transcription factor that plays an important role in the maintenance of pluripotent transcription factor network. Sox2 is known to co-operate with Oct3/4 in activating Oct3/4 target genes (Yuan, Corbi et al. 1995). Some papers have demonstrated that there are ESC-specific enhancers that contain binding sites for Oct3/4 and Sox2, including those present in Nanog (Boyer, Lee et al. 2005; Kuroda, Tada et al. 2005; Rodda, Chew et al. 2005) and Rex1 genes, that, in turn, is also directly regulated by Nanog (Shi, Wang et al. 2006). Interestingly, both Oct3/4 and Sox2 genes possess enhancers that are activated by Oct3/4-Sox2 complex in a stem-cell-specific manner (Tomioka, Nishimoto et al. 2002; Chew, Loh et al. 2005; Okumura-Nakanishi, Saito et al. 2005). Thus, an alteration of Sox2 expression levels in ESCs induces uncontrolled differentiation with the same phenotype observed for Oct3/4 misregulation (Ivanova et al., 2006). Recent studies have enabled to integrate all these key transcription factors, in an intrinsic core-regulatory circuit that maintains ESCs in the pluripotent state in vitro (Figure 1.2).

Klfs: In recent years the crucial role of Kruppel-like factor (Klf) family in the ESCs has been studied. Klfs are zinc finger transcription factors that regulate many biological processes, including proliferation, differentiation, development and apoptosis (McConnell et al., 2007). It has been demonstrated that Klf2, Klf4 and Klf5 have an ESC-specific expression with high levels of transcripts in undifferentiated ESCs, that drastically decrease when differentiation occurs (Jiang et al., 2008; Parisi et al., 2008). Klfs have also a crucial role in reprogramming of somatic cells. Indeed, Klf4 is one of the four transcription factors used to induce somatic cell reprogramming (Takahashi and Yamanaka, 2006). Moreover, Nakagawa et al. have demonstrated that Klf2 and Klf5 could replace Klf4 in 'Yamanaka

cocktail' to induce reprogramming (Nakagawa et al., 2008). In Klfs family, Klf5 is emerged to have a key role during blastocyct development in vivo as well as in ESC stemness in vitro. In fact, Klf5 works by activating self-renewal promoting genes and, at the same time, by inhibiting the expression of differentiation-related genes (Parisi et al., 2010). Klf5 suppression induces an aberrant differentiation of ESCs also in undifferentiated culture conditions (Ema et al., 2008; Parisi et al., 2008). In addition, it has been shown that Klf5 ectopic expression is able to sustain stemness also in differentiating conditions (Parisi et al., 2008; Ema et al., 2008), thus demonstrating the crucial role of Klf5 in ESCs.

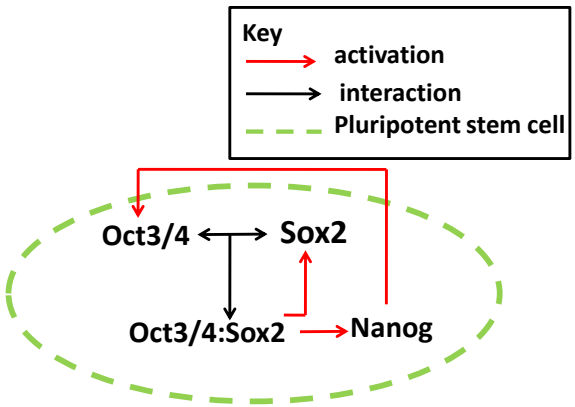


Figure 1.2: A transcription factor network controlling ESC self-renewal and differentiation. Transcription factor network in pluripotent ESCs. Positive-feedback loops between Oct3/4, Sox2 and Nanog maintain their expression and promote continuous ESC self-renewal (Adapted by Niwa 2007).

1.3 Epiblast Stem Cells (EpiSCs)

Epiblast Stem Cells (EpiSCs) are pluripotent cells isolated from post-implantation embryos. EpiSCs recapitulate the defining properties of their in vivo tissue of origin (Baoet al., 2009; Brons et al., 2007; Guo et al., 2009; Tesar et al., 2007) and are capable to differentiate into cell types of all three embryonic germ layers as well as the germ lineage in vitro (Aoki et al., 2009; Hayashi and Surani, 2009; Tesar et al., 2007; Vallier et al., 2009). They can be expanded indefinitely in culture being maintained in an undifferentiated state by activin/Nodal and FGF signaling pathways which, when inhibited, rapidly induce EpiSCs differentiation into neurectodermal lineage (Brons et al., 2007; Guo et al., 2009; Tesar et al., 2007). The regulation of Activin/Nodal pathway is a distinguishing characteristic of the mouse ESC and EpiSCs states. Indeed Oct4, Nanog, and Sox2 are expressed at lower level in EpiSCs than in ESCs, while Fgf5 and Brachyury are exclusive markers of Epiblast state (Hanna et al., 2010; Lanner and Rossant 2010). Some papers showed that EpiSCs share defining features with human ESCs which are derived from human pre implantation embryos (Brons et all., 2007; Tesar et all., 2007), including gene expression profile and epigenetic status. Based on this observation it seems that EpiSCs and human ESCs are in a ‘’primed’’ state on the road to differentiation, while mouse ESCs are in a naïve, more primitive state (Nichols and Smith, 2009). However, Najm et al., and Tesar et al., demonstrated that mouse blastocysts can also give rise to EpiSCs. Utilizing modified derivation protocol, without exogenous growth factors such as LIF, FGF2 or activin, the authors derived ESC lines from blastocysts from non-permissive strains of mice. This suggested that the type of pluripotent cell lines that are most readily derived from blastocysts is a primed EpiSC-like cell line. Recently, numerous studies have revealed that the ESC state is maintained by a dynamic mechanism characterized by reversible differences in the sensitivity to self-renewal and susceptibility to differentiation of ES cells population. This behavior is named ‘’metastable condition’’ that ensure indefinite self-renewal and at the

same time predisposes ESCs to EpiSC differentiation . These works demonstrated that a crucial role in this mechanisms is played by Otx2, a transcription factor essential for multiple steps of brain development and neuronal differentiation (Simeone et al., 1992; Simeone et al., 2002; Simeone et al., 2011). Otx2 antagonizes ground state pluripotency and promotes commitment to differentiation. It is required for ESC transition into EpiSCs and, subsequentely, to stabilize the EpiSC state by suppressing the switch of mesendoderm to neural fate in cooperation with Bmp4 and Fgf2. However to govern precisely the differentiation towards a specific fate, it is important to understand the molecular mechanisms that regulate pluripotent cell self-renewal and differentiation. For this reason we performed a screening of a shRNA library to identify factors governing ESC fate. Among these factors we found HMGA2.

