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THE CELL CYCLE GENES REGULATION OF IPS CELLS AND THE ROLE OF SWI/SNF CHROMATIN REMODELING ENZYMES DURING THEM DIFFERENTIATION

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ABSTRACT

The pluripotent cell state can be achieved by ectopic expression of some transcription factors that can reprogram somatic cells to achieve pluripotency. (Takahashi, K., and Yamanaka, S.; 2006; Takahashi, K., and Yamanaka, S.; 2007). One of the purpose of these studies is to identify which genes are modulated during the early stages of differentiation of iPS .We analyzed the expression of some proteins that regulate the cell cycle by Western blot: cyclin A, cyclin E, CDK2, the family members Rb (Rb105, p130 and p107), some subunits of the complex SWI / SNF (BRM and BRG-1) histone deacetylases ,mSin3A, and E2F2 and E2F4. The results show that the expression of cyclin A and cyclin E proteins are present in high quantities in undifferentiated iPS and their expression increase at 1h and then decreases at 3h, 6h, 24h in iPS induced to differentiate with retinoic acid (RA). In this studies I have been found that the level of CYC E in iPS is less than cES. As a further control, were carried out analysis of RealTime-PCR The data of immunoprecipitation shows that the interaction of BRG-1-HDAC-1 (data not shown) is strongly stimulated in the presence of RA 5uM for 1h and little without RA. The undifferentiated ESCs express this protein very weakly. The results indicate the presence of complex Rb/Id2 in ES and iPS (undifferentiated) samples.

Another objectives of my thesis was to study the regulation of gene expression during the cyclic E1 "self-renewal" of mouse iPS. This result confirms that during self-replication of the CES a multiprotein complex binds to the promoter sequence of CERM cyclin E1 is

expressed constitutively and without cell cycle periodicity in the iPS. Brefly in IPS treated with LIF and RA the CERC2 complex is made by P130-E2F4-CYCE-Rb proteins suìimilarly to what observed for the ESC cells. I have obtained differentiated cardiomyocytes from Embryonic Bodies Furtermore the FACS analysis has shown that the number of cardiomyocytes was about 8% of the total cells in the Embryonic bodies. My studies on the differentiative effect of ascorbic acid show that iPS cells behave as ES cells. In fact, ascorbic acid at a concentration of 50 μg/ml final has a stimulant effect on the differentiation of EB. The results with the treatment of iPS withAscorbic Acid indicate that this chemical induce the differentiation in cardiomyocytes .In fact I have shown that the level of CYC A, CDK2 and Oct 4 are decreasing following this treatment.

These results obtained has been very interesting and revealed the importance of the expression of cyclin E and of the Rb/E2F pathway during the early stages of differentiation in both ES cells into iPS. In pluripotent iPS and ES cells induced to differentiate, the length of the G1 phase of cell cycle is greatly increases, and at the molecular level, the complex cyclin E/Cdk2 kinase activity increased from the constitutive kinase activity in undifferentiated cells to a dependent activity as observed during the early stages of differentiation in the cell cycle.. In addition, the regulated activities of the cyclin E/Cdk2 complex results in the activation of the pathway Rb/E2F which in turn requires a cell cycle dependent transcriptional control of target genes for transcription factor E2F.

Overall, the results obtained in these studies supports the model that the cell cycle of ES and iPS cells abbreviated with a short G1 phase, is functionally linked to pluripotency.

In conclusion the iPS cell have a similar cell cycle regulation than ES cells.

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Dedication

I dedicate this work to my Mother, friends and colleagues who have encouraged me to work towards my goals and never give up.

1 INTRODUCTION

1.1 ANALYSIS OF THE CELL CYCLE IN ES CELLS.

Study of the pluripotency of embryonic stem (ES) cells aims to investigate the mechanisms of activation of proliferative signals, and transcription of the "checkpoint" that control the cell cycle. The level of proliferation of ES cells is unusually high. This reflects the structure of the cell cycle that lacks gap phases (G) complete and basically consists of stages of DNA replication (S phase) alternating with chromosome segregation (M phase) (Burdon T.,et. Al. 2002). In detail the phases G1, S and G2 / M represent 20, 70 and 10%, respectively, of the total cycle. Based on the proportion of cells that are in S phase and the length of the cell cycle (11-12 hours), it was estimated that the ES cells is about 5.5-6 hours in S phase and for about 1.5 hours in G1 phase (Savatier et al., 1994 and 1995). For this reason, the cell cycle regulation in ESC is fundamentally different from that of other somatic cells. The division by the ESC is led unusually high activity of the complexes cdk2/ciclina A and E, which appears to be constitutived. The only kinase activity that shows periodicity in the cell cycle is associated with cyclin B, which is highest in mitosis (Stead et al., 2002).

Numerous experiments have demonstrated the role of E2F TF target gene in the control of cell cycle genes regulated particularly when cells leave the G0 phase and enter in the cycle. To study the role of E2F TF binding sites in the regulation of cell cycle, Le Cam et al.,(1999) have investigated the promoter of cyclin E1 in differentiated cells. In this study on quiescent mouse fibroblasts they have discovered that the activity of cyclin E1 gene

promoter is controlled by a DNA sequence defined as "cyclin E repressor module (CERM). The CERM is located downstream of the transcription start site and consists of a variant of the canonical E2F sites flanked by a sequence AT rich. Both sequences are necessary to bind a complex of high molecular weight E2F called cyclin E repressor complex 1 (CERC1) that prevents the transcription of cyclin E1. When the fibroblasts begin to proliferate, the complex CERC1 dissociates from CERM sequence and cyclin E1 transcription is induced. Similar studies have shown that even in quiescent K562cells , CERM regulates the repression of CCNE1 gene in the period between the end of mitosis and late G1 phase through its association with a high molecular weight E2F complex called CERC2 (Polanowska et al ., 2001). The repression of cyclin E1 gene is not sufficient to block the kinase activity of the complex: it requires the involvement of protein Cip / Kip, which inhibit specifically the catalytic subunit of CDK2. More recently, Dalton and White et al (Stead, 2002) have investigated by Chip analysis the Cyclin E promoter in ES cell during differentiation. Briefly they have found the p107 is recruited on E2F4 binding sites in the Cyclin E promoter during the changes in Cyclin E1 transcription during differentiation.

1.2 INDUCED PLURIPOTENT STEM CELLS

Previous work had already indicated that individual transcription factors, when overexpressed or deleted, could induce cell fate changes in somatic cells. For example, overexpression of the transcription factor MyoD induced the conversion of fibroblasts into myogenic cells (Davis, Weintraub et al. 1987). Mouse B cells were shown to reprogram into progenitors that can give rise to the hematopoietic lineage when Pax5 was removed (Nutt, Heavey et al. 1999). In ESCs, the activation of transcription factors can lead to the differentiation into a certain lineage. The indication that transcription factor overexpression can induce cell fate changes started attempts to reprogram cell types to other lineages including the induction of pluripotency from differentiated cells.

1.2.1 MOUSE INDUCED PLURIPOTENT STEM CELLS

Several transcription factors and genes are important for maintaining the pluripotency and ES cell phenotype in early embryos and ES cells. In 2006, Takahashi *et al.* selected 24 genes as candidates for factors that induce pluripotency in somatic cells base done the hypothesis that these factors play a role in the maintenance of ES cell identity (Takahashi and Yamanaka 2006). A βgal-neomycin cassette was inserted into the mouse Fbx15 gene by homologous recombination. Fbx15 was used as a marker for pluripotency. The 24 candidates were all together introduced into mouse embryonic fibroblasts (MEFs) from Fbx15βneo/βneo embryos by retroviral transduction. This resulted in neomycin resistant

colonies with morphology similar to ES cells. Critical candidates to induce the pluripotency were determined by excluding individual factors from the pool of transduced genes. Ten genes were identified of which the individual absence resulted in poor or no colony formation. Transduction with the combination of these 10 factors produced more ES cell-like colonies that all 24 genes together did. When individual factors were excluded from the 10-pool transduction into MEFs, only a few or no colonies were formed when the factors OCT4, SOX2, KLF4 or c-MYC were not present. Takahashi *et al.* showed that these four factors together could produce a similar number of colonies to the ones observed with the pool of 10 genes. This demonstrated that induced pluripotent stem cells (iPS cells) could be derived from MEFs by the transduction with the transcription factors OCT4, SOX2, c-MYC and KLF4. Global gene-expression profiles of ES cells, iPS cells and Fbx15βneo/βneo MEFs revealed that iPS cells are closely related to ES cells but not to fibroblasts. It is for these reasons that in this thesis we have studied the cell cycle genes regulation of ips cells and the role of SWI/SNF chromatin remodeling enzymes during them differentiation. Takahashi *et al.* also showed the pluripotency of iPS cells by the differentiation into all three germ layers during teratoma formation after a subcutaneous injection of iPS cells into nude mice. Using tail tip fibroblasts instead of MEFs from Fbx15βneo/βneo mice led to the same result. However, no chimeric mice were obtained. However, when endogenous Nanog expression was used for the selection of iPS cells instead of Fbx15, chimeric mice could be obtained (Maherali, Sridharan et al. 2007; Okita, Ichisaka et al. 2007). Using this selection stategy, iPS cells were generated that have a lot of characteristics similar to ES cells including feeder-independent growth while

maintaining the ES-like morphology in contrast to Fbx15 selected iPS cells (figure 4) (Takahashi and Yamanaka 2006). Maherali *et al.* investigated the epigenetic changes of reprogrammed fibroblasts and found that pluripotency genes were demethylated in iPS cells whereas this was only partially the case in Fbx-15 selected iPS cells. The obtained homogeneous cell lines were derived from a selection of 3 weeks postinfection whereas the heterogeneous lines were selected 1 week postinfection suggesting that delayed selection strategy increases the chance of a pure population of iPS cells (Maherali, Sridharan et al. 2007). The detection of global hypomethylation in female iPS cells suggests a similar epigenetic state to that of female ES cells. X-inactivation is regulated by two noncoding transcripts Xist and its antisense transcript Tsix. Undifferentiated cells have two active X chromosomes that both express Tsix to repress Xist expression. Upon differentiation Xist becomes upregulated on one of the X chromosomes followed by this chromosomes inactivation and Tsix expression disappears. The Nanog-GFP MEFs were shown to have one Xist RNA-coated inactive Xchromosome, whereas no Xist could be detected in iPS cells where Tsix was present in high levels indicating that the four transcription factors are able to induce X-reactivation and erase the chromatin modifications specific for the inactivated chromosome (Maherali, Sridharan et al. 2007). Furthermore, it was also reported by Maherali *et al.* that X-inactivation was random in differentiated iPS cells. 94.4 % genes in Nanog-selected iPS cells had a methylation pattern identical to that of ESC's,whereas only 0.7% had a more MEF-like pattern. This suggests that reprogramming can reverse the epigenetic memory of a somatic cell to that of ESCs (Maherali, Sridharan et al. 2007). However, Okita *et al.* reported the development of neck tumors in chimeric mice

derived from the Nanog-iPS cell line that died soon after birth (Okita, Ichisaka et al. 2007). In these tumors the retroviral expression of c-MYC is reactivated whereas the expression of the transgenes remained low in normal tissues.

1.3 ES AND IPS CELL CYCLE

Cell cycle regulation is another unique feature that distinguishes ES cells from differentiated cells. ES cells transit through cell cycle much faster than differentiated cells mainly due to a shortened G1 phase. In mouse embryonic fibroblasts (MEFs), the G1 phase lasts 15–20 h and temporally accounts for more than 80% of the cell cycle. However, in both mouse and human ES cells, G1 lasts 2–4 h and temporally accounts for only 15–20% of the cell cycle. This unique cell cycle pattern is further characterized by hyperphosphorylated RB protein, constitutively high activity of cyclin E and A-associated kinases, and a lack of expression of major CDK inhibitors (Stead et al., 2002). Upon differentiation, the ES cell-cycle pattern quickly switches to a MEF-like pattern (Savatier et al., 1996). The role of a shortened G1 phase in maintaining pluripotency is not clear, though the exclusivity of this unique cell cycle among cells that are pluripotent suggests it is important. Another difference between ES cells and somatic cells is the activity of telomerase, where ES cells, as well as many adult stem cells, show a much higher telomerase activity. Similar to ES cells, iPS cells exhibit a cell cycle with a shortened G1 phase (Maherali et al., 2007) and elevated telomerase activity (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). The study of processes responsible for the undifferentiated state of ESC and iPS aims to investigate the mechanisms of activation of proliferative signals, and transcription of the "checkpoint" that control the cell cycle.

The level of proliferative CES is unusually high. This reflects the structure of the cell cycle that fails to complete phases G and basically consists of stages of DNA replication (S phase), alternating with chromosome segregation (M phase). In detail the stages G1, S and

G2 / M represent 20, 70 and 10% respectively of the total cycle. On the basis of the proportion of cells that are in S phase and the length of the cell cycle (11-12 hours), it was estimated that the ESC is about 5.5-6 hours in S phase and for about 1.5 hours in G1 phase (Savatier et al., 1994 and 1995). The high proliferative rate of the ESC seems to be independent of the route of regulation of Rb but related to the activity of some of the specific Cdk transition G1 / S that does not show periodicity in the cell cycle as the complex CDK2 / cyclin A1 and E1.

The only kinase showing periodicity in the cell cycle is associated with cyclin B, which is highest in mitosis (Stead et al., 2002). In addition, the kinase activity associated with Cdk4 is not detectable, cyclin D1 is present in low quantity and cyclin D2 is not expressed. This indicates that the mitogenic signals, particularly those transduced by receptor tyrosine kinases, are not coupled to the machinery of the cell cycle (Burdon et al., 2002) because it does not determine the expression of cyclin D.

Although present in limited quantities, the complex containing cyclin D1 and cdk4 can prevent the inhibitory action of p27 on cyclin complex E1/Cdk2 but do not contribute to constitutive phosphorylation of Rb (Burdon et al., 2002). Moreover, the absence of early G1 phase cyclin-dependent D shortens the G1 phase.

The complex CDK2-ciclina E1 constitutively active throughout the cell cycle promotes the transition from M directly to the late G1 phase in an inactive state while maintaining the pocket proteins Rb and p107.

As a consequence, E2F transcription factors are not subject to repression by pocket proteins and their genes are transcribed independently of the position of the loop. The repression of

cyclin E1 gene is not sufficient to block the kinase activity of the complex and it is necessary protein Cip / Kip that specifically inhibit the catalytic subunit of CDK2. In fact, the absence of inhibitors of CDKs, such as p21Cip1, p27Kip1 and p16INK4a as well as non-sensitive to the complex of cyclin D3-Cdk6, p16INK4a (Stead et al. 2002; Faast et al. 2004), seem to contribute to the absence of a checkpoint between the phases G1 / S in mouse ES cells.

Recently studies have shown that transcription factors Oct4 and Nanog play important roles in maintaining the undifferentiated state of mouse ES cells (Zhang W.W. et al., 2011). Oct4 (Pou5f1), belongs to the POU (Pit-Oct-Unc) transcription factor family. Oct4-null embryo dies at embryonic day 3.5 and the blastocyst are composed mainly of trophectodermal cells without the ICM. It has been proposed that, Oct4 controls pluripotency of ES cells in a dose-dependent manner (Niwa et al., 2000). A twofold induction of Oct4 led to ES cell differentiation into primitive endoderm and mesoderm. Loss of Oct4, on the other hand, triggers differentiation into trophectoderm lineages. These observations indicate that the appropriate level of Oct4 is critical for the maintenance of ES cells. Two independent studies confirme the essential roles of Nanog in ES cell maintenance and embryonic development (Chambers et al., 2003; Mitsui et al., 2003). Hence, Nanog is required for the ICM formation and primitive ectoderm development (Mitsui et al., 2003) because Nanog-null embryo fails in the formation of primitive ectoderm, and dies at embryonic day 4.5. Interestingly, over-expression of Nanog allows ES cells to bypass its dependence on the LIF and BMP signaling pathway (Chambers et al., 2003; Mitsui et al., 2003). These results

demonstrate the indispensable roles of Nanog in early embryonic development and ES cell maintenance.

Cyclin-dependent kinase 1 (Cdk1) along with cyclin B are involved in cell cycle regulation in eukaryotic cells. Deletion of Cdk1 leads to early embryonic lethality, suggesting its critical role in early embryonic development (Santamaria et al., 2007). Repression of Cdk1 resulted in the differentiation of trophoblast stem cells into giant cells. Furthermore, inhibition of Cdk1 caused rapid apoptosis of the ES cells (Ullah et al., 2008). These findings suggest that Cdk1 could be involved in the maintenance of the unique undifferentiated state of early stem cells. It was recently shown that Cdk1 is a member of the Oct4 interactome in mouse ES cells (Wang et al., 2006). Interestingly, many members of the Oct4 interactome network are necessary for the maintenance of self-renewal and pluripotency (Wang et al., 2006).