1.4 The High Mobility Group (HMG) factors

The high mobility group (HMG) proteins are abundant, heterogeneous, non-histone components of chromatin that lack a transcriptional activity *per se,* but act by orchestrating the assembly of transcription factors complexes, thus resulting in a positive or negative regulation of gene expression (Wolffe et al., 1994; Ashar et al., 2010). The members of the HMGA family of proteins, HMGA1 (HMGA1a and HMGA1b, HMGA1c) and HMGA2, contain three N-terminal "AT-hook" motifs, through which they bind preferentially to AT-rich sequences, and induce conformational changes to promote the recruitment of transcription factors to specific complexes. HMGA members are highly expressed during embryogenesis; their expression becomes more restricted as fetal development progresses with low or undetectable expression in adult (Zhou et al., 1995; Chiappetta et al., 1996) and becomes abundant in malignant cells in vitro and in vivo, where they have been extensively studied (Fusco and Fedele, 2007).
The high expression of HMGA proteins during embryogenesis suggests that they fulfill important roles in development. Indeed, the phenotypic characterization of mice knocked out (KO) for each of the

HMGA genes revealed that these proteins play crucial roles in development (Fusco and Fedele, 2007), indeed it has been recently reported that the HMGA1/HMGA2 double KO mice show embryonic lethality (Federico et al., 2014). However, the physiological role of HMGA factors in the early steps of development is still unknown as well as the transcription regulatory mechanisms they are involved in.

1.5 Mechanisms of action of HMGA proteins

HMGA protein group favors the formation of multi-subunit protein-DNA complexes by modifying chromatin structure. The mechanisms of action of HMGA proteins could be summarized in:
1. Macromolecular complexe formation in which HMGA directly bind to DNA on AT-rich sequences, modify its conformation and facilitate the binding of transcription factors on their own consensus sequences, as in the best studied case of human β-interferon gene (IFN-β) (Thanos and Maniastis, 1992).
2. Protein -protein interactions with transcription factors inducing changes in their DNA binding affinities; for example HMGA2, can interact with the transcriptional repressor p120E4F and this interaction results in disruption of p120E4F binding to the cAMP response element (CRE) site of the cyclin A gene promoter and subsequent activation of cyclin A gene transcription (Tessari et al., 2003).
3.Chromatin remodeling. In this context, the highly AT-rich sequences have high affinity for the nuclear matrix and organize genomic DNA into topologically distinct loop domains that are important in transcription (Saitoh and Laemmli, 1994). So, HMGA is involved in the dynamic changes of chromatin structure playing a dominant role in the regulation of gene transcription.

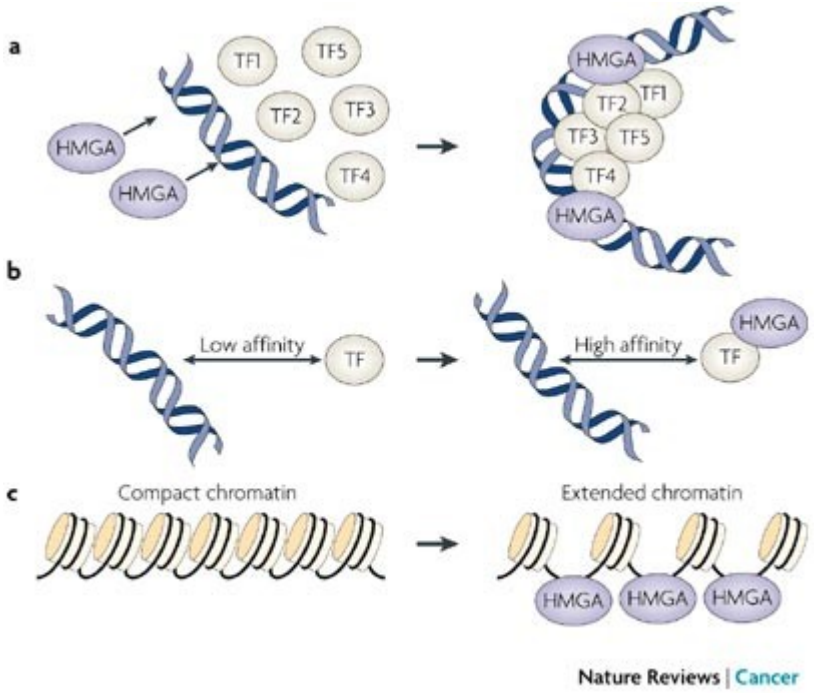


Figure 1.5: Mechanisms of action of HMGA proteins. a) HMGA proteins can modulate or assemble macromolecular complexes directly binding to the DNA. In doing so, HMGA proteins modify DNA conformation facilitating the binding of transcription factors (TF). **b)** HMGA proteins also influence gene expression through direct protein-protein interaction with TF inducing changes in their DNA-binding affinity. **c)** HMGA proteins have the ability to alter chromatin structure. (Fusco and Fedele, 2007).

1.6 Scientific hypothesis and aim of the work

The deep knowledge of the mechanisms that govern ESC fate is fundamental for both basic research and cell replacement therapy. As mentioned above, in this context some years ago we developed a systematic approach based on the screening of a shRNA library to identify factors governing ESC fate. HMGA2 was found among the genes whose suppression impairs ESC differentiation. These preliminary results, together with the consideration that HMGA2 is a chromatin remodeler and as such a regulator of gene expression, indicated that HMGA2 might have a crucial role in the control of ESC differentiation. Thus, the main aim of my thesis was the identification and characterization of HMGA2 role in governing ESC fate. To reach this aim I have addressed the following tasks:

- analysis of the expression profile of Hmga2 in undifferentiated ESCs and during differentiation;
- characterization of the phenotype due to the suppression (silencing) or the absence (iPS cells KO for HMGA2) of HMGA2 in the regulation of pluripotent stem cell fate;
- identification of the genes influenced by the chromatin remodeling activity of HMGA2;
- identification of the genes controlling HMGA2 expression.

2. Materials and Methods

2.1 ESC culture, monolayer differentiation and transfection

E14Tg2a (BayGenomics) mouse ESCs were maintained on feeder-free gelatine-coated plates in the following medium (ESC 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) and 10^3 U/ml leukemia inhibitory factor (LIF) (Euroclone).
For monolayer differentiation, ESCs were trypsinized into a single cells suspension, collected by centrifugation and resuspended in the following differentiation medium: Knockout Dulbecco's minimal essential medium supplemented with 10% Knockout Serum Replacement (both from Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 2 mM glutamine (Invitrogen), 100 U/mL penicillin/streptomycin (Invitrogen). Then, the cells were plated at low density ($3x10^3$ cells/cm^2) on gelatin-coated dishes and differentiation medium was changed on alternated days.
For transfection ESCs were plated at $6x10^4$ cells/cm^2 the day before the transfection. The transfection was performed by using Lipofectamine 2000 (Invitrogen) with siRNAs (Invitrogen) following manifacturer's instructions.

2.2 SFEBs differentiation and generation of EpiSCs

ESC differentiation into neuroectoderm was induced though SFEB formation. SFEBs were induced by placing $1x10^6$ ESCs in 100-mm Petri dishes in the followingdifferentiation medium: GMEM supplemented with 2 mM glutamine, 1 mM sodiumpyruvate, 1 × nonessential amino acids, 0.1 mM β-mercaptoethanol and 10% KSR. The formation of EpiSCs was induced adapting the methods of Hayashi et al., and Nakaki et al. In brief, ESCs were dissociated into a single-cell suspension with 0.05%Trypsin-EDTA at 37 °C for 5 min. Individual cells were then seeded in fibronectin-coated dishes at a density of $2.5x10^5$ cells/cm2 in ESC culture condition, and after 18 h

the medium was switched to the following EpiSC medium: 1 vol of DMEM/F12 combined with1 vol of Neurobasal medium, supplemented with 0.5% N2 supplement, 1% B27supplement, 1% KSR, 2 mM glutamine (Invitrogen), 20 ng/ml Activin A (R&D Systems),and 12 ng/ml bFGF (Invitrogen). Within 2 days in these conditions the cells undergo morphological transformation (including flattening, diminished cell-cell interactions and formation of cellular protrusions) and express epiblast markers.

2.3 RNA extraction, retro-transcription and real time PCR

Total RNA was extracted by using TRI-Reagent (Sigma). The first-strand cDNA was synthesized according to the manufacturer's instructions (M-MLV RT kit; New England Biolabs). Real Time RT-PCR was carried out with QuantStudio 7 Flex (Applied Biosystems) using Power SYBR Green PCR Master mix (Applied Biosystems). The housekeeping GAPDH mRNA was used as an internal standard for normalization, using $2^{-\Delta Ct}$ method. Gene specific primers used for amplification are:

Oct3/4-f: 5'-AACCTTCAGGAGATATGCAAATCG-3'
Oct3/4-r: 5'-TTCTCAATGCTAGTTCGCTTTCTCT-3'
Nanog-f: 5'- TCAGAAGGGCTCAGCACCA-3'
Nanog-r: 5'- GCGTTCACCAGATAGCCCTG-3'
Rex1-f: 5'- GCAGTTTCTTCTTGGGATTTCAG-3'
Rex1-r: 5'- CTAATGCCCACAGCGAT-3'
Dax1-f: 5'- AGATGGAGAAAGCGGTCGTA-3'
Dax1-r: 5'- AAGCCAGTATGGAGCAGAGG-3'
Klf4-f: 5'- ACTCACACAGGCGAGAAACCTTAC-3'
Klf4-r: 5'- TCAGTTCATCGGAGCGGG-3'
Gapdh-f: 5'-GTATGACTCCACTCACGGCAA-3'
Gapdh-r: 5'-TTCCCATTCTCGGCCTTG-3'

2.4 Antibodies and western Blot analysis

Undifferentiated and differentiated ESCs were lysed in a buffer containing 1 mM EDTA, 50 mM Tris-HCl (pH 7.5), 70mMNaCl, 1% Triton and protease inhibitor cocktail (Sigma), and analyzed bywestern blot. The following primary antibodies were used: mouse Oct3/4 (1 : 2000 Santa Cruz Biotechnology), rabbit Nanog (1 : 1000 Calbiochem-EMD Biosciences, La Jolla, CA, USA) mouse GAPDH (1 : 1000 Santa Cruz Biotechnology), goat Sox1 (1 : 100 Santa Cruz Biotechnology), rabbit Otx2 (1 : 500 Abcam) rabbit HMGA2 (1: 500 Cell Signaling) ; HMGA2 (1:500 antibody provided by A.Fusco et al.,). Antibody protein complexes were detected by HRP-conjugated antibodiesand ECL (both from Amersham Pharmacia, Milan, Italy).

2.5 Immunofluorescence

Undifferentiated or monolayer differentiated ESCs were fixed in 4% paraformaldehyde and permeabilized with 0.2% TX-100 in 10% FBS /1% BSA in 1X PBS for 15 min at room temperature. Thus the samples were incubated primary antibody. Following primary antibodies incubation, cells were incubated with appropriate secondary antibodies .

SFEBs were washed once with PBS 1× and fixed in 4% paraformaldehyde over night at 4°C with gentle rotation. The next two days dehydration was performed with increasing percentage of EtOH. The fourth day after one wash in Toluene for 40' at RT the samples were included in paraffin blocks. IF on slices was performed following a standard protocol. Briefly, the slides were washed two times in Xilene at RT for 3' and then rehydrated with a series of washes in EtOH at decreasing percentage. Then permeabilization was performed with 0.2% TX-100 for 5' followed by 2 washes in 1x PBS for 2'. Then, unmasking was performed in Citrate Buffer 1x. The non-specific block was performed by treating in 10% FBS/1% BSA/0.1% Tween 20/ 1x PBS for 2-3h at RT followed by primary antibodies

incubation, washes and secondary antibody hybridization. Nuclei were counterstained with Dapi (Calbiochem). The following primary antibodies were used: anti Oct3/4 (1:200, Santa Cruz), anti-βIII-tubulin (1:400, Santa Cruz) and anti-Sox1 (1:100, Santa Cruz). The secondary antibodies used are: anti-mouse Alexa 594 and anti-goat Alexa 488 (1:400, Molecular Probes). Images were captured with an inverted microscope (DMI4000, Leica Microsystems) or with a confocal microscope (LSM 510 META, Zeiss).

2.6 Chromatin immunoprecipitation (ChIP)-qPCR analysis

For ChIP-qPCRanalysis, ESCs untrated, differentiating EpiSCs and SFEBs were cross-linked with 1% formaldehyde for 10 min at room temperature and then with125 mM glycine. The chromatin was then sonicated to an average DNA fragment length of 500–1000 bp. Soluble chromatin extracts were immune-precipitated using an anti-Oct4 (Santa Cruz Biotechnilogy), anti-Otx2 (Millipore) anti-HMGA2 (Cell Signaling) antibody. Appropriate IgGs were used as negative control. Supernatant obtained without an antibody was used as an input control. After qPCR, the amount of precipitated DNA was calculated relatively to the total input chromatin and expressed as percentage of total chromatin or as fold enrichment relative to untreated samples. Oligonucleotide pairs are:
Oct3/4-f: 5'-CTGGCCAGTGAGTCACCAAA-3'
Oct3/4-r: 5'-AAGTATGCCTGCAGCCCAG-3'
HMGA2-f: 5'-TGGTCCTTTTGCAGACTGGAT-3'
HMGA2-r: 5-'GCACTGGTATTCACAACTGCC-3'
CR4-f:5'- GGAACTGGGTGTGGGGAGGTTGTA-3'
CR4-r:5'-AGCAGATTAAGGAAGGGCTAGGACGAGAG-3'
Otx2-f: 5'- TCAAGACCGCAAACTGCTCA-3'
Otx2-r:5'- TCAAGACCGCAAACTGCTCA-3'

2.7 iPS generation and culture

The formation of iPS wt e HMGA2 KO was induced adapting the methods of Nakagawa et al., 2007. In brief, Mouse pMXs-based retroviral vectors for Oct4, Sox2 and Klf4 were transfected into Plat-E cells plated to $4.5x10^6$ cells per 10 cm^2 dishes by using Lipofectamine 2000 (Invitrogen) following manifacturer's instructions.Twenty-four hours after transfection, the medium was replaced. Virus-containing supernatant was mixed with ratio of 1:1:1 and incubated in the virus/polybrene (8μg/ml) for 24 hours on Plat-E. MEFs wt e KO HMGA2 were plated $2x10^5$ cells per 9,6 cm^2 well and infected for twice with TFs transduced virus mix. After 24 hours from second infection, each well MEFs was split in 10 cm^2 plate in DMEM (Sigma) combined with 2 mM glutamine (Invitrogen). The next day medium were changed with ESC medium condition contained 15% FBS (Hyclone). The medium was change every 2 days. To 21^{th} day, appeared iPS clones were picked and seeded in 2 cm^2 well on mytomicin treated MEFs. Obtained wt and HMGA2 KO iPS clones were maintained on feeder-gelatine-coated plates in the following medium (iPS medium): Glasgow Minimal Essential 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), 15% FBS (Hyclone), 10^3 U/ml leukemia inhibitory factor (LIF) (Euroclone), and *2i* (MEK and GSK3 inhibitors) PD0325901 3μM and CHIR-99021 1μM (Selleckchem) respectively.