1.4 CELL-CYCLE CONTROL DIFFERS IN DIFFERENTIATED CELLS AND ES AND IPS CELLS

The pluripotent stem cells undergo unlimited self-renewal and differentiation in all types of body cells. Stem cells specifically regulate the structure of their cell cycle and this allows them to proliferate rapidly, including the expression of cell cycle regulators in the G1 phase of the cycle, and then quickly transition into S phase. In addition, stem cells because of the role of embryonic development (ES) must maintain genomic integrity and prevent the acquisition of mutations that would be transmitted to many cell lines. ES cells also express high levels of DNA repair proteins. Similarly, ES cells, induced pluripotent stem (iPS), are ready to proliferate and show a lack of cell cycle arrest G1 / S, extreme sensitivity to DNA damage, and a high level of expression of the repair genes DNA. The mechanisms that regulate the cell cycle in ES cells and genomic integrity iPS cells are similar, although not identical (Momcilovic O. et al 2011). Proliferation of differentiated mammalian cells is controlled primarily by regulating the progression through the G1 phase and entry into S phase. The (RB) retinoblastoma protein and its relatives P107 and P130 are responsible for controlling the G1 / S (Fig. 2). Phosphorylation regulates the activity of RB: hypophosphorylated (G1-specific) RB inhibits the expression of genes that are required for entry into S phase sequestering the E2F family of transcription factors. During the progression through the G1, RB is phosphorylated sequentially by cyclin complexes and cyclin dependent kinase (CDK). Phosphorylation by cyclin and cyclin D/CDK4 D/CDK6 induces a partial release of E2F, which is sufficient to activate the transcription of cyclin E and cdc25A genes. The cdc25A phosphatase removes phosphates from CDK2 inhibitors,

and the resulting cyclin E/CDK2 complex then completes the RB phosphorylation, leading to full release of E2F and activation of target genes and entry into S phase (Harbour, JW and Dean, DC 2000;, Bartek, J. and Lukas, J. 2001).

A second initiative involves the participation of the protooncogenes c-myc, which directly stimulates the transcription of genes encoding cyclin E and cdc25A to generate cyclin E/CDK2 kinase complex (Bartek, J. and Lukas, J. 2001) (Fig. 2). The tumor suppressors p16INK4a and p27Kip1 inhibit the cyclin and cyclin D/CDK4 D/CDK6 E/CDK2 and cyclin, respectively. They are activated in response to various growth inhibitory signals, such as contact inhibition and senescence and terminal differentiation (Sherr, CJ and Roberts, JM,. 1999). Embryonic stem cells have a short G1 phase of about 1.5 hours during which hypophosphorylated RB is virtually undetectable (Savatier P, et.al 1994) Thus, RB is likely to be rephosphorylated immediately after mitosis in embryonic stem cells, the contrary to differentiated cell types. In addition to RB, the ES cells expressing P107 (Robanus Maandag E-1998), but not p130.

1.5 THE CYCLIN E FAMILY

Two types of cyclin E have been recently described: cyclin E1 and cyclin E2, which are closely homologous. These show almost identical patterns of expression during embryonic development and in a similar way to adult tissues. This suggests that the two cyclins E control overlapping phases in the progression from G1 to S. Alternatively, the co-expression of cyclins E may represent a mechanism of redundancy to ensure the normal entry into S phase (Geng et al., 2001).

Unlike cyclins D, which are induced by external factors, the expression of cyclins E is controlled by endogenous mechanisms and is highest in the transition G1 / S. Furthermore, these proteins may drive the progression into S phase even in the absence of Rb, or at least for one round of cell division when is suppressed the transactivation capacity of E2F. This suggests that cyclin E promotes S phase in different ways and at least one of the critical events controlled by cyclin E is independent of the complex E2F/Rb (Lukas et al., 1997). Other specific events of S phase may be regulated by the complex cyclin E/cdk2. These include the phosphorylation of proteins involved in DNA replication, activation of the phosphatase Cdc25A, the regulation of transcriptional programs through transcription factors such as ID2, ID3, BAF155 and SWI / SNF, the modulation of events of splicing and transcription genes for histones (Moroy and Geisen, 2004). However, mice double knockout cyclin E1-/ - E2-/ - are viable, suggesting that E-type cyclins are not absolutely required for embryonic development in mice. On the other hand the E type cyclin are

essential for the proper development of extraembryonic tissues, such as the placenta (Geng et al., 2003).

1.6. THE FAMILY OF TRANSCRIPTION FACTORS E2F

The family of E2F transcription factors is essential for temporal control of gene expression during the cell cycle in mammals, exerting both positive and negative effects. Indeed, the E2F binding sites are found in various promoters of genes whose products are required for the biosynthesis of nucleotides, such as dihydrofolate reductase (DHRF) and thymidine kinase (TK), for DNA replication (DNA polymerase α and Cdc6), for cell cycle progression (cyclin E, cyclin D1, c-myc, c-myb, and cdc2) (Wells et al., 2000). The activity of E2F is regulated mainly by association with different proteins.

The E2F proteins form heterodimers with DP family members (DP1 and DP2). The functional specificity of the complex E2F/DP is determined by E2F subunits, however, heterodimerization with DP proteins dramatically increases the DNA binding, transactivation and regulation by the Rb family members. Additionally, DP proteins are associated with all E2F in the cell (Zheng et al., 1999) (figure 1).

The family of E2F transcription factors can be divided into three distinct subgroups based on sequence homology and functional characteristics (Dyson, 1998). Studies of "knockout" gene made it possible to identify the distinct roles of E2F family members in the development and physiology of the rat.

The domains for DNA binding and dimerization are conserved in all E2F, E2F6 but lack the C-terminal sequences responsible for transactivation and protein binding "pocket". The activity of this protein is thus independent of control by the Rb family members. E2F6 can repress transcription by binding the protein and the binding protein YY1 Ring1 (RYBP), a

component of the Polycomb complex (PCG) (Trimarchi and Lees, 2002).In conclusion, every member of the same subgroup have different biological functions that are the result of differences in their expression, activity and / or regulation in vivo.

1.7. THE REGULATION OF ACTIVITY BY THE E2F PROTEINS "POCKET"

The activity of E2F is negatively regulated by protein "pocket". The Rb gene was the first tumor suppressor to be identified. Alterations in the Rb gene are caused by deletions or missense mutations that result in a truncated, nonfunctional, protein, or its complete absence. These changes are associated with different types of human cancer, including familial retinoblastoma, osteosarcoma, lung cancer, cervical cancer, prostate cancer, breast cancer and some forms of leukemia. P130 and p107 mutations, however, are rarely associated with tumors. The different biological properties probably reflect differences in the way of regulating E2F (Giovanni A et al. 2000).

The various members of the protein "pocket" is associated with E2F at different stages of the cell cycle: p130/E2F complexes are mostly found in quiescent or differentiated cells, the complex p107/E2F are prevalent in cells in S phase and complexes Rb / E2F are present both in resting cells and in actively dividing cells (Dyson, 1998).The activity of Rb and other family members is regulated by phosphorylation and is expressed in a cascade control of E2F, in which each member of the family of E2F has a definite role for cell cycle progression (Fig. 1).

In this model, E2F4 and E2F5 is translocated into the nucleus after binding to DP, p107 and p130. The phosphorylation of Rb, p107 and p130, by the cdk of the G1 phase, leading to the destruction of the repressor complexes containing E2F4 and E2F5. The result is a de-repression of several genes involved in controlling cell growth, including E2F1 and E2F2

genes. This de-repression allows the accumulation of E2F1 and E2F2 (and probably E2F3) which function as transcriptional activators of genes important for S phase, including cyclin E and cdk genes. These generate additional kinase activity, which completes the phosphorylation of Rb and facilitates the accumulation of E2F and the beginning of DNA replication. Finally, E2F1 and E2F2 activate cdk inhibitors (p18 and p19), the function of which is important for cell cycle progression. DP1 and DP2 phosphorylation, by cyclin complex A/cdk2 causes loss of activity of E2F1, E2F2 and E2F3 at the end of S phase (De Gregori et al., 1997).

The binding domain of E2F to Rb and various family members is located at the C-terminal 18 amino acids and includes the transactivation domain. This explains how the Rb family members inhibit the transcription of E2F target genes. In fact E2F/DP the complex, bound to DNA, promotes the transcription of target genes through the transactivation domain. The complex with Rb linked to that site is unable to recruit the transcriptional machinery. Alternatively, Rb may recruit different proteins to the promoter, which affect chromatin structure, and actively repress transcription.These include the histone deacetylases (HDACs), two components of the ATPase complex SWI / SNF (BRM and BRG-1) and histone methyltransferases (MTAs, such as SUV39H1) (Trimarchi and Lees, 2002).

The histone deacetylases remove acetyl groups from octamer histone tails, facilitating the condensation of nucleosomes in chromatin. Gene expression is inhibited as well, since it blocked access to the promoter of transcription factors (Harbour and Dean, 2000). In addition, recent studies show that E2F1 can be acetylated and that this increases the binding of the complex E2F/DP DNA (Martinez-Balbas et al., 2000). Therefore inhibition of E2F

activity by the overall Rb / HDAC deacetylation can occur even for the same E2F, thereby reducing their ability to bind to DNA.

The complex SWI / SNF chromatin remodeling participates in adjusting the structure of nucleosomes and their position on the promoter. In particular, cooperate with Rb to block the activity of E2F transcription (Trouche et al., 1997). Recent evidence shows that Rb can recruit HDAC and SWI / SNF together in a single complex, in particular the complex HDAC-Rb-SWI/SNF repressor acts at the checkpoint of the cell cycle in G1 and S phase in the complex Rb-SWI/SNF (Zhang et al., 2000).

The formation of complexes containing the SUV39H1 histone tails allows the modification of the adjacent, this means that the effects of gene silencing to be propagated throughout the locus (Trimarchi and Lees, 2002).

In conclusion, the response mediated by the E2F transcriptional activation can be both a repression, depending on the context in which the promoter bind E2F. The complexity of these mechanisms, due to the formation of a variety of multiprotein complexes, suggests a multiplicity of functions. It is not yet clear whether the individual E2F have distinct roles in controlling cell growth or, alternatively, different complexes containing Rb family members, may direct transcription factors to different E2F target genes or to the same genes but at different times (De Gregori et al., 1997).

The difficulty of this analysis is due to the fact that most of the cells contains all of the E2F and all members of the Rb family. However, in vivo studies using chromatin immunoprecipitation (Wells et al., 2000), show that most of the target genes of E2F is bound by specific members of the E2F family. In addition, the DNA binding by the E2F

proteins and "pocket" is a promoter-specific and depends on the phase of the cell cycle. One hypothesis is that members of the Rb family, linked to the promoter, can be used both for the binding of repressor proteins in resting cells for the binding of activator protein in cells in S phase In any case, the target genes of E2F are not regulated by a single complex, static, probably, every cell has the potential to form different transcription complexes (Wells et al., 2000).

1.8 RB-MEDIATED INHIBITION OF E2F

Rb can repress transcription by at least two different mechanisms. First, it can bind transcription factors such as E2F and block their ability to activate transcription (Flemington et al. 1993; Helin et al. 1993). Second, the Rb–E2F repressor complex that forms at promoters can actively repress transcription (Bremner et al. 1995; Sellers et al. 1995; Weintraub et al. 1995). The balance between these two activities in vivo is still in question. In this section, we address the potential mechanisms through which Rb might inactivate E2F. Because Rb binds E2F within its transactivation domain (Flemington et al. 1993; Helin et al. 1993), it was assumed initially that it physically blocks E2F transactivation. This idea is supported by in vitro studies in which Rb inhibited transcriptional activation by E2F1 in the apparent absence of other corepressors (Ross et al. 1999). However, Rb may also inhibit E2F by recruiting chromatin remodeling enzymes, including the HDACs mentioned above. The HDACs are a family of at least seven different enzymes that remove acetyl groups from the tails of histone octamers, which appears to facilitate condensation of nucleosomes into chromatin. This, in turn, inhibits gene expression by blocking access of transcription factors to the promoter (Kingston and Narlikar 1999; Kornberg and Lorch 1999; Wolffe and Hayes 1999). In contrast to experiments in vitro, transfection assays in cultured cells have suggested that interaction with HDAC is required for the inhibition of E2F1 by Rb (Brehm et al. 1998; Magnaghi et al. 1998). Other studies have only shown a partial requirement for HDAC activity in the

Rb-mediated inhibition of E2F activity (Luo et al. 1998; Lai et al. 1999a). E2F1 has been shown to interact with the histone acetyl transferases p300/CBP and p/CAF (Trouche et al. 1996). Thus, it is possible that Rb-mediated recruitment of HDAC to E2F acts to offset this histone acetyltransferase (HAT) activity (Fig. 1). It has also been shown recently that E2F1 can be acetylated, which increases the binding of the E2F/DP complex to DNA (Martinez-Balbas et al. 2000). Therefore, recruitment of HDAC to E2F via Rb may inhibit E2F activity by deacetylation of the protein, thereby inhibiting its binding to DNA. Rb can also interact with BRG1 and BRM—the two ATPase components of the human SWI/SNF chromatin remodeling complex, which is discussed in more detail below (Dunaief et al. 1994; Singh et al. 1995). Some results have shown that overexpression of BRG1/BRM can facilitate Rb-mediated inhibition of E2F1transcriptional activity (Trouche et al. 1997); however, other studies have found that E2F activity is inhibited efficiently in cells that are BRG1/BRM deficient (Weintraub et al. 1992; Zhu et al. 1993; Zhang et al. 2000). Thus the relative importance in vivo of these two potential mechanisms for inhibiting E2F transactivation—direct binding and masking of the E2F transactivation domain versus Rb-mediated recruitment of chromatin remodeling enzymes to inhibit E2F—is still unclear.

1.9 ACTIVE TRANSCRIPTIONAL REPRESSION BY RB

Binding of Rb and other pocket proteins to E2F does not simply inhibit E2F activity. The resulting Rb–E2F complex binds to promoters and actively represses transcription by blocking the activity of surrounding enhancers on the promoter (Weintraub et al. 1992; Hsiao et al. 1994; Johnson et al. 1994; Adnane et al. 1995; Bremner et al. 1995; Neuman et al. 1995; Sellers et al. 1995; Weintraub et al. 1995; Chow et al. 1996; Ferreira et al. 1998; Meloni et al. 1999). Whereas Rb requires sequences in the pocket and in the carboxy-terminal region to bind and inhibit E2F, the pocket alone is sufficient for active repression when Rb is tethered directly to a promoter (e.g., through a Gal4 DNA binding domain; Bremner et al. 1995; Sellers et al. 1995; Weintraub et al. 1995). This is explained by the recent finding that active repression by Rb is attributable, at least in part, to the recruitment of pocketbinding corepressors. Perhaps the best studied of these corepressors are the chromatin remodeling enzymes. Modification of chromatin structure is an important mechanism for regulating gene transcription (Felsenfeld 1992; Kingston and Narlikar 1999). One manner in which chromatin structure can be altered is by acetylation of histones. HATs have been shown to act as transcriptional coactivators that alter chromatin structure, thereby allowing transcription factors access to the promoter. In contrast, HDACs have been associated with transcriptional inhibition and are found in corepressor complexes (Alland et al. 1997; Grunstein 1997; Hassig and Schreiber 1997; Hassig et al. 1997; Heinzel et al. 1997; Laherty et al. 1997). Three of the seven known HDACs (HDAC1–

HDAC3) interact with Rb, and Rb can bind simultaneously to HDAC and E2F, allowing recruitment of an HDAC–Rb–E2F repressor complex at promoters of cell cycle genes (Brehm et al. 1998; Luo et al. 1998; Magnaghi et al. 1998; Lai et al. 1999a; Chen and Wang 2000; Dahiya et al. 2000). In two recent studies, Rb mutants that have amino acid substitutions in the LXCXE-binding site showed reduced binding to HDAC1 and HDAC2, but not HDAC3 (Chen and Wang 2000; Dahiya et al. 2000). The impaired interaction with HDAC1 and HDAC2 had no effect on the ability of the Rb mutants to inhibit transcriptional activation by E2F, but these mutants were unable to actively repress some genes and unable to maintain growth arrest. In addition, it has been shown that recruitment of an HDAC–Rb–E2F complex can actively repress transcription and regulate histone acetylation at the promoter (Luo et al. 1998).

1.10 CHROMATIN REMODELING: THE ROLE OF MULTIPROTEIN COMPLEX SWI / SNF CONTAINING BRM AND BRG-1

In the course of evolution the complex SWI / SNF has been stored for promoting events of chromatin remodeling and it is important for activation and for transcriptional repression of genes involved in the regulation of cell proliferation (Muchardt C. and Yaniv, M., 2001) . The chromatin-remodeling complexes are multiprotein complexes. The genes that encode complex SWI / SNF were initially characterized in Saccharomyces cerevisiae. The SWI genes or "faulthy mathing type switching" and SNF or" Sucrose Non-Fermenting "were defined as transcriptional activators involved in the regulation of a group of inducible genes (CWM Roberts and Orkin SH, 2004). In this organic complex SWI / SNF is composed by 11 proteins assembled into a complex of 2MDa. This complex acts transcriptional activity, affecting the structure of chromatin in the vicinity of the promoters of target genes (M. Carlson and BC Laurent, 1994). In mammals have been identified proteins homologous to many members of the complex SWI / SNF in yeast.

In Drosophila SWI2/SNF2 homologue is known as Brahma or brm (Tamkun JW et al., 1992). The gene encoding this protein was initially identified as a suppressor of Polycomb mutations. The protein resembles the brm complex SWI2/SNF2 throughout its length and contains a protein similar to yeast, a bromodomain type helicase and a domain with an ATP-binding site. As in yeast, brm is associated with a large complex that also contains SNR-1, the Drosophila homologue of SNF5. Even in mammalian cells have identified two

proteins closely related to SWI2/SNF2. These proteins, known in humans and mice hbrm/hSNF2alfa and BRG-1/hSNF2 Beta as mbrm and mBRG-1 (190 kDa) were both associated with the so-called including complex mammalian SWI / SNF containing at least eight proteins including homolog of SNF5 (hSNF5/IN1), BAF 155 and BAF 170, two homologous subunits of yeast SW13 e BAF a homologue of the 60 subunits of yeast SWP73 (Muchardt C. and Yaniv, M., 1993).

The multiprotein complex in mammalian SWI / SNF complex is composed of subunits that contain the BRG-1 or brm-1 activity with adenosine triphosphatase (ATPase) as central to hydrolyze ATP. BRG-1 and mbrm-1 are homologues of yeast SWI2/SNF2 ATP ase. There are two types of ATPases can be grouped into different subfamilies according to whether they contain a bromodominio (the SWI2/SNF2 subfamily), two pairs of cromodomini (the ISWI subfamily). All the members of different subfamilies seem to be part of a multi-subunit complex that can remodel nucleosomes by ATP-hydrolysis and increase or decrease the effects of repressive chromatin (SIf. S. et al., 2001). Within this complex, the two related protein BRG-1 and brm-1 are mutually exclusive indicating that co-exist at least two versions of the complex SWI / SNF in mammalian cells (Roberts, C. W. M. and Orkin H., 2004). Similar to their counterparts in yeast, the complex SWI / SNF purified in mammalian differentiated cells possess chromatin remodeling activity. The remodeling of chromatin is a process that participates in cell cycle control by acting directly on the structure of DNA. Chromatin consists of DNA wrapped in strings of nucleosomes (octamers of histone proteins, namely of two molecules of each histone H2A, H2B, H3 and

H4). Thanks to the nucleosomes, the chromatin structure in order to achieve higher order levels of organization that increase the compaction ratio.

The adjustment of the compaction ratio of nucleosomes structures is critical to the control of gene expression and is regulated by two mechanisms. The first mechanism is based on the modification of amino-terminal histone tails. The most studied covalent modification involves acetylation. This post-translational modification is the addition of acetyl groups mediated by histone acetyl transferase (HAT) on the lysine residues present N-terminal histone tails. The acetylation of histones cause relaxation of chromatin, facilitating access to transcription factors and the de-acetylation mediated by histone deacetylase makes the chromatin less accessible to transcription factors. The acetylation seems therefore more involved in the phenomena of transcriptional activation, in contrast to the deacetylation which causes repression of transcription. Recently, methylation has also played an important role in transcriptional repression. In fact, the histone methyltransferase SUV 23H1 seem involved in the phenomena transcriptional repression. The addition of a methyl group on lysine residues 9 Histone H3, by SUV39H1 creates a binding site with high affinity for the HP1 protein (heterochromatin protein 1) making the area of DNA, which was bound, transcriptionally inactive. The second mechanism depends on the complex SWI / SNF uses the energy released from ATP hydrolysis to move nucleosomes along the DNA helix facilitating the access of transcription factors. The complex SWI / SNF interacts with the chromatin is able to form stable areas of accessibility in the distribution of ordered nucleosomes to allow transcription factors access to their target sites.

It was also suggested a possible direct interaction of the complex SWI / SNF with RNA polymerase II holoenzyme to locate the start point of transcription (Owen-Hughes T., Workman JM 1996). The alteration of the ATP-dependent nucleosome is targeted to the histones made recognizable by modification of the enzyme complexes. The most common changes involve covalent bond formation by acetylation, phosphorylation, methylation and ubiquitination of histone molecules and / or non-covalently in the action of enzymes that remodel chromatin using the energy released by the hydrolysis of ATP (Roberts C.W.M. S. and Orkin. H., 2004). In some cases it is then that the complex SWI / SNF and HAT may cooperate for the transcriptional regulation (Roberts C.W.M. and Orkin S.H., 2004). Moreover, the complex SWI / SNF can remodel chromatin causing alterations of the position and shape of the nucleosomes in an energy-dependent hydrolysis of ATP generated. This affects the stability of the octamer of nucleosome causing slippage along the same DNA strand or the transfer of the octamer in regions adjacent to those of transcriptionally active DNA, increasing the accessibility to the transcriptional machinery. Recently, it has also been shown that the complex SWI / SNF are involved in regulating gene expression, also important because they can recruit transcription factors that make the structure even more complex (W. Wang et al., 1996). In differentiated cells is well documented interaction between pRb and BRG-1 or brm-1, which can only occur when pRb is in its hypophosphorylated form (Roberts C.W.M. and Orkin S.H., 2004). This suggests that SWI2/SNF2 proteins associated with Rb have an important role in tumor suppression. THE BRG-1 subunit of the SWI2/SNF2 multiprotein complex is involved in controlling cell proliferation and is a likely candidate in the role of a tumor suppressor.

The mutant BRG-1 - / - is lethal at an early stage blastocysts, and heterozygous for the mutation in BRG-1 + / - induce rhabdoid tumor, an aggressive form of cancer in children that affects various types of tissues. The reintegration of the gene BRG-1 is able to stop the rapid proliferation of cancer cells (CWM Roberts and Orkin SH, 2004). It is believed that the arrest of the growth of cancer cells for the inclusion of BRG-1 is in part due to decreased expression of target genes of the E2F family transcription factors such as cyclin E, but even more by the increased mRNA transcription of the cyclin-dependent kinase inhibitors (CDI) as p21 and p15 (H. Kang et al., 2004). In differentiated mammalian cells the regulation of cell proliferation activities attributed to BRG-1 seems to depend on its ability to interact with the tumor suppressor pRb, thereby suppressing the expression of target genes dependent on E2F. pRb can bind both BRG-1 and brm. The BRG-1 complex / brm appears to regulate the phosphorylation of pRb through modulation of cyclin-dependent kinase inhibitor p21.

It has been shown that the complex HDAC-Rb-SWI/SNF repress the target genes of E2F such as cyclin E, cyclin A and cdc2 blocking the cycle at G1 phase. The activation of cyclin D/CDK4-6 complex in differentiated cells in response to mitogenic stimuli cause the phosphorylation of Rb. This allows the release of HDAC and the S-phase transition. The complex Rb-SWI/SNF is so able to activate the expression of cyclin E, whereas the expression of cyclin A is still repressed. Cyclin E forms a complex with CDK2 that can phosphorylate Rb and cause the release of the complex SWI / SNF, the activation of the expression of cyclin A and entry into G2 phase. The association ordered in time of the

BRG-1 with complexes HDAC and pRb allows the sequential activation of cyclin E and cyclin A by inducing progression of the cell cycle.

All the purified complexes contain different subunit except the complexes SWI2/SNF2 where BRG-1 and brm are subunite highly conserved. The similarities between the subunits are also found in the case of the human histone deacetylase complex remodeling of nucleosomes (NuRD) containing Mi-2 as ATP ase center. Nurd contains closely related subunits, which include HDAC-1 and -2 proteins associated to retinoblastoma (RBA) Rb and p46, and p48 itself (Sif, S. et al 2001), As mentioned above, if acetylation is a type of chromatin modification that triggers the gene expression, histone deacetylation results in the repression of gene expression. It was in fact shown that brm and BRG-1 are able to work on recruiting other chromatin complexes such as HDAC-1 and 2 and their counterparts co-repressors of yeast Sin3, and mSIn3A mSin3B. It has been proposed that the repression of specific promoters may be permitted by the recruitment of the complex HDAC/mSIn3A BRG-1/brm-1. This complex also contains Rb HDAC/mSin3A AP46, Rb AP48 and mSin3A associated proteins such as SAP18, and SAP30, whose function is unknown (Sif, S. et al., 2001).

By studying the interactions of individual fractions purified from the complex SWI / SNF that brm-1 is capable of co-precipitated with HDAC-1, HDAC2, Sin3A, and Rb AP48 and BRG-1 co-precipitate with small amounts of HDAC-2 and mSIn3A AP48 Rb but not with HDAC-1. This different composition of protein complexes associated with brm and BRG-1 may explain the different functional characteristics of these proteins.

In fact it seems that BRG-1 has a greater ability to hydrolyze ATP and remodel the chromatin and then working both in the conformation of the nucleosome and altering its position. BRM chromatin remodeling can only change the order in the position of nucleosomes (Sif S . et al., 2001). The expression of BRG-1 is ubiquitous at all stages of development from the earliest pre-implantation embryo, whereas the expression of brm is absent in these stages and increases at later stages in the embryo at the stage of blastula . At this stage the cells leave the cell cycle to be targeted to cell differentiation.

1.11 **SWI/SNF-BRG1 REGULATES SELF-RENEWAL AND OCCUPIES CORE PLURIPOTENCY-RELATED GENES IN EMBRYONIC STEM CELLS**

The SWI/SNF-Brg1 chromatin remodeling protein plays critical roles in cell-cycle control and differentiation through regulation of gene expression. Loss of Brg1 in mice results in early embryonic lethality, and recent studies have implicated a role for Brg1 in somatic stem cell self-renewal and differentiation. However, little is known about Brg1 function in preimplantation embryos and embryonic stem (ES) cells. Il gruppo di Kidder B. L. , 2009, report that Brg1 is required for ES cell self-renewal and pluripotency. RNA interference-mediated knockdown of Brg1 in blastocysts caused aberrant expression of Oct4 and Nanog. In ES cells, knockdown of Brg1 resulted in phenotypic changes indicative of differentiation, downregulation of self-renewal and pluripotency genes (e.g., *Oct4, Sox2, Sall4, Rest*), and upregulation of differentiation genes. Using genome-wide promoter analysis (chromatin immunoprecipitation) they found that Brg1 occupied the promoters of key pluripotency-related genes, including *Oct4*, *Sox2*, *Nanog*, *Sall4*, *Rest*, and *Polycomb group* (*PcG*) proteins. Moreover, Brg1 co-occupied a subset of Oct4, Sox2, Nanog, and PcG protein target genes. These results demonstrate an important role for Brg1 in regulating self-renewal and pluripotency in ES cells.

1.12 **REGULATION OF CYCLIN E1 GENE BY THE COMPLEX REPRESSOR CERC2**

In somatic cells the regulation of G1 / S is governed by sequential activation of pRb and cyclin complexes / Cdks. Precisely pRb forms a complex with E2F, recruiting HDAC and SWI / SNF, able to repress the transcription of genes involved in G1 / S cell cycle such as the gene encoding the cyclin E1 CCNE1. When, in response to appropriate stimuli proliferative, pRb is phosphorylated and inactivated by the complex cyclin / Cdks, the cells complete the G1 phase and progress into S phase. The phosphorylation of Rb by cyclin complex D1/Cdk4-6 promotes the release of HDACs from the domain "pocket" of pRb. The removal of HDAC leads to increased acetylation of a specific nucleosome located at the site of initiation of transcription of cyclin E1 and its subsequent activation. The activation of the promoter of cyclin E1, which occurs during the G1 / S decreases in S phase and disappeared during the period between the end of mitosis and the late G phase. The expression of cyclin E1 reflects transient activation of gene promoter CCNE1 (Cyclin E1 gene) that occurs during the G1 / S and then fall in the S phase and disappear during the period between the end of mitosis and late G phase.In quiescent mouse fibroblasts has been shown that the promoter activity of cyclin E1 gene is controlled by a DNA sequence defined as "cyclin E repressor module" (CERM), located downstream of the transcription start site and consists of a variant of the canonical E2F sites and a sequence rich in AT. Both sequences are necessary to bind a complex of high molecular weight E2F called "cyclin E repressor complex 1" (CERC1) that prevents the transcription of cyclin E1. When

fibroblasts begin to proliferate, the complex dissociates from the sequence CERC1 CERM and is induced transcription of cyclin E1.CERC1, even if it contains E2F4/DP1 pocket and the protein p130, E2F complexes differs from canonical in that it has additional components as observed through a glycerol gradient sedimentation slowed (Le Cam et al., 1999). Similar studies have shown that, even in proliferating K562 cells, the repression of the gene regulates CERM CCNE1 in the period between the end of mitosis and late G1 phase through its association with a high molecular weight E2F complex (Polanowska et al. 2001). Further studies have shown that this complex possesses histone deacetylase activity and was named CERC2.EMSA experiments performed with a DNA probe that spans the promoter region of the murine cyclin E1 gene (CERM probe) and nuclear extracts prepared from K562 cells blocked in G1, show that the complex CERC2 reacts with antibodies directed against p107, E2F4 , DP1, cyclin E1 and CDK2 but not with antibodies directed against Rb, p130 and E2F family members such as E2F1, E2F2, E2F3, E2F5 and cyclin A1. Ultimately CERC2 is a complex of high molecular weight containing G1-specific E2F4/DP1, p107, cyclic E1 and CDK2 (Polanowska et al., 2001). The repressive role of the CERC2 gene promoter of cyclin E1 requires conformational changes in chromatin structure.To investigate whether the complex contains CERC2 among its members and the HDAC complex SWI / SNF, other experiments were carried out EMSA and inhibitor TSA, which have shown that histone deacetylase activity exists in the K562 and BRG -1 and Brm are not associated with CERC2. In particular, the repression of the gene CCNE1 correlates with deacetylation of histones H3, H4, and methylation of arginine and lysine K9 of histone H4 R3 of the single nucleosome that includes CERM (Nielsen et al., 2001,).The

transcriptional activation of the gene CCNE1 during late G1 phase is determined by the reduction of methylation in the same region. Methylation of histone lysine methyl transferase is produced by lysine methylation of arginine SUVAR39H1 while the protein is left to the "R-methyltransferase" (PRMT). In mammals, nine proteins were identified PRMTs divided into two groups for their ability to catalyze one or two methylation reactions, both symmetric and asymmetric. In mammals the activity of PRMT1 and PRMT4 responsible for the formation of asymmetric dimethylarginine, is associated with gene activation (Bauer UM et al., 2002), while PRMT5 catalyzes the symmetrical dimethyl associated with transcriptional repression. It has recently been shown that arginine methyl transferase PRMT5 is part of the complex transcriptional repression of cyclin E1 promoter of the gene and its methyl-transferase activities sull'istone H4 is a prerequisite for the blockade of transcription and the consequent inhibition of cell proliferation (Fabbrizio E. et al., 2002).

1.13. **DIFFERENTIATION OF THE CES AND IPS.**

The removal of LIF from the culture medium of ESC is sufficient to induce differentiation programmed to form all types of cells which carry out embryonic development. This is possible in vitro through the formation of specific cellular structures called embryoid bodies (EB) for their similarity to post-implantation embryonic tissues. These cells contain the precursors of all three germ layers and mimic gastrulation and organogenesis early (Savatier et al., 1995). Alternatively, differentiation can be induced experimentally by adding the appropriate chemicals (such as retinoic acid in the case of this study) that address the ESC differentiation toward a specific system. For the incredible potential for differentiation in vitro, the ESCs and iPS are only one cell type to develop strategies for an efficient gene therapy. In addition, CES is an ideal system to study the mechanisms involved in the processes of early embryonic development and cell differentiation and tissue.From the molecular point of view, during the early stages of development, when cells must be targeted to specific lines, the differentiation of ESCs and iPS is associated with a rearrangement of the expression and gene silencing, processes that are intimately connected with the alteration of chromatin structure (Lee et al., 2004). In fact, the dynamic changes of chromatin structure facilitate or inhibit the access of transcription factors to DNA. At least two mechanisms can be used to alter or remodel the structure of chromatin: one involving multiprotein complexes SWI / SNF, which use the energy of ATP to change the position and / or conformation of nucleosomes, and the other involves the modification both

covalent DNA (cytosine methylation) and histone tails (phosphorylation, methylation and acetylation). In cells in active proliferation, the genes are regulated by the cyclic recruitment of HDAC and HAT but remain nell'eucromatina; the induction of differentiation leads to DNA methylation or histone H3, which triggers the repositioning of genes and nell'eterocromatina through their HDAC silencing (Fig. 3) (Ferreira et al., 2001).These processes were examined during in vitro differentiation of ESCs and iPS : histone acetylation, a marker dell'eucromatina, declines rapidly after induction of differentiation, and then be partially restored on the contrary, methylation of histone lisina9 H3, a marker of heterochromatin, increases following induction of differentiation. This is consistent with the model that the differentiation of stem cells is accompanied by a restriction of a series of genes that are expressed. The global deacetylation of histones is required for the differentiation of ESCs: the histone acetyltransferase and deacetylase transmit signals to initiate differentiation of the appropriate epigenetic modifications, as well as the elimination of the existing structure of chromatin and the establishment of a new pattern of global change is both gene-specific during the differentiation in vitro (Lee et al., 2004).Taken together, these results suggest that the structure of chromatin at the promoter of a gene key to differentiation is programmed early in development.