3. Results

3.1 Hmga2 suppression hampers mESCs differentiation

The screening of a shRNA collection, designed to target as many as possible mouse genes, allowed the identification of many genes whose suppression impairs ESC differentiation (Aloia et al.,2010). Among these genes we found HMGA2. Two shRNAs of our RNAi collection, targeting the HMGA2 mRNA, hampered the differentiation process of ESCs. The block of neuronal differentiation due to HMGA2 suppression was very evident when ESCs, transfected with specific shRNAs targeting HMGA2 mRNA, were differentiated in monolayer culture (Figure 3.1.1).

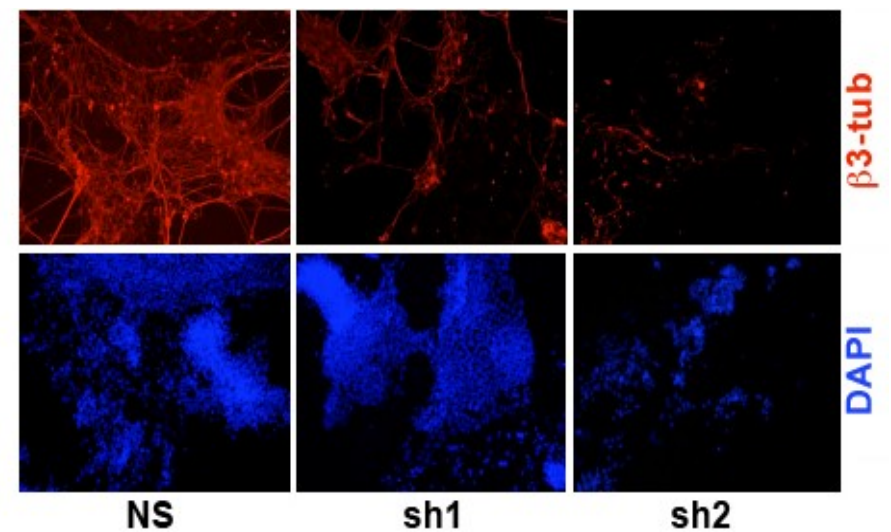


Figure 3.1.1: **The effect of HMGA2 suppression**. Staining with β3-tubulin antibody in the ESC transfected with two independent shRNAs targeting HMGA2 and induced to differentiate into neurons. The presence of post-mitotic neurons was detected by immunostaining with the antibody for βIII tubulin at 7 days of differentiation.

The evident effect of HMGA2 suppression on ESC differentiation was confirmed in a second experimental setting, that is serum free embryoid bodies (SFEBs). In the absence of serum, ESCs aggregate to form bodies starting to express neural-specific markers, like Sox1, around day 2 of differentiation. As shown in figure 3.1.2, the inner part of the SFEBs contains small groups of cells still positive for the stemness markers, like Oct4 and Nanog. The silencing of HGMA2 by siRNAs resulted in a decrease of Sox1 positive cells and an increase of the cells still positive for pluripotency markers at 4 days of SFEB differentiation.

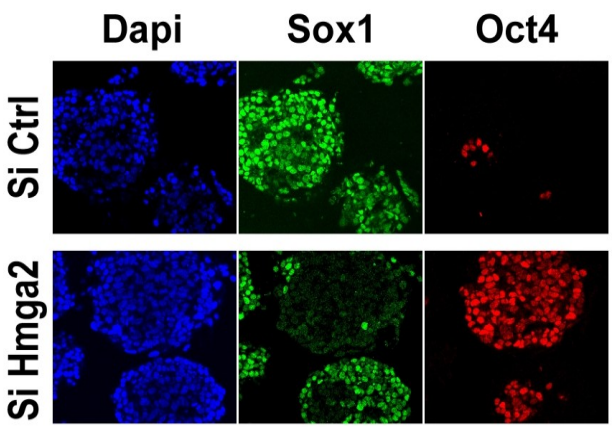


Figure 3.1.2: HMGA2 silencing hampers ESC differentiation. ESCs transfected with siRNA Hmga2 and siNS were induced to differentiate as SFEB and after 4 days the presence of stemness (Oct3/4) and neuroectodermal (Sox1) markers was analyzed by immune-staining.

HMGA2 protein was not detectable in undifferentiated ESCs but it appeared soon after the induction of differentiation (day 2, corresponding to the epiblast stage), accumulated up to day 4 and disappeared with the development of post-mitotic neurons (day 7). In this differentiation condition, Oct4 levels rapidly declined, becoming actually undetectable at day 4, while Otx2 was strongly induced at day 2 and maintained elevated levels of expression up to day 7 (Figure3 1.3).

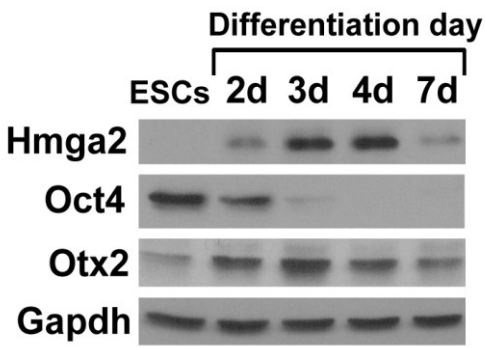
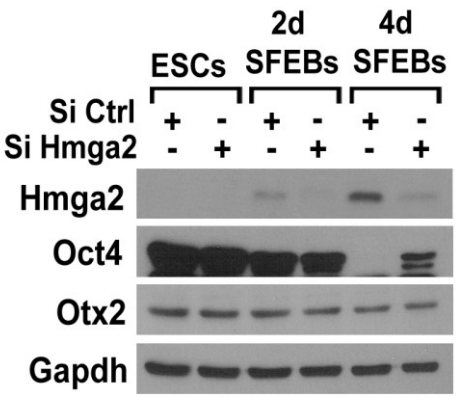


Figure 3.1.3: **Expression profile of HMGA2, Oct4 and Otx2 during ESC differentiation.** Western blot analysis shows a time course of endogenous protein expression for HMGA2 compared with Oct4 and Otx2 protein trend during ESC differentiation.

The expression profile of HMGA2 KD cells induced to differentiate into SFEBs was significantly different. The protein levels of Oct4 was maintained at higher levels up to day 4, while Otx2 protein did not significantly change (Figure 3.1.4A). Rex1 and Klf4, specific markers of undifferentiated ESCs, was turned off, as soon as ESCs differentiated. These genes were expressed at very low levels in control-transfected SFEBs, whereas their expression resulted significantly high in SFEBs derived from HMGA2 KD ESCs (Figure 3.1.4B). These results suggested that the suppression of HMGA2 may hamper the first step of ESC differentiation: the transition from ESCs into EpiSCs.