1.14 **RETINOIC ACID AS AN INDUCER OF DIFFERENTIATION**

Retinoic acid (RA), a derivative of vitamin A (retinol), has an important role as an endogenous factor during embryogenesis, morphogenesis and cell proliferation. It is known that RA has two distinct roles during the early stages of cell differentiation: it inhibits myogenesis and cardiogenesi, while simultaneously promotes neurogenesis and adipogenesis (zur Nieden et al., 2004). The RA may also regulate growth and differentiation of a wide variety of pre-malignant and malignant cells, both in vivo and in culture.

In CES, the RA can induce differentiation into cells ectoderm, mesoderm and endoderm, in a time-dependent and concentration. Under certain conditions, ESCs differentiate into extraembryonic cells, similar to the parietal endoderm (Chen and Gudas, 1996).The extensive self-replication of CES depends on the balance between signals that promote the proliferation and differentiation. In CES, the addition of RA induces differentiation in part by blocking the signals transduced by LIF (Tighe and Gudas, 2004).The main mechanism by which RA regulates the differentiation, is expressed through the action of nuclear receptors for RA (RAR and RXR), expressed in ESCs and during embryonic development. In the presence of different isomers of RA, RAR and RXR receptors form heterodimers that activate the transcription of specific genes for differentiation, including the homeotic genes. The RA may also indirectly regulate the expression of many genes involved in pre-and postnatal development (Chen and Gudas, 1996).

1.15 METASTABLE STATES OF PLURIPOTENCY

The term “metastability” has been previously used to describe transient changes within ICM like ES cell populations resulting from oscillations in Nanog or Stella gene expression (Chambers et al., 2007; Hayashi et al., 2007). The goup of Jacob Hanna (Hanna J et al 2009) apply this term to describe the interconversion between two distinct pluripotent states in NOD and 129 mouse strains. Them results suggest that the ICM and EpiSC pluripotent states may be in a “metastable” equilibrium dictated by the genetic background where exogenous factors can convert one state into another. Thus, one may consider the two states of pluripotency, the ICM/ES cell-like state and the epiblast/EpiSC cell-like state, as two different levels of pluripotency. Exogenous factors such as c-Myc and Klf4 in combination with Oct4 and Sox2 can induce the ICM-ES like state from somatic cells. However, the stability of the ES cell state is determined by the genetic background: while ICM-ES cells or iPS cells derived from a “permissive” genetic background such as 129 or C57BL/6 are stable once established in the presence of Lif, the ES cell like state of iPS cells or of ICM derived pluripotent cells of the ”non-permissive” NOD background remains unstable with the maintenance of the pluripotent state depending on the continuous expression of the exogenous factors in addition to Lif/Stat3 signaling. Inactivation of the transcription factors or removal of the inhibitors causes the ES like NOD cells to assume an EpiSC-like state, characterized by reduced pluripotency. Inter-conversion between these states can be controlled by the absence or presence of the same factors. Several lines of evidence support the notion that the conversions between the different pluripotency states are due to cells being inefficiently induced to successfully convert from one state to another, rather than

due to selection for rare pre-existing cells constantly present in heterogeneous stem cell populations. First, evidence for direct reprogramming of EpiSCs into iPS cells is supported by the observations that EpiSC cells do not convert spontaneously into ES like cells and that all derived Epi-iPS cell lines carried integrated viral transgenes. Second, the EpiSC to ES cell conversion requires multiple passages in defined media and continuous transgene induction, which is similar to generating iPS cells from somatic cells (Jaenisch and Young, 2008)). Third, it is unlikely that NOD ES cultures carry already rare EpiSC-like cells since the NOD iPS or ES lines were passaged routinely by trypsinization, which does not allow propagation of the EpiSC cells. Finally, the NOD EpiSC-like iPS cell line carried an identical Sox2 integration as its parental Dox dependent NOD iPS line indicating a clonal relation. An important question remains why only a small fraction of the NOD ES cells convert into an EpiSC state. One possibility is that after removal of the exogenous stimuli, the EpiSC state becomes one of several epigenetic states that can be acquired by the NOD ES cells upon differentiation.

1.16 CONTROL OF EMBRYONIC STEM CELL METASTABILITY BY L-PROLINE CATABOLISM

The molecular mechanisms controlling mouse Induced Pluripotent Stem Cells (iPS) metastability, i.e. their capacity to fluctuate between different states of pluripotency, are not fully resolved. The Casalino L., group (2011) has for first induced EPISC from CES using as inducer a fisiological esposition at L-Prolyn. I developed and used this platform, for induce the iPS to differentiate in EPISC. L-Pro, force iPS toward a novel epiblast stem cell (EpiSC)-like state, in a dose- and time-dependent manner. Unlike EpiSCs, L-Pro-induced cells (PiCs) contribute to chimeric embryos and rely on leukemia inhibitor factor (LIF) to self-renew. Furthermore, PiCs revert to ESCs or differentiate randomly upon removal of either L-Pro or LIF, respectively. Remarkably, PiC generation depends on both L-Pro metabolism (uptake and oxidation) and Fgf5 induction, and is strongly counteracted by antioxidants, mainly L-ascorbic acid (vitamin C, Vc). ESCs↔PiCs phenotypic transition thus represents a previously undefined dynamic equilibrium between pluripotent states, which can be unbalanced either toward an EpiSC-like or an iPS phenotype by L-Pro Vc treatments, respectively. All together, our data provide evidence that iPS metastability can be regulated at a metabolic level just as the CES.

2. MATERIALS AND METHODS

2.1. ES AND IPS CELL CULTURE

The cell cultures used in this study are:

- Mouse embryonic stem cell lines (ES CGR8)
- Mouse Induced Pluripotent Stem Cell (iPS #202)

2.1.1 LINES OF MOUSE EMBRYONIC STEM CELLS CGR8 AND IPS.

A cell line used in the experiments of this study, is called CGR8 (Exponentially growing germ-line feeder-independent embryonic stem cell line Competent) and is derived directly from the blastocyst inner cell mass of a mouse. These are embryonic stem cells (ESCs) and in vitro retain the capacity to differentiate into all mature somatic phenotypes when induced by appropriate signals. The CGR8 can be grown indefinitely in culture in an undifferentiated state in the presence of leukemia inhibitory factor (LIF) (Savatier et al., 1995). Another cell line used in this study is iPS #202 .The CGR8 and iPS #202 grow in the culture medium GMEM ("Glasgow's Modified Eagle's Medium", BioMed), containing sodium bicarbonate (3.7 g / l) and complemented with fetal calf serum (FCS, 10%), L-

glutamine (2 mM) , non-essential amino acids (1%), sodium pyruvate (1 mM), 2β-mercaptoethanol (0.1 mM), penicillin (100U/ml), streptomycin (100mg/ml) and LIF (1000U/ml). These cells are adherent and are grown in 10 ml Petri dishes, thin layer of gelatin (0.2%). The cES grow exponentially in an incubator at 37 ° C with 7.5% CO2 and, in general, you must change the culture medium every two days. Reached confluence, the cells are counted, collected and analyzed for protein extraction, in the case of this study.

2.2 MICE

Isl1-Cre mice were generously provided by Sylvia Evans (University of California–San Diego, La Jolla, CA, USA) (24). The conditional Cre reporter mouse lines R26R-LacZ and R26RYFP were generated by Philippe Soriano (Mt. Sinai School of Medicine, New York, NY, USA) (25) and Frank Costantini (Columbia University Medical Center, New York, NY, USA) (26), respectively. Isl1-Cre/R26R double-heterozygous mice were generated by crossing single-heterozygous mice. Mice are in a mixed 129 _ C57Bl/6 background.

2.3 CELL CULTURE

For isolation of dermal fibroblasts, skin from newborn Isl1- Cre/R26R mice and human dermal biopsies were minced into 2-mm pieces, placed on culture dishes, and incubated in Quantum 333 medium (PAA, Pasching, Austria). Cells migrating out of the explants were passaged in DMEM containing 10% FBS and used at passage 4 for iPS cell induction. Mouse iPS cells were grown on mitomycin-C-treated murine embryonic fibroblasts (MEF feeders) in standard ES medium [DMEM supplemented with 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM _-mercaptoethanol, 50 U/ml penicillin, 50 _g/ml streptomycin,and 0.1 _g/ml leukemia inhibitory factor (LIF)] containing 15% knockout serum replacement (KSR; Invitrogen, Karlsruhe, Germany) for 5 passages and then maintained in ES medium containing 15% FBS. Human iPS cells were grown on MEF feeders in human ES medium consisting ofDMEM/F12 supplemented with

20% KSR, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 0.1 mM _-mercaptoethanol, 50 U/ml penicillin, 50 _g/ml streptomycin, and 10 ng/ml human b-FGF (R&D System, Minneapolis, MN, USA). Human research subject protocol was approved by the institutional review boards and the ethics committee of both the Klinikum rechts der Isar and the Technical University of Munich.

2.4 RETROVIRAL PRODUCTION AND IPS CELL INDUCTION

Dermal fibroblasts were isolated from 3-d-old Isl1- Cre/R26R-indicator double-heterozygous mice and retrovirally transduced with either 4 transcription factors, Oct4, Sox2, Klf4, and c-Myc, or with only 3 in the absence of c-Myc. As expected, 3–4% of parental fibroblasts were already positive for the marker genes LacZ or YFP before transduction because Isl1 expression has been reported in melanocytes of the skin. Nevertheless, after retroviral infection, only 5 of 123 picked clones that generated cell lines with ES-like morphology were LacZ or YFP positive indicating that Isl1-derived skin cells are not more amenable to reprogramming. We excluded these clones from the present study and performed all further analysis on the marker negative ones. Thirty of 35 tested clones presented characteristics of iPS cells such as expression of ES cell markers (alkaline phosphatase and Nanog), reactivation of the endogenous pluripotency genes (*Oct4, Sox2, Klf4,c-Myc, Rex1,* and *Nanog*), and silencing of retroviral transgenes.

iPS#202 (Moretti et al 2010) were maintained on mitomycin-treated MEF feeder cell layers, cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Invitrogen), supplemented with 15% fetal bovine serum (FBS; GIBCO, Invitrogen), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol,5 mg/ml penicillin, 100 mg/ml streptomycin, and 10^3 U/ml leukemia inhibitory factor (LIF, Chemicon International). Subsequently, iPS#202 cells were replated and cultured without MEFs on 0.1% gelatin-coated 100-mm tissue culture dish (IWAKI, Bibby Scientific) for at least three passages in Glasgow Minimum Essential Medium (GMEM; GIBCO, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 5 mg/ml penicillin, 100 mg/ ml streptomycin, and 10^3 U/ml LIF. Medium was renewed every day.

2.5 CHEMICAL TREATMENT OF IPS AND CES WITH RETINOIC ACID .

The protocol used in this study to induce differentiation of ESCs provides for the removal of LIF and retinoic acid treatment. This treatment produces a change in the cell cycle of the ESC in response to retinoic acid, the proportion of cells in G1 phase increased progressively with a concomitant decrease of cells in S phase (Savatier et al., 1995). The first day, cell cultures that reached confluence in the presence of LIF, were treated with trypsin and grown in a medium devoid of LIF in low density. The next day, is added to the culture medium, retinoic acid (Sigma, $0{,}5x10^{-6}$M) to accelerate the differentiation. This treatment lasts for 72 hours. On the third day, the culture medium was replaced with fresh medium, deprived of retinoic acid, to minimize the effects of apoptosis due to long exposure. The cells undergoing differentiation, are grown in these conditions for a day. The cells are counted and collected for protein analysis, in the case of this study.

2.6 EMBRYONIC BODIES INDUCTION (ASCORBIC ACID TREATMENT)

Mouse and human ES/iPS cells can differentiate into various cell types, including cardiomyocytes, neuronal cells, and embryonic erythrocytes . However, the efficiency of cardiomyocyte differentiation is poor and the differentiated cells are a heterogeneous mixture of various types of cells. To improve the efficiency of cardiomyocyte differentiation, the directed differentiation of ES/iPS cells into cardiomyocytes was induced by the supplementation of signaling molecules, such as Ascorbic Acid (final concentration 50μg/ml).

It has so far been reported that human cardiomyocytes could be induced from pluripotent stem cells by several methods. Using an embryoid body formation assay, human ES/iPS cells can differentiate into beating cardiomyocytes in the presence of fetal bovine serum .Under the serum-free conditions, with the supplementation of several cytokines, including Activin A and BMP4, the embryoid body can efficiently differentiate into cardiomyocytes.

The hanging drop method provides uniform sizes of EBs by dispensing equal numbers of ES cells in physically separated droplets of media suspended from the lid of a Petri-dish. This method offers a similar environment for forming individual EBs within each drop *via* gravity-induced aggregation of the cells. For this reason, this technique has been used to generate plentiful cell types such as neuronal cells, hematopoietic cells, cardiomyocytes , vascular cells and chondrocytes. The hanging drop method is tremendously useful for appraisal of molecular mechanisms occurring in early embryogenesis

in any cell type**.** The hanging drop method is composed of two steps; the aggregation of ES cells in drops and maturation of aggregates to EBs in suspension culture using low adherence bacterial Petri-dishes. Several elements of the method may be troublesome such as losses of EBs during picking up the formed EBs by pipette and attachment of premature EBs on Petri-dishes. For this motif it has been used different cellular suspensions of $1x10^4$ $/10^5$ $/10^6$ cells/ml. After 2 day-old hanging drops transfer the EB to 60 mm petri dishes and adjust the volume to 4 ml with ES/iPS differentiation media. After 3 days incubation at 37°C the colture of EB were transferred in adhesion. Spontaneous contractile cardiomyocites start to appear after 3 days adhesion and can be easily identified by microscopy.

2.7 EPICS INDUCTION WITH L-PROLYN

L-Pro and L-Orn induce the phenotypic switch in a dose- and time-dependent manner (Casalino , 2011). Physiological concentrations of L-Pro in human and mouse plasma range from 0.1 mM to 0.5 mM. We thus used the Microscopy for evaluate the effect of increasing concentrations of both L-Pro on the colony phenotype of iPS #202s. Low amounts of L-Pro (30 mM) already induced the formation of a significant fraction (25%) of atypical (flat or mixed shape) cell colonies compared with control (4%–8%). The results were then validated in manual assays and, remarkably, up from 75 mM L-Pro, the majority of the colonies (85%–90%) displayed a clear-cut EpiSC-like morphology, after 4 days in culture. Thus, physiological concentrations of L-Pro were sufficient to trigger the phenotypic switch of iPS in EPISC phenotype. In my studies I have used a final concentration of L-Pro of 150 μM.

2.8 THE CELL COUNTING AND SAMPLING

The samples of ES cells treated with LIF alone and those cells treated with other differentiation agents are taken at time varies depending on the type of sampling treatment. The cell sampling consists of a series of washes in PBS. The cells are then mechanically detached from the Petri Dish taken with the help of 1 ml of medium or PBS. A small aliquot of cells resuspended in a few μl of PBS or culture medium is collected and diluted with the addition of Trypan Blue dye (1X). This staining technique is fundamental to distinguish dead cells when counting those lives. In fact, the cells lost due to death due to lysis of cell membrane allow the entry of the dye staining assuming a typical blue while live cells appear as small transparent particles observed by phase contrast microscopy. Counting of the cell is made by applying the Burker chamber with factor dilution of 1:10000 and counting only the living cells present in a grid . Following the count the right number of cells at different dilution factors is multiplying by the dilution factor of the dye, the dilution factor of the Burker chamber and the total volume. After the cell count samples were centrifuged at low rpm to separate the liquid suspension containing cellular debris by live cells that are deposited on the bottom, forming a pellet (precipitated). Pellets obtained from nuclear protein extracts can be analyzed in various ways.

2.9 ANALYSIS OF NUCLEAR PROTEIN IN CES AND IPS

The nuclear proteins, which includes several transcription factors taken into account in our studies may be extracted from the cells separately from cytoplasmic proteins. The number of cells from which we start to get the nuclear protein extracts is about $2x10^6$-$5x10^6$ cells. The protocol used is as follows: after 2 washes in 1X PBS most of the cells is removed mechanically by Petri and collected in 1 ml of PBS. Centrifuge for 10 seconds in a refrigerated microcentrifuge at 4 ° C resulting in a pellets. After eliminating the supernatant , the pellet is dissolved in 400 μl of cold Buffer A (a solution that allows cell lysis by keeping intact nuclei made by 10 mM HEPES pH 7.9, 1.5 mM MgCl, 10 mM KCl, 0.5 DTT mM, 0.2 mM PMSF and a mixture of inhibitors of the protease (20X)). The samples are incubated on ice for 10-15 minutes and then centrifuged again at a speed of 10,000 rpm for a few seconds to delete the supernatant (containing membranes, cytoplasmic proteins and cellular debris) and get the nuclei that precipitate to the bottom. The nuclei have to be dissolved in 20-40 μl of cold buffer C (20 mM Hepes pH 7.9, 25% glycerol, 420 mM Na Cl, 1.5 mM MgCl 2, EDTA 0.2 mM, 0.5 mM DTT, 0.2 mM PMSF and a mixture of protease inhibitors (20X)). Lysed in buffer C nuclei are incubated on ice for 20 minutes and centrifuged in a refrigerated microcentrifuge set to 4 ° C at 13,000 rpm for 10 minutes. The supernatant containing the protein is recovered and frozen in liquid N2. The samples are stored at -80 ° C.

2.10 COLORIMETRIC ASSAY FOR QUANTITATIVE PROTEIN ANALYSIS

Proteins extracted from each sample were quantified by spectrophotometer readings at a wavelength of 595 nm in order to determine their optical density (OD). To perform this assay a small quantity of each protein sample is added to the chemical reagent Bio-Rad (1X), resulting in a change in colour intensity of the protein concentration .This colour change is measured in a spectrophotometer to obtain the OD reading for each sample. In parallel, a standard curve is constructed showing the ordinate values of the OD as a function of the concentration values on the abscissa. The OD values come from the reading of samples of known concentration of bovine serum albumin (BSA) treated with the same reagent. Concentration μg / μl of each protein sample is obtained by interpolating the value of his reading of the OD with the standard curve.

2.11 ACRYLAMIDE ELECTROPHORESIS WITH SDS (SDS PAGE)

This technique allows to separate the proteins according to molecular weight, non-native conditions for the presence of Sodium Dodecyl Sulphate (SDS). The anionic detergent SDS, which binds tightly to the protein amino acid canceling the charges. The technique takes advantage of the migration of proteins in an acrylamide gel, subjected to an electric field. It takes two types of gel ("stacking" and resolution) layered over one another, placed in a vertical chamber. The gel of "stacking" (40% acrylamide, Tris 2M pH 6.8, 10% SDS, 10% APS and TEMED), placed on top, allowing all to get along with protein samples in gel resolution, regardless of their molecular weight. The gel resolution (40% acrylamide, 1.5M Tris pH 8.8, 10% SDS, 10% APS and TEMED) has a concentration variable, depending on the molecular weight of the protein to be separated, in general, the more concentrated gels are more well-separated proteins of low molecular weight. The protein samples for analysis (~ $1x10^6$ cells) are diluted in a loading solution (0.5 M Tris pH 6.8, 10% SDS, 50% glycerol, 0.05% β-mercaptoethanol, 0.05% Bromophenol Blue) and denatured for 5 minutes at 98 ° C. Next to the sample, it is running a standard of known molecular weights. The electrophoresis takes place for about an hour and thirty minutes at a constant voltage of 180V, in a running buffer (Running Buffer1x: 25 mM Tris, 192mm Glycine, SDS 0.1%). The protein of interest is then detected by Western blot, by reaction with specific antibody.

2.12 CES AND IPS GENE EXPRESSION ANALYSIS THROUGH WESTERN BLOTTING

This system allows to transfer macromolecules from a gel in which the electrophoretic separation has occurred, an immobilizing membrane. The proteins are transferred from the gel to the membrane while maintaining the form and level of dissemination acquired at the end of electrophoresis. The membrane used is a nitrocellulose (Hybond). The transfer is a grid in which the gel in contact with the membrane is placed between two sponges and two sheets of paper Wattmann 3mm. The transfer buffer, proteins with MW> 20KDa, is composed of 50 mM Tris, 380 mm glycine, 0.1% SDS, 20% methanol. The transfer occurs at 80 mA overnight. The membrane is thereafter treated with Blotto (1x TBST, milk powder 5% fat) for 2 hours at room temperature to block nonspecific sites of the nitrocellulose. Subsequently, the membrane is incubated with the primary antibody directed against the protein of interest, in blotto and agitation. The incubation times vary from two hours to overnight, depending on the antibody being used. We shall, therefore, three washes, each for 10 minutes with 1x TBST (150 mm NaCl, Tris-HCl pH7.6, 0.1% Tween-20) at room temperature, stirred. The membrane is then treated to an hour with the secondary antibody (diluted 1:3000) directed against the primary antibody. The secondary antibody is conjugated with horseradish peroxidase, allowing detection. This is obtained by chemiluminescence, using ECL (chemiluminescence Existing Detection Reagents) and autoradiography.

2.13 USED ANTIBODIES.

Rb monoclonal antibody (IF-8: sc-102 Santa Cruz), polyclonal Rb (C-15: sc-50 Santa Cruz), p107 polyclonal (C-18 Santa Cruz), p130 polyclonal (C-20 Santa Cruz), E2F1 polyclonal (C-20: sc-193 Santa Cruz), E2F2 polyclonal antibody (L-20: sc632 Santa Cruz), E2F3 monoclonal antibody (clone PG37 Upstate Biotechnology), E2F4 monoclonal antibody (Ab-1, Oncogene), polyclonal E2F5 (E-19: sc-999 Santa Cruz), polyclonal Oct4 (Santa Cruz H-134), HDAC-1 polyclonal (H-11: sc-8410 Santa Cruz), HDAC-1 monoclonal antibody (H-51: sc-7872 Santa Cruz), HDACs -2 polyclonal (H-54: sc-7899 Santa Cruz); brm monoclonal antibody (Transduction Laboratories clone 24), Sin3A monoclonal antibody (Transduction Laboratories clone 2); RBBP monoclonal antibody (Clone 12 Transduction Laboratories), anti-acetyl-histone H3 and H4 polyclonal (Upstate), polyclonal cyclin E (Upstate), polyclonal Id2 (C-20X: sc-489 Santa Cruz), polyclonal ID4 (L-20: sc-491 Santa Cruz).

2.14 IMMUNOPRECIPITATION WITH DYNEABEADS

This techniques consist in three steps: Binding of Antibody (Ab), Immunoprecipitation of Antigen (Ag), Elution of Ab/Ag complex.

Binding of Antibody (Ab):

Completely resuspend Dynabeads by pipetting or rotating on a roller (5 min) and transfer 50 µl Dynabeads to a tube, place on magnet and remove supernatant. Remove tube from magnet and resuspend the Dynabeads in 200 µl Ab Binding & Washing Buffer containing the Ab of choice. *(Typically 1 - 10 µg Ab, the optimal amount needed will depend on the individual Ab used).*

Incubate 10 minutes with rotation at room temperature. and place tube on magnet and remove supernatant. Remove tube from magnet and wash the Dynabeads-Ab complex by resuspending in 200 µl Ab Binding & Washing Buffer.

Immunoprecipitation of Antigen (Ag)

Place tube on magnet and remove supernatant and add the Ag-containing sample (typically 100 - 1,000 µl) to the Dynabeads-Ab complex and gently resuspend by pipetting. Incubate 10 minutes at room temperature with rotation. Place tube on magnet, transfer supernatant to a clean tube. Wash the Dynabeads-Ab-Ag complex 3 times, using 200 µl Washing Buffer for each wash. Mix gently by pipetting. Resuspend the Dynabeads-Ab-Ag complex in 100

µl Washing Buffer and transfer the suspension to a clean tube. Place tube on magnet and remove supernatant.

• Elution of Ab/Ag complex *(alternatives A: denaturing or B: non-denaturing)*

Gently resuspend the Dynabeads-Ab-Ag complex in 20 µl Elution Buffer. Incubate 2 minutes at room-temperature. Place tube on magnet and transfer supernatant/sample to a clean tube.

2.15 EMSA (ELECTROPHORETIC MOBILITY SHIFT ASSAY)

There are differences in electrophoretic mobility between the DNA-free protein, which migrates in a polyacrylamide gel more quickly, and a protein complex bound to DNA, which migrates more slowly. The DNA fragment was radioactively labeled with 32P at 5 ', while the proteins are not marked. This technique is very sensitive because the DNA probes used have a high specific activity and because the conditions of polyacrylamide gel shift the balance in favor of complex formation, resulting in detection of even weak interactions.

2.15.1 MARKING END IN 5 '(CERM)

The oligonucleotide used in this study corresponds to the sequence of the gene promoter CCNE1 (CERM probe). The sequence is 5'-CAG CTC GAT GCT GAC ATT GGG TTT AAA TGT CCC GCT CGA AGT CAT C -3 '.The labeling reaction the 5 'end of an oligonucleotide is the transfer of one atom of radioactive phosphorus isotope (γ-32P)-ATP to the 5'-terminal OH. This reaction is catalyzed by T4 polynucleotide kinase. For this reaction are used 16ng of oligonucleotide to which are added 4µl Forward Reaction buffer 5x, 3µl of (γ-32P) ATP, 2µl of enzyme T4 polynucleotide kinase (10U/µl), in a final volume of 20µl. The reaction is carried out for one hour at 37 ° C.
The nucleotides not incorporated in the probe are eluted in a column chromatographic kits supplied by Qiagen (QIAquiek Nucleotide Removal Kit).
The specific activity of the probe is measured by reading the cpm (counts per minute). 1µl

of probe is added to 5ml of liquid scintillation (Packard Opti-Fluor) and the reading is made to a β-counter (Beckman LS 5000 TD), a device able to measure only the radiation type β.

2.15.2 ANNEALING AND ACRYLAMIDE GEL ELECTROPHORESIS ON (CERM)

The reaction of "annealing" is the association between the sequence and its radiolabeled complementary sequence with the formation of a duplex. The sense sequence complementary to the CERM is called CERM wt wt antisense: 5'-GAT GAC TTC CGG GAG GAC TCC ATT TAA AAA CAG CGT CAT CGA GCT G-3 '.
The probe, previously labeled, the complementary sequence is added in amounts 10 times higher (160ng), so that the two sequences are annealed completely, and 1x kinase solution (kinase buffer: 50 mM Tris pH 7.4, 10mM MgCl2, 5mM DTT). The reaction is carried out for two minutes at 85 ° C and allowed to cool overnight.
A similar reaction is conducted for the formation of a radioactively labeled duplex (oligo sense 16ng, 160ng oligo antisense, 1x kinase solution), used for studies of competition. The nuclear or total protein extract (~ 5x105 cells) is incubated for about 20 minutes at room temperature in a solution containing: DTT (10 mM), NaCl (0.5 M), Ficoll-Tris pH 7.4 Poly d (IC) (0.5 g) and double-stranded oligonucleotide (30000 cpm) in a final volume of 20µl.
The paper by "supershift" is achieved by incubating the protein extract with the appropriate antibody, for about 15 minutes at room temperature before adding the solution containing the binding oligonucleotide labeled. The DNA-protein complexes are loaded in a polyacrylamide gel, non-denaturing 4% (40% acrylamide 19:1, 5x TBE, 10% APS,

TEMED).

The electrophoresis takes place in a 0.25 x TBE running buffer (25 mM Tris, 25 mM boric acid, 0.63 mM EDTA) at 4 ° C, for about three hours at a constant voltage of 360Volt. The gel is then dried for about an hour.The DNA-protein complexes were visualized by autoradiography. You need at least 4 days of exposure at -80 ° C.

2.15.3 MARKING END IN 5 DHFR PROMOTER

The oligonucleotide used in this study, corresponding to the binding site for E2F, is derived from the promoter of the dihydrofolate reductase (DHFR sense wt). The sequence is 5'-GGCTGCGATTTCGCGCCAAACTTG-3 '. The sequence containing a mutated site (DHFR mut sense), unable to bind E2F, has been used in the marking: 5'-GGCTGCGATTGCTCGACCAACTTG-3 '.

The labeling reaction the 5 'end of an oligonucleotide is the transfer of one atom of radioactive phosphorus isotope (γ-32P)-ATP to the 5'-terminal OH. This reaction is catalyzed by T4 polynucleotide kinase. For this reaction are used 16ng of oligonucleotide to which are added 4µl Forward Reaction buffer 5x, 3µl of (γ-32P) ATP, 2µl of enzyme T4 polynucleotide kinase (10U/µl), in a final volume of 20µl. The reaction is carried out for one hour at 37 ° C. The nucleotides not incorporated in the probe are eluted into a chromatographic column, supplied by Qiagen kit (QIAquiek Nucleotide Removal Kit). The specific activity of the probe is measured by reading the cpm (counts per minute). 1µl of probe is added to 5ml of liquid scintillation (Packard Opti-Fluor) and the reading is made to a β-counter (Beckman LS 5000 TD), a device able to measure only the radiation type β.

2.15.4 ANNEALING AND ACRYLAMIDE GEL ELECTROPHORESIS ON (DHFR)

The reaction of "annealing" is the association between the sequence and its complementary radioactively labeled with the formation of a duplex. The sequence complementary to the sense DHRF wt wt is called DHFR antisense: 5'-CAAGTTTGGCGCGAAATCGCAGCC-3 '. The complementary sequence is called all'oligonucleotide mutated DHFR mut antisense: 5'-CAAGTTGGTCGAGCAATCGCAGCC-3 ' The probe, previously labeled, the complementary sequence is added in amounts 10 times higher (160ng), so that the two sequences are completely appaino, and 1x kinase solution (kinase buffer: 50 mM Tris pH 7.4, 10mM MgCl2, 5mM DTT). The reaction is carried out for two minutes at 85 ° C and allowed to cool overnight.

A similar reaction is conducted for the formation of a radioactively labeled duplex (oligo sense 16ng, 160ng oligo antisense, 1x kinase solution), used for studies of competition. The nuclear or total protein extract (~ 5x105 cells) is incubated for about 20 minutes at room temperature in a solution containing: DTT (1 mM), KCl (50 mM), MgCl2 (5mM), Ficoll-Hepes (20 mM), poly d (IC) (0.5 g) the oligonucleotide double-stranded (30000 cpm) in a final volume of 20µl. The sequences used in this essay are: wt DHFR, for the identification of complexes containing the E2F and DHFR mut, as a control for the specificity of binding. As a further control for the specificity of binding, competition reactions were performed in which radioactively labeled DNA is included in higher concentration (~ 100 times more) of the labeled DNA.

The "supershift" is achieved by incubating the protein extract with the appropriate antibody, for about 15 minutes at room temperature before adding the solution containing the binding oligonucleotide labeled.

The DNA-protein complexes are loaded in a polyacrylamide gel, non-denaturing, 4% (40% acrylamide 19:1, 5x TBE, 10% APS, TEMED). The electrophoresis takes place in a 0.25 x TBE running buffer (25 mM Tris, 25 mM boric acid, 0.63 mM EDTA) at 4 ° C, for about three hours at a constant voltage of 300Volt. The gel is then fixed in a 10% solution of acetic acid and 20% methanol and dried for about an hour, in a "gel drier." The DNA-protein complexes were visualized by autoradiography. You need at least 4 days of exposure at -80 ° C.

2.16 REAL TIME PCR

2.16.1 RNA EXTRACTION

To the Pelleted cells add one milliliter Tryzol of up to 500 million cells, pipetting more times to homogenize the pellet.

Then Incubate 5 'at 25°C and add 0.2 ml of chloroform per ml of Tryzol. Close the sample and manually shaking the tubes for 15 sec and incubate at room temperature for 2 -3 min.Centrifuge 120000 rpm for 15 'at 2-8 ° C. After centrifugation the mixture separates into a lower red phenol chloroform phase, an 'interphase and a colorless aqueous phase.The RNA remains in the aqueous phase.The volume of the aqueous phase is about 60% of the volume of tryzol used.

2.16.2 RNA PRECIPITATION

Precipitate the aqueous phase with stirring isopropyl alcool. Use 0.5 ml of isopropyl alcool per 1 ml of Tryzol. Incubate samples at room temperature for 10 minutes and centrifuge at 12000 rpm for 10min. at 2-8 ° C . RNA gel-often falls to the bottom and the wall of the eppendorf.

2.16.3 WASHING OF RNA

Remove the supernatant and wash the pellet with 75% ethanol, using 1 ml per sample. Mix the sample by vortexing and centifuge to no more than 7500 rpm. for 5 'at 2-8 ° C. Dry the sample to the air and dissolved in RNase free water and incubate at 55 - 60 ° C for 10 min. Put on ice and quantify.

2.16.4 RNA QUANTIZATION

RNA can be quantified using absorption of light at 260 and 280 nm (A260/280). Ideally, this ratio should be close to 2 for high quality nucleic acid.

2.16.5 RETROTRANSCRIPTION

The reaction of reverse transcriptase (RT) was carried out on 1mg of total RNA per sample, into the Gene AMP PCR sistem 9700, in PCR Buffer II (PerkinElmer) diluted to 1x, containing 5 mM MgCl 2, 10 mM DTT, random hexamers (pdN6) 5 mM, 1 mM dNTP, RNase inhibitor (1 U / ml) and RT (10 U / ml). After denaturation of the samples at 70 ° C for 10 minutes, we proceeded to the following reaction: 25 ° C for 10 minutes, 42 ° C for 45 minutes, 99 ° C for 3 minutes. Rate of double-stranded cDNA, was used as such in real-time quantitative PCR experiments.

2.16.6 QReal Time RT-PCR

In order to accurately analyze Pouf5f1 and SOX2 mRNA levels, a QRT-PCR quantification method was performed according to the TaqMan Gene Expression Assays from Applied Biosystems. Samples were normalized by evaluating GAPDH expression.

3 THESIS RESEARCH OBJECTIVE

Embryonic stem cells (ES) cells and human induced pluripotent stem (iPS) have captured the imagination of the scientific community for the last twelve years because of the tremendous potential that they offer for regenerative medicine, drug discovery, toxicology, and modeling of diseases. All these potential applications depend on a thorough understanding of the basic biology of these fascinating cells.