A



B)

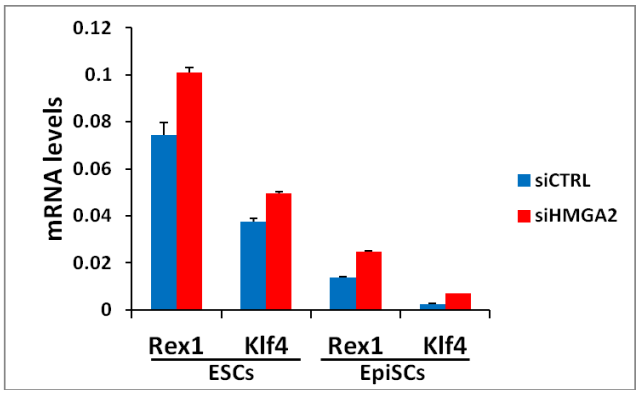


Figure 3.1.4: HMGA2 suppression hampers the ESC differentiation. A) ESCs transfected with siHMGA2 were differentiated into SFEBs. Oct4 and Otx2 expression profile was evaluated by Western Blot analysis at 2 and 4 days in SFEBs KD HMGA2. **B)** Rex1 and Klf4 mRNA levels measured by q-PCR in EpiSCs derived from HMGA2 silenced cells compared to the control (siCTRL).

3.2 Hmga2 is necessary to mESCs differentiation into EpiSCs

To explore the possibility that HMGA2 KD was interfering with the transition from ESCs into EpiSCs, we induced ESC differentiation in culture conditions that stabilizes the EpiSC state (Hayashi et al., and Nakaki et al 2013). Two days after the induction of differentiation of wt cells, Rex1 and Dax1 decreased while Fgf5, a specific marker of the epiblast, started to accumulate. The establishment of the EpiSC phenotype was also characterized by the decrease of Oct4 and Nanog and by the accumulation of Otx2 (Acampora, 2013). The silencing of HMGA2 altered this differentiation phenotype. Indeed, Fgf5 levels were lower in KD cells compared to wt cells, while Oct4 and Nanog continued to be expressed at high levels. Moreover, while Rex1 and Dax1 are actually undetectable in wt EpiSCs, they are still expressed in HMGA2 KD cells. The expression of Otx2 again was unchanged upon HMGA2 KD (Figure3.2.1). In summary, these results confirm the possibility that HMGA2 silencing is hampering the transition from ESCs into EpiSCs.

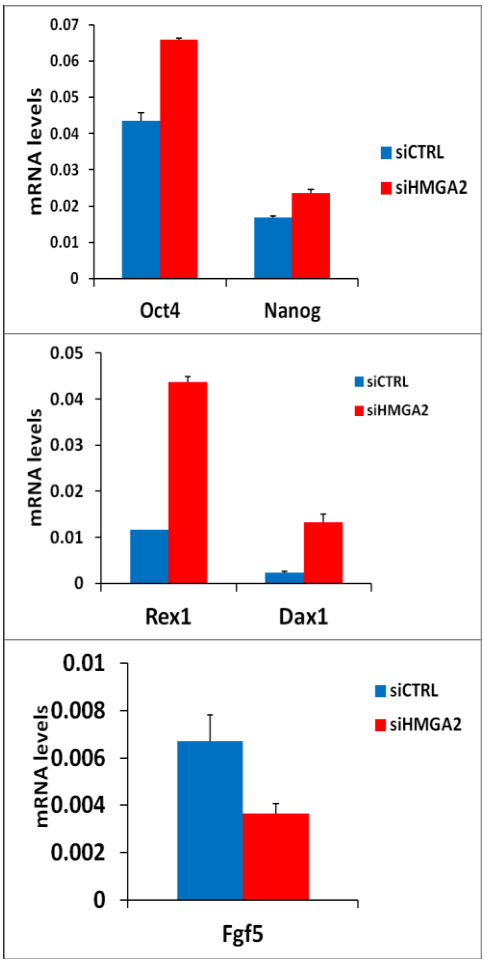
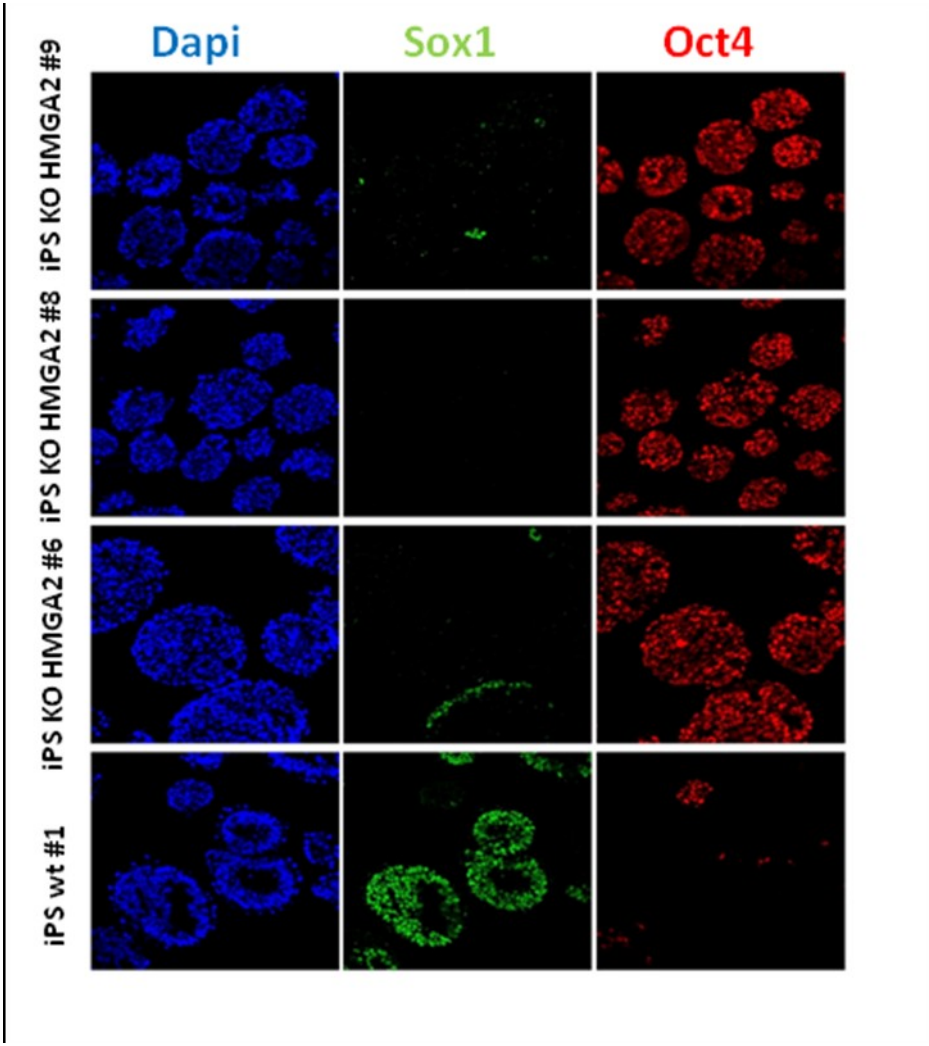