Reprogramming somatic cells towards ground state pluripotency, has recently allowed to obtain iPS cells directly from adult fibroblasts and many different types of adult tissues. In the presence of leukemia inhibitory factor (LIF), iPS cells can be maintained in culture indefinitely in an undifferentiated state. The absence of LIF in the culture medium or treatment with retinoic acid (RA) alters the expression of a specific set of genes that regulates the cell cycle in both ES cells iPS. For this reason we decided to study in detail the early events of differentiation of iPS cells and compare the results to recent published data for ES cells by the Functional Genomics in Embryonic Stem Cell (FunGenES) consortium database (Schultz et al., 2009 PLoS One. 2009 Sep 3;4(9):e6804. Briefly, the aim of my PhD thesis was to investigate the cell cycle during the reprogramming of iPS cells because it is still unclear whether the cell cycle of the iPS cells presents unique characteristics compared to the cell cycle of ES cells. This study is of considerable importance for understanding the molecular mechanisms that control the unlimited capacity of self-renewal and differentiation potential of iPS. During the three-year course of my International Ph.D. in Stem Cell Research, XXIV cycle, my research studies on iPS have been done in collaboration with the Centre for Stem Cell Research, University of Milan

(**UniStem)**, directed by Prof.ssa Elena Cattaneo. In this collaborative studies ,I have learned the principal techniques adopted for the iPS generation by mouse fibroblast and I was able to develop techniques for teratoma and iPS cell differentiation. Moreover through a theoretical-pratical "Stem Cell Differentiation Training Course" at the Stem Cell Fate (SCF) Laboratory at the Institute of Genetics and Biophysics Adriano Buzzati-Traverso (Naples) I have learned experimental procedures for culturing and differentiating ES cell lines to cardiomyoicytes and neurons. All together, the UniStem and SCF Lab, has allowed my research studies at the Istituto Superiore di Sanità (Rome) to develop new tools for the identification/characterizatio of iPS cell cycle modulators and molecules involved in early lineage commitment in iPS cells.

In particular, my thesis research objective has been focalized on two principal experimental investigation:

1) Phenotypic Cellular Analysis of the iPS cells during self renewal and in early stage of differentiation:
    i) Analysis of the iPS induction in cardiomiocytes through Ascorbic Acid
    ii) In vivo analysis and molecular differentiation studies on the effect of ascorbic acid in the iPS embryonic bodies
    iii) Cellular Analysis of iPS induction in EPISC through L Proline in vivo and in vitro
    iv) Study of Retinoic Acid Effect on the iPS cellular phenotype.
2) Identification of Specific Modulators that control the cell cycle during the self-renewing or early differentiation in iPS cells.

i) Study of gene expression regulators (cyclin E, cyclin A, CDK2, Rb/E2F, HDACs, Id, Sin3A) that control the cell cycle in iPS cells treated with or without LIF and after treatment with retinoic acid. during neural differentiation.

ii) Expression analysis of the genes specifying SWI/SNF chromatin remodeling complexes in ips cells during differentiation with RA.

iii) the study of complex interactions of E2F/Rb and SWI/SNF with some chromatin remodeling enzymes (eg E2F/Rb/HDAC, E2F/Rb/brm) during differentiation

iv) Biochemical characterization of complex E2f/Rb/HDAC1/mSin3A , ID2/RB , BRG-1/CYCE in undifferentiated iPS and in cells induced to differentiate by treatment with retinoic acid;

v) Molecular differentiation studies of the effect of L-Prolyn on the iPS cells metastability

vi) Molecular differentiation studies of the effect of ascorbic acid on the iPS cells

vii) Analysis of the composition of the CERC2 transcription factor complex that regulates the expression of cyclin E during the various phases of the cell cycle of iPS cells induced to differentiate.

viii) The study of CREB complex in undifferentiated iPS and in cells induced to differentiate into neuronal line with Retinoic Acid

These results obtained has been very interesting and revealed the importance of the expression of cyclin E and of the Rb/E2F pathway during the early stages of differentiation in both ES cells into iPS. Overall, the results obtained in these studies supports the model that the cell cycle of ES and iPS cells abbreviated with a short G1 phase, is functionally linked to pluripotency.

4.1 EXPLOITING EXPERIMENTAL PROCEDURES FOR CULTURING AND DIFFERENTIATING IPS CELLS TO CARDIOMYOCYTES AND NEURONS

4.1.1 GENERATION OF IPS CELLS FROM DERMAL FIBROBLAST OF MICE

The generation of iPS cells used in this PhD thesis has been done in collaboration with the Center of Stem Cell Research, University of Milan, directed by Prof. Elena Cattaneo.

Briefly, dermal fibroblasts were isolated from mice and retrovirally transduced with either 4 transcription factors, Oct4, Sox2, Klf4, and c-Myc, or with only 3 in the absence of c-Myc (figure 1). After retroviral infection, only 5 of 123 picked clones generated cell lines with ES-like morphology.. Thirty of 35 tested clones presented characteristics of Ips cells such as expression of ES cell markers (alkaline phosphatase and Nanog), reactivation of the endogenous pluripotency genes (*Oct4, Sox2, Klf4, c-Myc, Rex1,* and *Nanog*), and silencing of retroviral transgenes. Once transplanted into nude mice, 4 out of 4 iPS cell lines tested produced tumors that consisted of various tissues of all 3 germ layers, indicating their pluripotency (data not shown).

4.1.2 IN VITRO DIFFERENTIATION OF EMBRYONIC BODIES GENERATED BY IPS: EFFECT OF ASCORBIC ACID (AA).

The metabolic and biochemical properties of AA have been extensively studied. The major biochemical function proposed for AA has been its antioxidant properties. Surprisingly, two additional lines of research indicate that AA may be involved in embryonic/cell differentiation. The first regards Schwann cell differentiation and myelination. It has been demonstrated that AA is needed in Schwann cell/axon co-cultures to induce myelin formation. (Carey et al., 1987; Eldridge et al., 1987; Plant et al., 2002) The second line of research has been initiated more recently and involves embryonic stem cell (ES) differentiation. Using high throughput screening of a chemical library, Takahashi *et al*. found that AA was the only molecule present in the library that promoted differentiation of murine ES in cardiomyocytes. The identification and isolation of a cardiac precursor cell population is expected to provide a source of cells for tissue regeneration, while also providing valuable insight into cardiac development. For this reason, I decided to investigate the effect of ascorbic acid on iPS cells.

Briefly, my pioneering research study on differentiative effect of ascorbic acid shows that iPS cells behave like ES cells (fig. 4). Ascorbic acid at final a concentration of 50 μg/ml has a stimulatory effect on the differentiation of EB. The EB treated with this compound are more uniform, and the cells within the embryoid bodyes seem closer to each other like a tissue. These data are in agreement with the results obtained by Takahashi group (2003) which indicate that ascorbic acid markedly increases the expression of sarcomeric myosin

and α-actinin in a dose-dependent manner in ES cells. All together these results confirms that AA induces cardiac differentiation in ES and iPS cells.

4.1.3 COOPERATIVE EFFECT OF SEEDING DENSITY AND THE ASCORBIC ACID EFFECT ON THE DIFFERENTIATION OF EB DERIVED IPS

Using microarray analysis, Shin et al.,2004 found that the overexpressed genes responding to AA treatment of ES cells belonged to gene families involved in neurogenesis, maturation and neurotransmission.

Mouse embryonic stem (ES) cells can be differentiated into neural lineage cells, but the differentiation efficiency remains low (Zhou et al., 2008). The quality of embryonic bodies (EBs) is an important factor that influence the neural differentiation efficiency of mouse ES cells: Good quality of EBs were consistently originated from a suspension culture density of 1 $x10^5$ ES cells/ml in a serum-free chemically defined neural inducing medium and they exhibited a smooth round shape, with a dark central region surrounded by a light band. Such EBs are capable of attaining high neural differentiation efficiency (Figure 4 panels A, A', B ,B', C,C'). However, poor quality EBs were instead originated from a suspension culture of 1 x 10^6 ES cells/ml serum-free chemically defined neural inducing medium and this EBs exhibited an irregular shape or they were adherent to the bottom of the dish displaying low neural differentiation efficiency. The second factor that effects the quality of EBs is the seeding density: a low seeding density (5 EBs/cm2) induced cells to differentiate into a more caudalized subtypes compared to the cells obtained from high seeding density (20 EBs/cm2).

During my research project in obtaining EBs–derived iPS I have followed experimental procedures according to Zhou et al., 2008. My results revealed that the EB from iPS behave

like the EB-derived ES. In fact, it has been found that at low-density seeding the EB are rounder in appearance but at high density tend to have a more irregular shape. When the iPS #202 were dissociated and cultured in a suspension at 1 x 10^5 cells/ml medium, EBs were spontaneously formed and they became cystic 2 days later. After 3 days of culture, AA was added into the medium, and incubation was continued for 4 days. The EBs gradually became more compact with a smooth, round shape, with the central region becoming dark and surrounded by a light band EBs showing these characteristics were considered to be of good quality. When the iPS #202 cells were suspended at >1x 10^6 cells/ml medium, the EBs were irregular in shape (Figure 4 C and C'), and some adhered to the bottom of the Petri dish or formed connections with each other. Such EBs are considered of poor quality. The effect of AA was to increase the speed of EB differentiation and to induce the classic round shape of EB. Moreover the EB treated with Ascorbic Acid are more compact and regular. These findings provided fresh insight into the neural induction of mouse iPS cells.

4.1.4 INDUCTION OF IPS-EPICS CELLS BY TREATMENT WITH L-PROLYN.

This study has been performed under the supervision of the Stem Cell Fate Laboratory at the Institute of Genetics and Biophysics in Naples.

Many studies have been focused on the development of new tools and methodology to identify small molecules/extrinsic factors able to modulate the pluripotent state of ES cells. Recently, Casalino et al., 2010, using a novel automation platform, the Cell Maker, to screen a library of metabolites, provide evidence that L-proline (L-Pro) and L-ornithine (L-Orn), are key regulators of ES cells metastability, forcing the ES cells toward a novel epiblast stem cell (EPISC)-like state

Following the same methodology, but using iPS cell, I have found that the iPS cells have a behavior similar to ESC when induced with L Prolyn at 150 μM for 7 days (figure 8). In fact iPS assumed a morphology similar to EPISC when treated with this aminoacid. Among iPS LIF and EPICS the second seem different, more heterochromatic nuclei and many more cells. Finally, in EPICS LIF- the nuclei seem smaller and heterochromatic, whereas in EPISC +LIF nuclei are larger and heterochromatic (figure 8A).

The WB specific for Oct4 have revealed that I was able to obtain the induction of EPISC by iPS with treatment with L Prolyn 150 μM (final concentration) for 1h : in fact the level of Oct4 in EPISC cells is less respect to the iPS control (ips +LIF) (figure 21G).

4.1.5 TREATMENT OF IPS WITH RETINOIC ACID (COMMITMENT TOWARD NEURAL DIFFERENTIATION)

The addition of specific chemicals in the culture medium directs the iPS to undertake different lineage -specific cell types. Specifically, Retinoic Acid directs mouse iPS mainly to neuronal differentiation (figure 7). The following experiments on the treatment of the IPS with RA are very useful to understand the molecular behavior of cell cycle genes during differentiation and compare it with the same studies on cES; these results are very useful to realize if the iPS behaviour is comparable to ES. Moreover the study of different types of differentiation induced in iPS by specific factors is very important to clarify the molecular mechanisms responsible for the exit of iPS from the "self renewal". RA treatment that allows a better differentiation has a duration of about 24h-72h. To understand the role played by cell cycle proteins and multiprotein complexes that remodel chromatin in mediating the transition from undifferentiated iPS to differentiated ones, we analyzed the variation of gene expression in iPS cells treated with leukemia inhibitory factor (LIF) and retinoic acid. The characteristics of stem cells are preserved in mouse iPS in a culture medium in which LIF is added (figure 6). In contrast, mouse iPS cells deprived of LIF tend to spontaneously to differentiate into different cell types.

To analyze the differences of expression in the ESC and iPS of genes that regulate the cell cycle and to study the biochemical characterization of protein complexes potentially

involved we better characterize the iPS in vitro. In particular, the cell line used was ips #202, derived from transduction of the “Yamanaka” four factors. Moreover we analyzed the expression profile of some protein the regulate the cell cycle in the iPS. Treatment with retinoic acid consistently alters the activity and expression of proteins of the cell cycle and chromatin remodeling in the CES. For this reason we investigated in detail the early events in the iPS cells after chemical treatments. IPS cells # 202 are were placed 3-4 days in a culture medium containing LIF, which ensures the maintenance of stem cell. These cells before treatment are maintained without LIF. After a series of washes in PBS, the retinoic acid is added to a final concentration of 5 x 10-7 M. Each treatment is repeated three times to assess the reliability of the results obtained. The culture medium of iPS were added the following chemicals: Retinoic Acid (RA) in a final concentration of 5 x 10-7 M for kinetic analysis at several times.

In particular, for subsequent testing we used Retinoic acid in conditions, which induce neural differentiation (figure 7). This chemical agent affects the expression of a wide variety of genes and results in the decondensation of specific regions of chromatin with anti-proliferative effects. The purpose of these studies is to identify which genes are modulated during the early stages of differentiation of iPS .

4.2 IPS DIFFERENTIATION :MOLECULAR ANALYSIS

4.2.1 IPS TREATMENT WITH GMEM MEDIUM WITHOUT LIF FOR INDUCING DIFFERENTIATION

Experimentally the exit from the "self-renewal" of the iPS can be induced through the removal of LIF from the culture medium or with the addition of appropriate molecules (BMP, Id1) or chemicals (inhibitors of kinases and in our case the Retinoic Acid). From the molecular point of view exit of the "self-renewal" of the iPS is associated with an alteration of chromatin structure, and by modulation of the cell cycle genes.For this motive we considered appropriate to investigate the effects of treatment of iPS using the GMEM medium without LIF by Western blot analysis.This studies are very important to understand what are the genes modulated and realize what happen from molecular point of view during these treatment. Moreover is very useful to compare these results with those obtained using ES cells to understand the differences in the behavior of these genes in these two cell lines.

The iPS # 202 were treated with culture medium enriched with factors GMEM without the addition of LIF for induce the differentiation. The LIF is a chemical compound that prevents cell differentiation of iPS. I have analyzed the protein expression of the Oct4 gene at 0, 2, 6, 10 days without the addition of LIF in the culture medium (Figure 12A). At day 2 we notice a decrease of about 80% of the protein than control LIF ($p< 0,01$). The expression at days 6 is slightly increased of about 20% than day 2 ($p<0,05$). And

surprisingly at 10 days after removal of LIF, there is a substantial increase in the expression of the gene Oct4(of about 30% than control LIF). The most interesting observation is that even at 10 days the expression of this gene after removal of LIF is increased compared to cells grown in the presence of this chemical (p< 0,001). **O**ther WB experiments were performed to study the expression of Cyclin E (figure 10B) . Through this analysis it was found that at days 2 after treatment without LIF there is an expression increased of cyclin E (of about 2,5 times than control LIF) (p <0.05). At days 6 there is a decrease not significant, while at days 10 there is a statistically significant increase of about 2 times than LIF (p <0.01). Evidently there is a endogenous stimulation that leads to increased expression of this gene. The CYC A (figure 12C). gene behaves in the same way as the CYC E gene. In fact, at day 2 there is a statistically significant increase in the expression of this cyclin of about 1,6 tmes than LIF (P<0,01) . At Day 6 a significant decrease of about 50% than LIF (P<0,01) and a significant increase on day 10 (of about 30% than LIF) (P<0,001) The study of HDAC1 accumulation led to the conclusion that the accumulation of this protein is not modulated by the removal of LIF in the culture medium. In fact we observe only a slight increase in protein expression at 10 days (data not shown).