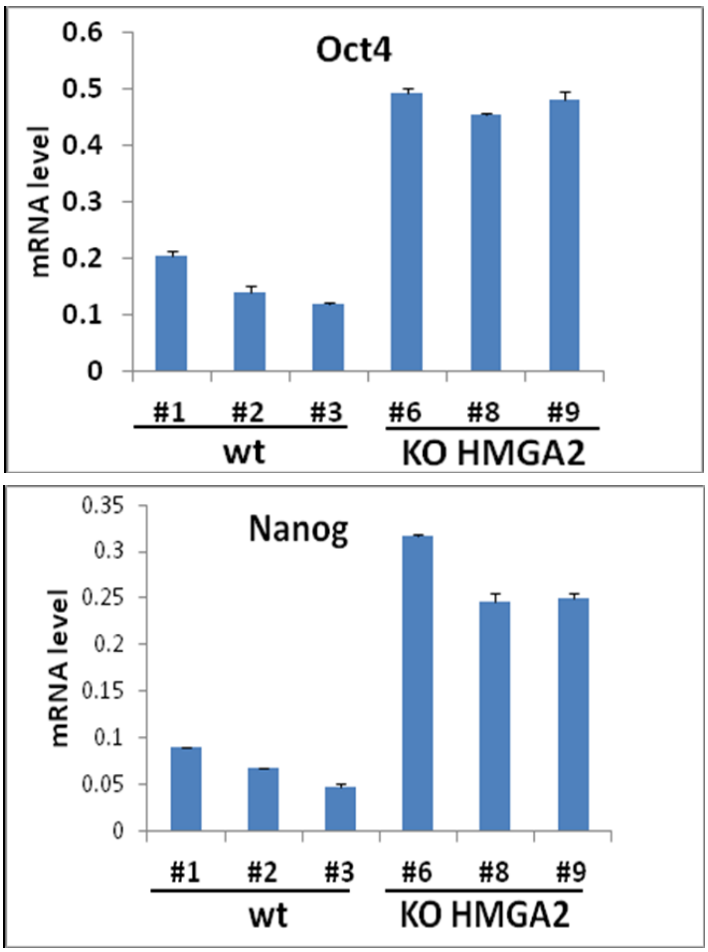
Figure 3.2.1: HMGA2 silencing hampers the transition from ESC to EpiSCs. siHMAG2 was transfected in ESCs and then cells were induced to differentiated in EpiSCs. The panels show the expression of stemness markers (Oct4, Nanog, Rex1 and Klf4) and a specific epiblast marker Fgf5.

However, HMGA2 knock down by RNA interference did not allow us to definitively address the question of whether this protein is necessary for the transition from ESCs into EpiSCs. This is due to the transient nature and to the heterogeneity of the silencing upon transfection with siRNA in the cells. Therefore, to easily explore the function of HMGA2 in the early steps of pluripotent stem cells differentiation, we have generated iPS cells from embryonic fibroblasts (MEFs) derived from HMGA2 knock out embryos. The iPS cells were obtained by over-expressing OCT3/4, Sox2 and Klf4 using retroviral infection as previously reported (Nakagawa et al., 2007). The HMGA2 KO iPS cells appeared to be not distinguishable from the wt iPS cells with a normal pattern of expression of pluripotency markers. However, when these cells were induced to differentiate into neuroectoderm through SFEB formation, a dramatic phenotype appeared, characterized by an almost complete block of differentiation. As shown in figure3.2.2A, in contrast with the high number of Sox1 positive cells observed in differentiated wild type iPS cells, almost all the HMGA2 KO cells remained positive for Oct4, with only very few cells expressing Sox1. To confirm that the block of differentiation due to the absence of HMGA2 occurs soon after the differentiation induction, that is when undifferentiated iPS clones become EpiSCs, we induced EpiSC formation of several and independent the iPS clones. We have found that the HMGA2 KO clones shows an evident inability to develop into EpiSCs (Figure 3.2.2B). All together, these results demonstrated that HMGA2 is necessary *in vitro* for the transition from undifferentiated ESCs/iPS cells into EpiSCs.

A)


Dapi
Sox1
Oct4
iPS KO HMGA2 #9
iPS KO HMGA2 #8
iPS KO HMGA2 #6
iPS wt #1

B)


Oct4
mRNA level
0.6
0.5
0.4
0.3
0.2
0.1
0
#1
#2
#3
#6
#8
#9
wt
KO HMGA2
Nanog
mRNA level
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
#1
#2
#3
#6
#8
#9
wt
KO HMGA2

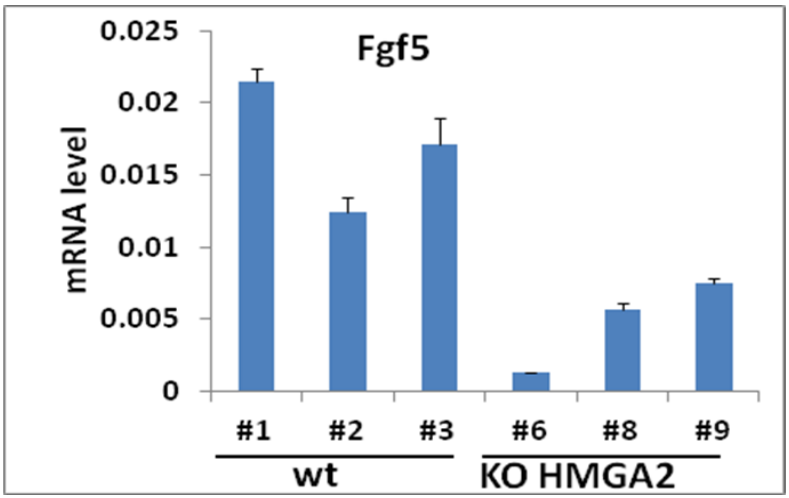


Figure 3.2.2: HMGA2 is necessary for the transition from ESC to EpiSCs. A) Immunostaining of a representative iPS HMGA2 KO clone generated from KO HMGA2 MEFs compared with iPS wild type after 4d of SFEB differentiation for the presence of stemness (Oct4) and neuroectoderm (Sox1) markers; **B)** several and independent iPS cell clones were differentiated into EpiSCs and then the expression levels of stemness (Oct4 and Nanog) and epiblast marker Fgf5 were analyzed.