4.2.2 PROTEIN EXPRESSION ANALYSIS OF THE CELL CYCLE GENES DURING TREATMENT OF IPS CELLS WITH RETINOIC ACID

The protein extracts obtained from iPS, treated with both LIF and with RA (0,5uM) after 1h, 3h, 6h, and 24h, were analyzed by Western blot for the presence of some proteins that regulate the cell cycle: cyclin A, cyclin E, the family members Rb (Rb105, p130 and p107), some subunits of the complex SWI / SNF (BRM) histone deacetylases ,mSin3A, and E2F2. In all experiments, to control the proper differentiation of iPS and iPS with RA, we used the gene Oct4, a marker specific to pluripotent iPS, which is turned off in differentiated cells. Several studies have demonstrated that cyclin D1, which regulates the G1 / S transition in differentiated cells, has a low level of expression in the CES (Savatier et al., 1995). In order to understand which complexes cyclin / cdk are used by iPS during their rapid proliferation, we studied the expression of cyclin A and cyclin E (figure 13). The results show that these proteins are present in undifferentiated iPS and their expression increase and then decreases in iPS induced to differentiate with RA. Oct4 protein level (figure 11A) was used as a marker of the undifferentiated state. The level of expression of this protein should decrease during differentiation . We found the Oct4 level is indeed high in iPS treated with LIF but surprisingly increased following treatment with RA at 1h (1,6 times than LIF) , and 3h (2,2 times than LIF) (the increasing is significative: $p< 0,05$).; at 6h slightly decrease (than 6h of about 20%) ($p<0,05$) and at 24h even decrease not significativelly ($p>0,05$) (figure 11A). In this studies I have been found that the level of CYC E in iPS (figure 11B).is less of 20% than cES ($p< 0,05$). In particular after treatment with RA 0,5 μM the expression of Cyclin E increase at 1h of about 40 % ($p< 0,05$) (than

the control iPS LIF) and then start to decrease at 3h (of 20% than 1h), 6h after treatment with RA. At 24h the expression is minimal (of about 40%) than the control LIF ($p < 0,001$) It has been found that in iPS the level of CYC A (figure 13C). is 25% lower in iPS than cES ($p< 0,05$) This study shows that during differentiation toward the neural commitment (through treatment of iPS with RA 0,5 uM) the level of CYC A surprisingly increases at 1h of about 60 % than LIF ($p< 0,05$) and then decreases at 24h treatment of 80% as compared with control LIF ($p< 0,01$). Accordingly the CYCA gene expression during differentiation is similar to CYCE. The treatment with RA not influence the expression of CDK2 which protein expression level are approximately constant (data not shown). It is significative instead ($p<0,05$) the lower level of CDK2 in iPS than ES. In figure 14 A it shown that the expression of mSin3A in iPS is more of about 2,5 times than ES LIF. Moreover the protein expression level of mSin3A in iPS treated with RA is increased (more 30%) after 1h of treatment ($p<0,001$). After 3h and 6h there is a decreasing statistically not significative than control LIF. Even at 24 h there is a decreasing of mSIn3A but is not significative ($p>0,05$) The gene BRM (data not shown). instead after treatment with RA increase in its protein expression. This results means that the brm is increasing when the ips start to differentate In conclusion, treatment of iPS with RA modulates the expression of some proteins that control the cell cycle (specifically cyclin A, cyclin E,mSIn3A and brm). Moreover, genes as HDAC-1 (figure 14B) are constitutively expressed even after RA treatment (are not modulated by this treatment). These results suggest a change in qualitative and quantitative analysis of protein complexes involved in the control of unlimited proliferation and subsequent differentiation of iPS. The analysis by Western blot revealed that the protein

HDAC-1(figure 14B) is highly expressed in undifferentiated ESCs and is lesser in iPS undifferentiated of about 10% ($p<0,01$). Instead in the iPS treated with RA the level of HDAC-1 is slighty constant. In particular HDAC-1 slightly increase at 1h and 3h ($p> 0,05$) and at 6h and 24h decrease to value similar at the control LIF although changes were not statistically significant (figure 14B)

4.2.3 EXPRESSION ANALYSIS OF THE GENES SPECIFYING SWI/SNF CHROMATIN REMODELING COMPLEXES IN IPS CELLS.

The SWI/SNF-Brg1 chromatin remodeling protein plays critical roles in cell-cycle control and differentiation through regulation of gene expression. Loss of Brg1 in mice results in early embryonic lethality, and recent studies have implicated a role for Brg1 in somatic stem cell self-renewal and differentiation. However, little is known about Brg1 function in preimplantation embryos and embryonic stem (ES) cells. Here we report that Brg1 is required for iPS cell self-renewal and pluripotency (data not shown). RNA interference-mediated knockdown of Brg1 in blastocysts caused aberrant expression of Oct4 and Nanog. In ES cells, knockdown of Brg1 resulted in phenotypic changes indicative of differentiation, downregulation of self-renewal and pluripotency genes (e.g., *Oct4, Sox2, Sall4, Rest*), and upregulation of differentiation genes. Using genome-wide promoter analysis (chromatin immunoprecipitation) we found that Brg1 occupied the promoters of key pluripotency-related genes, including *Oct4*, *Sox2*, *Nanog*, *Sall4*, *Rest*, and *Polycomb group* (*PcG*) proteins. Moreover, Brg1 co-occupied a subset of Oct4, Sox2, Nanog, and

PcG protein target genes. These results demonstrate an important role for Brg1 in regulating self-renewal and pluripotency in ES and iPS cells.

Studies by RT-PCR and immunofluorescence analysis showed that the gene BRG-1 is ubiquitously and constitutively expressed in mouse ESCs during embryogenesis. Instead, BRM gene transcripts is poorly expressed in undifferentiated ESCs. During murine embryonic development the expression of BRM is activated starting from the blastocyst stage (LeGouy et al., 1998). The results of our analysis by Western blot showed brm is slightly more present in iPS induced to differentiate with RA ($p > 0,05$) (data not shown), confirming previous studies on cES. Moreover brm is expressed 70% more in iPS than ES ($p < 0,05$) (data not shown).

The B-actin gene is ubiquitously expressed at high levels in each phase of the cell cycle. For this reason, its level of expression is constant for the same amount of cell extract loaded for each sample. The assay of beta-actin is useful to confirm that the modulation of genes studied revealed by analyzing the results of Western blot is genuinely due to the change in the level of expression or due to operator error in the preparation and loading of the extracts protein.

4.2.4. EFFECT OF TREATMENT OF IPS WITH RETINOIC ACID ON DIFFERENTIATIVE MARKERS (REAL TIME PCR ANALYSYS)

Oct4 protein level was used as a marker of the undifferentiated state, the level of expression of this protein should decrease. We found the Oct4 level is indeed high in iPS treated with LIF but surprisingly increases following treatment with RA at 1h, and 3h (the increase is significative: $p<0,05$)At 6h we observe a slight decrease ($p<0,05$) and at 24 h a further decrease ($p>0,05$) (figure 15)

As a further control, were carried out analysis of RealTime-PCR (figure 15). In particular, we evaluated the level of Oct4 and Sox2 mRNA, both markers of the undifferentiated state. The results shown in Fig.15 indicate that the treatment of iPS with RA after 3 h causes a increase in mRNA encoding for Oct4 as compare with the control LIF (increasing of 20%). Sox2 gene instead decrease about two fold after 3h of treatment with RA 0,5 μM. Instead after 1 day and 4 days of withdrawal of LIFwe observe a decrease in Oct4 mRNA while Sox2 mRNA is constant. The GAPDH gene was used for normalization of samples. These results confirm that during the period of treatment with RA the iPS begin to lose their characteristics of pluripotency. Surprisingly the protein and RNA expression of Oct4 increase after RA treatment; this suggest that at this concentration of RA (0,5 μM) this compound increase the pluripotency of the iPS.

4.2.5 INTERACTION OF THE COMPLEX SWI / SNF (BRM AND BRG-1) WITH HDAC1 DURING PROLIFERATION AND DIFFERENTIATION OF IPS: BRG-1 AND HDAC1 INTERACT IN IPS INDUCED TO DIFFERENTIATE WITH RA: RECIPROCAL IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS

Brg1 is known to associate with multiprotein coactivator and corepressor complexes to positively or negatively regulate transcription . Actively transcribed genes bound by Brg1 include genes such as *Oct4, Sox2,* and *Nanog* that are essential for iPS cell pluripotency, suggesting that Brg1 occupancy of pluripotency-related gene promoters positively regulates transcription and promotes iPS cell self-renewal.Together, these results implicate Brg1 in promoting expression of self-renewal genes and repression of lineage-specific genes in ES cells.

Recent studies have shown that Rb can form two different complexes containing HDAC-Rb-SWI/SNF and Rb-SWI/SNF repressors that regulate the cell cycle and control G1 / S transition. The clock cycle in G1 is blocked by the repressor complex activities HDAC-Rb-SWI/SNF. Rb can be phosphorylated sequentially by different complexes cyclin / Cdks during the cell cycle. In particular, the complex of cyclin D1/Cdk4-6 phosphorylates Rb allowing the dissociation of HDAC from the complex Rb-SW/SNF and this results in the activation of the expression of cyclin E. Increasing the level of expression of cyclin E allows cell proliferation. The recruitment of cyclin E/CDK2 facilitates phosphorylation of Rb on Ser 567, an acceptor site for phosphate groups A and B within the domain of Rb. This phosphorylation causes the release of transcription factors and E2F. HDAC-1 HDAC-

2 but not HDAC3 contain similar amino acid sequence to LXCXE that allows them to this histone deacetylase to interact with Rb. This pattern LXCXE is also characteristic of the transcription factors of E2F family and is the region that interacts with Rb. The recruitment of HDAC-1 and HDAC-2 by E2F through Rb can inhibit the activity of E2F themselves because of deacetylation. Finally, Rb AP48 appears to be a component of the complex Rb-HDAC-1 in mammalian cells. However, it is unclear whether this complex that includes either mBRG-1-1 or mbrm has a specific role in the regulation of the iPS cell cycle. This could be a key aspect in self-renewal of iPS and events in the early stages of differentiation. For this reason we studied the interactions SWI / SNF (BRG-1) with HDAC-1, and some protein machinery that controls the clock cycle of the iPS cells treated with LIF and RA.

Samples treated with LIF, or RA 5uM for 1h were immunoprecipitated with a polyclonal antibody specific for BRG1 (data not shown). The immunoprecipitates obtained were loaded onto a 8% SDS-PAGE gels and analyzed by Western blot using a polyclonal antibody specific for HDAC1. As positive controls, the same protein extracts not immunoprecipitated were loaded in the gel (data not shown). The data showed that the interaction of BRG-1-HDAC-1 is strongly stimulated in the presence of RA 5uM for 1h and little without RA. The undifferentiated ESCs express this protein very weakly.As a control, all samples were immunoprecipitated with the antibody NRS, which is not able to recognize specific proteins: no interaction was detected in these samples.

In the reverse experiment, the same total protein extracts were immunoprecipitated with a monoclonal antibody specific for HDAC1, resolved on 8% SDS-PAGE and analyzed by Western blot with a polyclonal antibody specific for BRG-1 (data not shown). As negative

control the same total protein extracts were immunoprecipitated with nonspecific IgG antibody.In conclusion the results confirm the presence of mutual complex BRG-1 / HDAC1 in RA and LIF. In particular this interaction is strengthened exactly during the differentiation in the neural way (in the iPS treated 1h with RA 5μM).

4.2.6 THE COMPLEX INTERACTION WITH E2F/RB WITH CHROMATIN REMODELING ENZYMES IN IPS TREATED WITH RETINOIC ACID.

Rb function depends, at least in part, on interactions with the E2F family of DNA-binding transcription factors (E2F) (Chellappan et al. 1991; Dyson 1998; Nevins 1998). E2F sites are found in the promoters of many genes that are important for cell cycle progression, and Rb appears to repress transcription of these genes through its interaction with E2F (Blake and Azizkhan 1989; Thalmeier et al. 1989; Dalton 1992; Ohtani et al. 1995). Recent findings in the Rb/E2F field are clarifying how this pathway regulates the transition from G1 to S phase at the molecular level. Other emerging results show that this pathway also regulates other parts of the cell cycle and that it may even have roles beyond cell cycle control. In this thesis, we review the current understandingof the mechanism of action of Rb and its roles in iPS cell cycle regulation, and during iPS differentiation with RA.

The balance between the levels of functionally active Rb protein levels and E2F may, in part, define a critical point for cell cycle progression in G1 / S. However, the complex formation E2F/Rb not only blocks the transcriptional activation mediated by E2F, but can also actively repress transcription. In this condition, the Rb promoter recruits several proteins that modify chromatin structure, such as histone deacetylase complex and the SWI / SNF. In particular, in differentiated cells, several studies show that Rb represses the E2F transcriptional activity more efficiently in the presence of brm (Trouche et al., 1997) and that cooperation between Rb and brm or BRG-1 can induce the arrest cell proliferation

(Muchardt and Yaniv, 2001). The dynamic changes of chromatin, mediated by these proteins may be a mechanism by which iPS regulate gene expression during differentiation and development.

In this regard, in the present work we have analyzed the interactions of complex E2F/Rb with enzymes that remodel the chromatin in undifferentiated iPS treated with LIF and iPS treated with RA.

In reciprocal immunoprecipitation, I have observed that E2F1 interacts with Rb during the cell cycle of iPS but this interaction is less important during the differentiation induced by treatment with RA 5uM for 1h. Also the other Immunoprecipitation IPRB/WB E2F2 confirm that this interaction is found in iPS treated with LIF but not in iPS treated with RA (data not shown).

4.2.7 ID2 INTERACTS WITH RB IN ES AND IPS TREATED WITH LIF

In vivo, a physical association between Id2 and pRb was seen in cross-linked extracts from SAOS-2 cells transfected with Id2 and pRb. Furthermore, the identification in normal cells of cell-cycle-regulated associations between Id2 and pocket proteins indicates that suppression of Id2 function by Rb is likely to be a general mechanism by which pocket proteins control progression of the cell cycle. The observation that Id2 (but not other Id proteins) is expressed in differentiating neuronal and haematopoietic cells(Neuman et al. 1993), and that these cell types are able to re-enter inappropriate proliferation and ultimately apoptosis in the absence of Rb is consistent with the idea that maintenance of the post-mitotic state requires physiological control of Id2 by Rb. Therefore, Id2 must target other factors during development and normal cell-cycle progression and these targets must be activated following Rb-mediated inactivation of Id2. Therefore, RB can be thought of as an upstream regulator of ID2, which, when lost, leads to gain of ID2 activity and inappropriate sequestration of E proteins. By binding to Rb, Id2 is able to abolish its growth-suppressing activity. In addition, it has been suggested that Id2 is a downstream target of Rb since rescue of embryonic lethality of Rb-/- mice by loss of Id2 observed. The relationship between the Rb-E2F interaction and the Rb-Id2 interaction is poorly understood and remains to be further clarified. Rb might simultaneously bind E2F and Id2 but alternatively E2F and Id2 might compete for binding to Rb.

For these reasons the experiments of Immunoprecipitation were conducted to evaluate the presence of complex Rb/Id2 in ES and iPS. Samples treated with LIF, immunoprecipitated with a polyclonal antibody specific for Id2, were loaded onto 12% SDS-PAGE gels and analyzed by Western blot with a polyclonal antibody specific for Rb (Fig.16A). In the reverse experiment, the same total protein extracts were immunoprecipitated with a polyclonal antibody specific for Rb, resolved on 8% SDS-PAGE and analyzed by Western blot with a polyclonal antibody specific for Id2 (Fig.16B). As positive controls, the same immunoprecipitated samples were loaded onto gels (Fig.16 A e B). As negative controls, all samples were immunoprecipitated with nonspecific NRS antibody (Fig.16 A e B). The results indicate the presence of complex Rb/Id2 in ES and iPS (undifferentiated) samples. Our data identify a role for Id2 in the regulation of cellular proliferation of iPS and suggest that the interaction between Id-2 and pRB is a molecular pathway over which synchronous changes in growth and differentiation are mediated in vivo.

4.2.8 HDAC1 CO-IMMUNOPRECIPITATES WITH E2F1.

In contrast to experiments in vitro, transfection assays in cultured cells have suggested that interaction with HDAC is required for the inhibition of E2F1 by Rb (Brehm et al. 1998; Magnaghi et al. 1998). Other studies have only shown a partial requirement for HDAC activity in the Rb-mediated inhibition of E2F activity (Luo et al. 1998; Lai et al. 1999a). E2F1 has been shown to interact with the histone acetyl transferases p300/CBP and p/CAF (Trouche et al. 1996). Thus, it is possible that Rb-mediated recruitment of HDAC to E2F acts to offset this histone acetyltransferase (HAT) activity. It has also been shown recently that E2F1 can be acetylated, which increases the binding of the E2F/DP complex to DNA (Martinez-Balbas et al. 2000). Therefore, recruitment of HDAC to E2F via Rb may inhibit E2F activity by deacetylation of the protein, thereby inhibiting its binding to DNA. Rb can also interact with BRG1 and BRM (Dunaief et al. 1994; Singh et al. 1995) the two ATPase components of the human SWI/SNF chromatin remodeling complex, which is discussed in more detail in this thesis. Some results have shown that overexpression of BRG1/BRM can facilitate Rb-mediated inhibition of E2F1transcriptional activity (Trouche et al. 1997); however, other studies have found that E2F activity is inhibited efficiently in cells that are BRG1/BRM deficient (Weintraub et al. 1992; Zhu et al. 1993; Zhang et al. 2000). Thus the relative importance in vivo of these two potential mechanisms for inhibiting E2F transactivation direct binding and masking of the E2F transactivation domain versus Rb-mediated recruitment of chromatin remodeling enzymes to inhibit E2Fis still unclear.

For this motive we studied the exixstence of this interaction in IPS cells. Nuclear extracts obtained by iPS treatment with LIF and RA 5uM were immunoprecipitated with the HDAC1(data not shown) and E2F antibodies. Normal Serum Rabbit was used as a control. HDAC activity associated with E2F was also analyzed in the reciprocal experiment. We observed that HDAC1 is associated with E2F only in iPS treated with LIF but with much less activity it is found in iPS treated with RA 5uM. The experiments above described indicate that HDAC1 can interact with E2F.

4.2.9 MSIN3A FORMS A COMPLEX WITH HDAC1 IN IPS INDUCED TO DIFFERENTIATE WITHOUTH LIF AND TREATED WITH RA: RECIPROCAL IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS.