3.3 HMGA2 gene expression is regulated by Otx2

Several papers have recently reported that Oct4 and Otx2 transcription factors play a crucial role in the regulation of ESC differentiation from the undifferentiated stem cell state into the post-implantation epiblast state (Acampora, 2013, Buecker, 2014, Yang, 2014). These results prompted us to examine the possibility the HMGA2 is under the control of these transcription factors. As reported above, HMGA2 accumulates upon the induction of ESC differentiation (Figure 3.1.3). We first asked whether the suppression of Otx2 or of Oct4 alters the expression profile of HMGA2 in ESCs. As shown in figure 3.3.1, HMGA2 protein was undetectable in both wt and Otx2 KO ESCs, while it was about 50% lower in Otx2 KO ESCs induced to differentiate into SFEBs. The HMGA2 mRNA showed the same behaviour of the cognate protein. On the contrary, HMGA2 mRNA significantly increased in undifferentiated Oct4 KD cells and the protein was about two-fold higher in Oct4 KD cells vs wt cells, upon the induction of differentiation. These results suggested that HMGA2 may be a direct target of Otx2 and Oct4. To explore this possibility, we examined the already published ChIP-seq data and we found that the HMGA2 gene is one of the candidate targets of both Otx2 and Oct4 in EpiSCs. Inspection of the DNA sequence, where Otx2 and Oct4 binding take place, demonstrated the presence of two bona fide cis-elements for these transcription factors. Thus, we analyzed the direct binding of Otx2 and of Oct4 to HMGA2 promoter by ChIP-qPCR in undifferentiated ESCs and in EpiSCs. We observed a significant interaction of Oct4 with the HMGA2 promoter in ESCs while in EpiSCs the binding of Otx2 and, to a lesser extent of Oct4, was very evident (Figure 3.3.2). Based on these data, we suggest that Oct4 and Otx2 may control HMGA2 expression during ESC differentiation in a negative and positive manner, respectively.

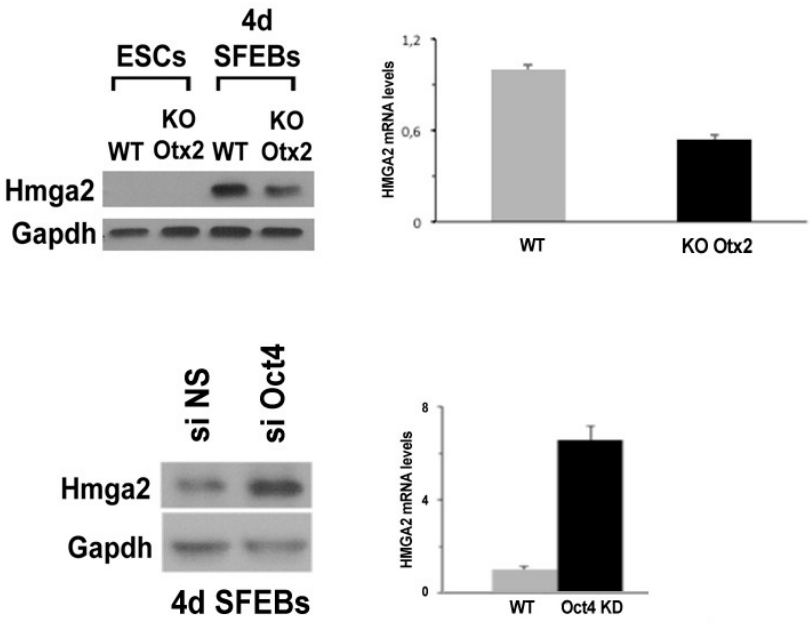


Figure 3.3.1: HMGA2 is a direct target of Oct4 and Otx2. Changes of HMGA2 expression levels were analyzed in ESC and in 4d SFEBs derived from Otx2 KO cells and Oct4 silenced cells by Western Blot and RT-PCR analysis.

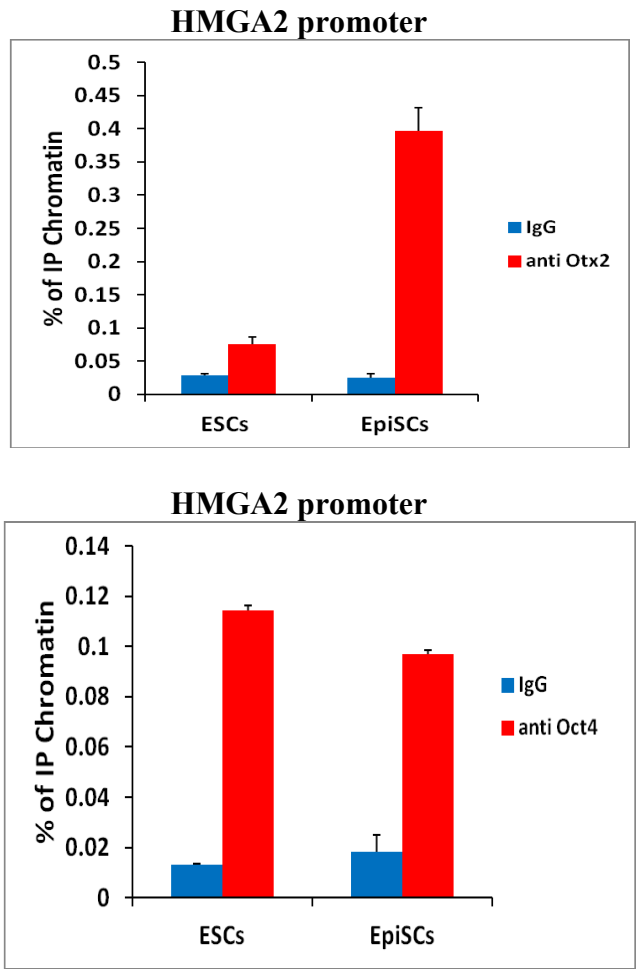


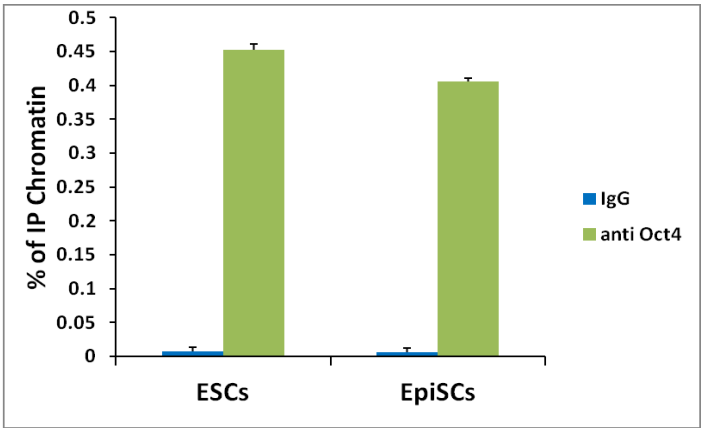
Figure 3.3.2. Oct4 and Otx2 control HMGA2 expression during ESCs differentiation. ChIP experiments were performed in wild tipe ESCs and EpiSCs to evaluate the Oct4 and Otx2 binding to HMGA2 promoter.

3.4 HMGA2 role in the control of Oct4 expression

As reported above, HMGA2 suppression hampers the formation of EpiSCs. In these conditions, the expression levels of Oct4 were maintained high by the suppression of HMGA2, while no changes were observed in the expression of Otx2, which accumulated to the same extent observed in wt cells. Thus, we asked whether HMGA2 directly regulates Oct4. Oct4 gene promoter is governed by an auto-regulatory loop, as it activates the transcription of its own gene, together with Sox2 and Nanog (Boyer et al., 2005). The results of ChIP experiments reported in Figure 3.4.1A showed that, as expected, Oct4 was associated with its promoter in ESCs. The extent of this binding slightly decreased in EpiSCs after the induction of differentiation. The same chromatin preparations used to analyze the binding of Oct4 were immune-precipitated with a HMGA2 antibody. We carried out the scanning of the genomic region upstream of the TSSs of the Oct4. While in ESCs no significant binding for HMGA2 was found, in agreement with the observation that HMGA2 is undetectable in these cells, HMGA2 antibody efficiently pulled down specific regions of Oct4 gene promoter in EpiSCs. (Figure 3.4.1B). The effects of HMGA2 on the transcription of Oct4 were examined by measuring the luciferase reporter transcription under the control of Oct4 promoter. The results shown in Figure 3.4.2 demonstrated that luciferase expression is significantly decrease in the cells where HMGA2 was overexpressed. These results indicate that HMGA2 is associated with Oct4 promoter and its binding parallels the down-regulation of Oct4 gene transcription, thus suggesting a repressor role for HMGA2 on these gene.

Oct4 promoter in ESCs and EpiSCs

A)



B)

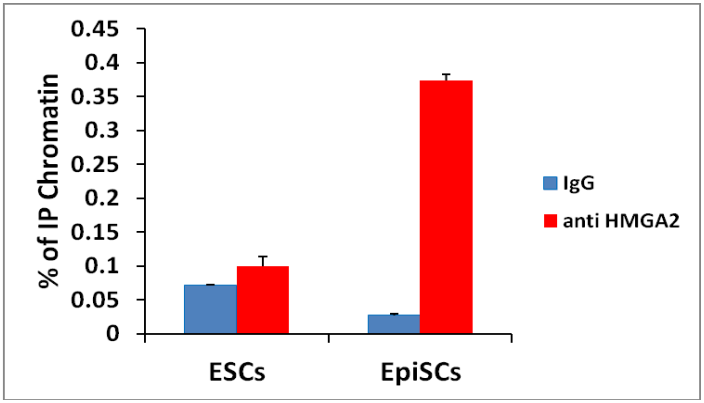


Figure 3.4.1: HMGA2 affects Oct4 transcription binding its promoter. A) ChIP experiment of Oct4 binding on its own promoter in ESCs and EpiSCs. **B)** HMGA2 binding on Oct4 promoter was examined by ChIP using the same chromatin preparation of anti Oct4-ChIP in A).

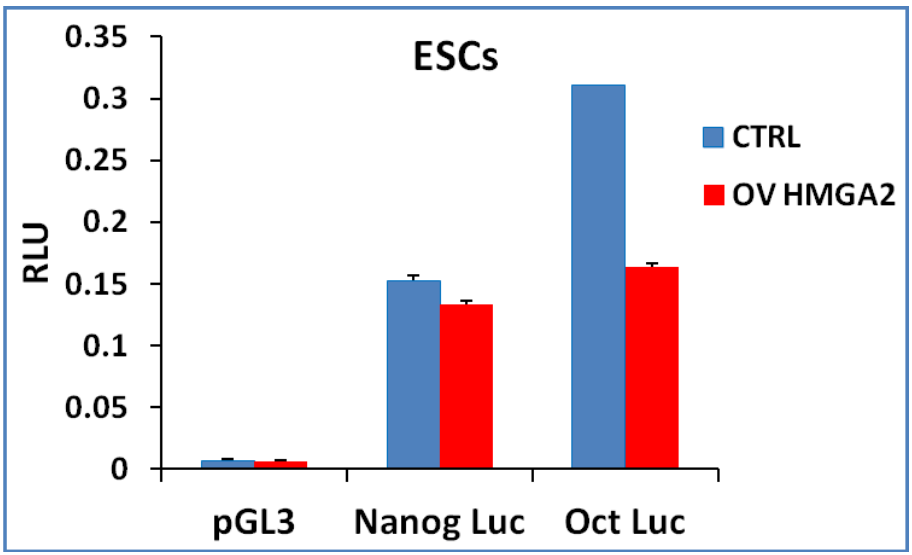


Figure 3.4.2: HMGA2 down-regulate Oct4 expression. Luciferase reporter cloned under control of Oct4 gene promoter was transfected in ESCs-Hmga2 overexpression resulted in a decrease of luciferase activity.

4. Discussion/Conclusions

ESCs derive from the inner cell mass of pre-implantation embryo and have the ability to self-renew in culture and to differentiate giving rise to all specialized cell types of the embryo. For these characteristics, they represent a powerful tool for the study of the development and for experimental medicine. For these reasons the complete understanding of the mechanisms underlying ESC decisions is necessary to provide new ESC-based developmental models and for clinical applications of these cells. A complex balance between different signals tightly regulates ESC state. All these signals converge on a complex transcriptional network that in turn regulates gene expression.

In the recent years, the regulatory mechanism that control the maintenance of pluripotent state has been extensively investigated. Indeed, many of the transcription factors (TF) involved in the establishment and in the maintenance of pluripotency are well characterized. Among these TFs there are Oct4 and Sox2, necessary to establish and maintain pluripotency (Niwa et al., 2000; Masui et al., 2007), Nanog, required to establish but not to maintain the ESC naïve state (Boyer et al., 2005), and several other TFs, like Klf2, Klf4, Klf5, Sall4, Gbx2, Tbx3, Essrb, etc, whose silencing severely affects ESC state. These factors and many others are interconnected in a complex network of reciprocal regulation, and even slight alterations of their concentration often perturb pluripotency and differentiation.

Few years ago, the laboratory of Prof. Russo undertook a project to identified factors governing ESC fate through a screening of a shRNA library. The work done during my doctoral thesis was aimed to analyze the effects of the suppression of a non-histonic chromatin factor HMGA2, identified through this screening, on ESC fate. HMGA2 along with other members of HMGA family, contains three N-terminal AT-hook motifs through which it binds preferentially to AT-rich sequences, and induces conformational changes to promote the recruitment of transcription factors to specific complexes. It lacks a transcriptional activity *per se,* but acts by orchestrating the assembly of transcription factor complexes also known as enhanceosome, thus

resulting in positive or negative regulation of gene expression (Wolffe et al., 1994; Ashar et al., 2010). The high expression of HMGA proteins during embryogenesis suggests that they fulfill important roles in development. However, the physiological role of HMGA factors in the early steps of development is still unknown as well as the transcription regulatory mechanisms they are involved in. We have demonstrated that, in condition promoting differentiation HMGA2 suppression maintains the undifferentiated phenotype, preventing ESCs transition into EpiSCs. A detailed analysis of the early steps of ESC differentiation, using HMGA2 KO iPS clones, gave the same results but with a more dramatic phenotype.

A recent paper reported HMGA2 as a putative target of Otx2 and Oct4 on the basis of ChIP-seq data (Yang et al., 2014). We have found that the expression of HMGA2 is regulated by Otx2 and Oct4 but while Otx2 promotes the expression of Hmga2, Oct4 acts as a suppressor. We have also demonstrated through ChIP experiment that a *cis* element in proximity of HMGA2 TSS interacts with Otx2 and Oct4 to regulate HMGA2 expression. Interestingly, we have also found that Hmga2 negatively regulates the expression of Oct4 gene during differentiation by directly binding to the regulatory region of its promoter. Finally, all together these results demonstrate that HMGA2 plays a crucial role in ESC fate determination, allowing the changes of gene expression program that lead ESCs to the exit from ground state.

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