Large networks of proteins govern embryonic stem (ES) cell and iPS pluripotency. Recent analysis of the critical pluripotency factors Oct4 and Nanog has identified their interaction with multiple transcriptional repression complexes, including members of the mSin3A-HDAC complex, suggesting that these factors could be involved in the regulation of Oct4/Nanog function. *mSin3A* is critical for embryonic development, but the mechanism by which the mSin3A-HDAC complex is able to regulate iPS cell pluripotency is undefined. It has been shown (Baltus A. G. et al. 2009) that the mSin3AHDAC complex positively regulates *Nanog* expression in ES cells through Sox2, a critical ES cell transcription factor and regulator of *Nanog*. They have identified the mSin3A-HDAC complex to be present at the *Nanog* promoter only under proliferating conditions concurrent with histone acetylation. For these motives we have studied the interactions between HDAC-1 and mSIn3A in mouse iPS using the nuclear protein extracts obtained from cell cultures treated with LIF or 6 days withouth LIF. In other experiment it has been used extracts of iPS treated with RA 0,5uM for 1h. All this samples were immunoprecipitated with polyclonal antibodies mSIn3A or HDAC1. The immunoprecipitates obtained were resolved by SDS-PAGE and analyzed by Western blot using specific polyclonal antibodies to the protein antibodies HDAC-1 or mSin3A. We have been able to precipitate the protein HDAC1 with antibodies anti-mSIn3A and in reciprocal experiment we used the antibodies anti-HDAC-1

to co-precipitate and get a band corresponding to the protein mSIn3A. As positive controls, the same immunoprecipitated samples were loaded into the gel (Figure 17A, column 1, 2 and Figure 17B column 1,2,3). As negative controls, all samples were immunoprecipitated with an NRS antibody that is able to recognize specific proteins (Figure 17 A e B).

The results show the formation of a HDAC1-mSin3A complex in iPS treated with LIF and wich becomes more abundant in iPS induced to differentiate without LIF (for 10 days) and iPS treated with RA 5 μM for 1h.

4.2.10 BRG-1 INTERACTS WITH CYCLIN E DURING THE DIFFERENTIATION INDUCED BY RETINOIC ACID.

The mammalian SWI/SNF chromatin-remodeling complex is essential for the multiple changes in gene expression that occur during differentiation. However, the basis within the complex for specificity in effecting positive *versus* negative changes in gene expression has only begun to be elucidated. The catalytic core of the complex can be either of two closely related ATPases, BRM or BRG1, with the potential that the choice of alternative subunits is a key determinant of specificity. Short hairpin RNA-mediated depletion of the ATPases was used to explore their respective roles in the well characterized multistage process of osteoblast differentiation. The results reveal an unexpected role for BRM-specific complexes. Instead of impeding differentiation as was seen with BRG1 depletion, depletion of BRM caused accelerated progression to the differentiation phenotype. BRM-specific complexes are present only on the repressed promoter and are required for association of the co-repressor HDAC1. These findings reveal an unanticipated degree of specialization of function linked with the choice of ATPase and suggest a new paradigm for the roles of the alternative subunits during differentiation.

SWI-SNF complexes have been implicated in transcriptional regulation by chromatin remodeling. For this motive we analized the probable interaction between the complex SWI/SNF/BRG1 and the CYC E. an essential cell cycle regulatory protein required for G1/S transition. BRG1 and BAF155, mammalian homologs of yeast SWI2 and SWI3, respectively, are found in cyclin E complexes and are phosphorylated by cyclin E-

associated kinase activity (Shanhan F., et al 1998). Our results suggest that cyclin E may modulate the activity of the SWI-SNF apparatus to maintain the chromatin in a transcriptionally permissive state. To further elucidate the role of cyclin E-cdk2 in growth control and in cell cycle transitions, we looked for novel proteins that associate with cyclin E within the cell. By immunoprecipitation analysis of iPS self renewal and induce to differentiate using antibodies against cyclin E, we identified the presence of the principal components of the SWI-SNF apparatus BRG-1.

In fact in the Immunoprecipitation experiment (data not shown) indicate that exist the interaction between BRG-1 and Cyclin E in iPS LIF sample but in an increasing manner in iPS treated with RA 5uM for 1h. This results shows that this interaction is stronger during differentiation towards neural commitment. This interaction would appear to be functionally significant, because cyclin E can abrogate the ability of BRG1 to induce growth arrest and perhaps is also important for the inducing of differentiation.

4.2.11 RB INTERACTS WITH HDAC1 IN IPS TREATED WITH RA

We present evidence that Rb forms a repressor containing histone deacetylase (HDAC) and the hSWI/SNF identified in higher eukaryotes, and, as in yeast, these nucleosome remodeling complex, which inhibits transcription of genes for cyclins E and A and arrests cells in the G1 phase of the cell cycle. Phosphorylation of Rb by cyclin D/cdk4 disrupts association with HDAC, relieving repression of the cyclin E gene and G1 arrest. However, the Rb-hSWI/SNF complex persists and is sufficient to maintain repression of the cyclin A and cdc2 genes, inhibiting exit from S phase. HDAC-Rb- hSWI/SNF and Rb-hSWI/SNF then appear to maintain the order of cyclin E and A expression during the cell SWI/SNF activity has been widely associated with cycle, which in turn regulates exit from G1 and from, S phase, respectively. Taken together, the above studies provide evidence of interplay between BRG1 (and thus hSWI/SNF) and Rb. However, the significance of hSWI/SNF to normal Rb function remains unclear. Here, we provide evidence that Rb can form a repressor complex with hSWI/SNF and HDAC and a second complex with only hSWI/SNF. These complexes appear to serve as distinct check- points in G1 and S phase, where we suggest that they E2F function to order the expression of cyclins and cdks during the iPS cell cycle.

Brefly nuclear protein extracts LIF, RA were immunoprecipitated with a monoclonal antibody specific for Rb, resolved in 8% SDS-PAGE and tested for the presence of HDAC1, by Western blot (Fig. 18 A). All immunoprecipitated samples were loaded into the gel to verify the expression of HDAC1 (Fig. 18 A). As negative controls, was carried

un'immunoprecipitazione with IgG, which does not recognize specific proteins (Fig. 18 A , column 5,6). It is shown that in the samples LIF and RA 0,5μM exist a complex Rb/HDAC1.On the other end in the samples RA 2uM and RA 5uM is not evident a complex between HDAC1 and Rb. In the reverse experiment, the same total protein extracts were immunoprecipitated with a monoclonal antibody specific for HDAC-1, resolved on 8% SDS-PAGE and analyzed by Western blot with a polyclonal antibody specific for Rb (Fig.18B). In conclusion the results show that the formation of a Rb/HDAC1 is significant in LIF and less in iPS induced to differentiate with RA.

4.2.12 PRELIMIAR RESULTS ON THE STUDY OF THE GENES THAT REGULATE THE CELL CYCLE DURING DIFFERENTIATION OF IPS INDUCED IN EPISC BY L-PROLYN.

From these preliminary data the treatment with L-Proline 150 uM for 72 h (important for the induction of EPISC) affects the level of expression of certain genes that regulate the cell cycle compared with iPS treated with LIF (figure 21 A,B,C,D,E,F,G). To understand the mechanisms that underlie induction of the EPISC, are analyzed through Western blot the expression profile of genes that regulate the cell cycle such as cyclin A (Figure 21A), the cyclin E (Figure 21B), the CDK2 (Figures 21C), mSIn3A (Figure 121D), BRM (Figure 21E), HDAC1 (Figure 21F) Oct4 (figures 21 G). All samples were normalized with a Western blot for beta actin, a protein expressed constitutively in all the cell lineages.As control has been used a line iPS treated with LIF. Cyclin complex in somatic cells occurs during S phase A1/CDK2 maintaining pRb in an inactive form, highly phosphorylated. In undifferentiated ESC this complex shows a costitutive kinase activity which is responsible of extensive autoreplication. As confirmation the levels of cyclin A detected by Western blot are high in undifferentiated ESCs. The results obtained shows that following treatment with L-proline of iPS, the levels of cyclin A (Figure 21A) undergo a slight reduction (range 20-40% lesser than LIF) in their protein expression (both in the samples treated and not treated with LIF) compared to those measured in undifferentiated iPS. The levels of expression of Cyclin E (Figures 21B) underwent a reduction (range 60-70% than LIF) (compared to undifferentiated iPS), more pronounced than that of cyclin A. The levels of

CDK2 (Figures 21 C) instead undergo a slight increase after induction with L-proline in the samples grown with EPISC LIF.Invece EPISC samples grown without LIF have a CDK2 protein expression similar to control samples undifferentiated iPS. The levels of CDK2 (Figures 21 C) instead undergo a slight increase after induction with L-proline in the samples EPISC grown with LIF (of about 20%) Instead EPISC samples grown without LIF have a protein expression of CDK2 increasing than iPS (of about 70%) .The WB analysis of specific mSIn3A (figure 21D) shows that this protein is more expressed in the samples EPISC treated with LIF and to a greater extent in samples EPISC not treated with LIF compared to those measured in undifferentiated iPS. It is significant that the expression levels of brm (figre 21E) increase dramatically in EPISC treated with LIF than iPS LIF (of about 9 times). Emerges from this analysis that treatment with L-proline greatly influence the level of protein expression of brm compared with iPS treated with LIF. The literature shows that BRM is not expressed during M phase and then increases greatly during the entry into G1 phase. Probably L-Proline induces an increase of brm in such a way that they enter in the G1 phase typically just as undifferentiated stem cells. The levels of Oct4 are lesser in iPS cells induced by L-Proline (of about 40-50% than control iPS LIF) (figure 21 G). As negative control the WB for HDAC1 show that this protein shows a similar level of expression in undifferentiated iPS and induced with L-proline (figure 21F).

4.2.13 PRELIMIAR RESULTS ON THE STUDY OF THE GENES THAT REGULATE THE CELL CYCLE DURING DIFFERENTIATION OF IPS BY ASCORBIC ACID.

Treatment with ascorbic acid induces the differentiation of iPS cells into cardiomyocytes, which is why it was decided to investigate in detail the early events of differentiation of iPS. In particular, these studies have been extended with the use of Ascorbic Acid (50 ug / ml) that modulates genes that control the cell cycle of iPS. This chemical induces increases the degree of induction and differentiation of embryoid bodies generated from iPS. The purpose of these studies is to identify which genes are modulated during the early stages of differentiation of iPS cells with a HDAC-control. It has been made preliminary analysis of Western Blot to test the effect of ascorbic acid on the iPS # 202 treated for a short time (1 h) with this chemical. In particular, we analyzed the level of expression of Cyclin A (Figure 22 A), CDK2 (Figure 22B) brm (Figure 22 C) HDAC1 (Figure 22E) and Oct4 (Figure 22 D). The genes of cyclin A after Ac. Ascorbic treatment (figure 22A) decrease of about 10 times than control LIF. The CDK2 expression (figure 22B) slightly decrease after treatment compared to control iPS LIF (of about 5%) . Instead, the brm gene (figure 22C). after induction with this chemical agent is increasing of about 2,2 times in its protein expression compared to LIF The results indicate that treatment with ascorbic acid does not affect the gene expression of HDAC1 compared to control LIF(figure 22E). It has also been used as a marker gene Oct4 undifferentiated state: the level of expression of this protein is indeed very high in iPS treated with LIF and decreases following treatment with ascorbic acid of

about 40% (figure 22D). These results confirm that during the period of treatment with ascorbic acid, the iPS # 202 begin to lose their characteristics of pluripotency.

4.2.14. CHARACTERIZATION OF COMPLEX REPRESSOR CERM / CERC BY EMSA IN CES AND IPS TREATED WITH LIF AND WITH RETINOIC ACID.

The regulation of G1 / S in somatic cells is governed by sequential activation and pRb complexes cyclin / Cdk. Precisely pRb forms a complex with E2F, recruiting HDAC and SWI / SNF, able to repress the transcription of genes involved in G1 / S cell cycle such as the gene encoding the CCNE1 cyclin E1.When, in response to appropriate stimuli proliferative, pRb is phosphorylated and inactivated by cyclin complexes / Cdk, the cells complete the G1 phase and progress into S phase. The phosphorylation of Rb by cyclin complex D1/Cdk4-6 promotes the release of HDACs from the domain "pocket" of pRb. The removal of HDAC leads to increased acetylation of a specific nucleosome located at the site of transcription initiation of cyclin E1 and its subsequent activation.The activation of the promoter of cyclin E1, which occurs during the G1 / S decreases in S phase and disappeared during the period between the end of mitosis and late G phase

In differentiated cells such as the K562, has been seen that the activity the promoter of cyclin E1 gene is controlled by a DNA sequence defined as "cyclin E repressor module" (CERM) through association with a complex of high molecular weight E2F-specific G1 called "cyclin E repressor complex 2" (CERC2). In proliferating cells in early G1 phase sequence is bound by the CERM CERC2 complex and cyclin E1 gene is not transcribed. In proliferating cells during S, G2, M, the dissociation of the complex CERC2 CERM sequence at the end of the G1 phase, is correlated with the induction of the transcription of cyclin E1. Recent studies have shown that undifferentiated ESCs are characterized by the

lack of complex cyclin D1 / Cdk4-6 and the absence of a cell cycle regulated by the activity of Rb. Although the CES express the pocket proteins, pRb and p107, these are kept in a hyperphosphorylated by G1 phase of the complex Cdk2/ciclina. The absence of the hypophosphorylated form of Rb, the only functionally active in sequestering the E2F, reflects the high proportion of cells in S phase and the high proliferative rate: E2F transcription factors are not subject to repression by pocket proteins transcribe the genes responsible for progression of the cycle. Constitutive kinase activity of Cdks during the cycle is closely related to the levels of expression of cyclins E1 and A1 do not show any periodicity. For this reason, an objective of my thesis was to study the regulation of gene expression of E1 during the cyclical "self-renewal" of mouse ESCs and iPS. In particular, studies were carried out EMSA in ESCs and iPS treated with LIF to study the complex CERC2 repressor that binds to the sequence of the promoter of cyclin E1 CERM in the period between the end of mitosis and late G1 phase in differentiated cells K562.My original studies through EMSA experiments shows the formation of different molecular weight bands that reflect the differences in electrophoretic mobility between the probe sequence marked CERM-free protein, which migrates in a polyacrylamide gel more quickly, and a protein complex related to radioactive probe, which migrates more slowly.In particular, the low molecular weight bands (indicated by square brackets) correspond to nonspecific complexes CERM-independent (termed NS), while the probe sequence marked CERM corresponds to high molecular weight multiprotein complexes that bind the site CERC2 CERM (Fig .19 A and B).The binding activity in all complexes is solely due to the complex CERC2, as evidenced by the addition of cold competitors that decrease almost

completely specific complexes (Fig. 19 A, column 2 and 3; Fig.19B, column 2 and 3). The results obtained indicate that in undifferentiated ESCs treated with LIF was found to contain a high molecular weight band corresponding to the complex CERC2 of differentiated cells. The results in figure 19A and B and C indicate that p130 is present in the complex CERC2 in ES and iPS treated with LIF and also in iPS induced to differentiate with RA 0,5 μM for 1h. . Other result confirms that during self-replication of the CES a multiprotein complex binds to the promoter sequence CERM cyclin E1, which is expressed constitutively and without cell cycle periodicity in the CES.The intensity of the band corresponding to the complex CERC2 in iPS undifferentiated cells treated with LIF, compared with that of the human CES undifferentiated treated with LIF line, emphasize a reduced function of the repressor complex in the CES (Fig 19 E and E').

In these very important studies, EMSA experiments were performed to investigate any differences in the composition of the complex CERC2 in undifferentiated ESCs and in iPS cells. Although different antibodies have been used, only the addition of the antibody specific for p130 determines the capacity reduction of the complex to bind the oligonucleotide and therefore a decrease in the intensity of the LIF band in the samples, stressing that p130 might be one of the components of CERC2 in iPS and CES (Fig. 19A and B and C column 5). To evaluate the effect of treatment with 0.5 μM RA for 1 hour on the composition of the complex CERC2 EMSA experiments were performed with antibodies specific for certain transcription factors such as P130, E2F4, and CYC E and Rb. The results obtained in the reaction with the p130 antibody showed that the intensity of the band decreases in LIF and RA samples (figure 19 A, B, C). It was subsequently studied the

composition of the complex in iPS CERC2 using antibody E2F4. In this experiment we observe a slight shift of the band with the probe wt but not in the mutated (where the band of the complex CERC2 does not exist), indicating that the interaction is specific (Figure 19 D and D'). This super shift can be seen in both iPS LIF that in iPS RA. Furthemore we also used the antibody specific for cyclin E and also in this experiment we observed supershift of the specific band for the CERC complex 2 (figure 19 E and E') in both samples LiF and RA. Figure 19 F and F' shows another EMSA experiment showing that Rb is more present in the extracts iPS treated with RA because there is a greater reduction of the specific band signal in this sample compared to the control LIF. This result shows that Rb is more present in complex CERC2 in RA samples than in undifferentiated iPS cells. In figure 19 G and G' is presented a experiment of EMSA studing the presence of CERC2 complex in iPS EPISC cell and in iPS induced to differentiate with RA.The results shown that also in iPS induced to differentiate in EPISC is present the CERC2 comples; also in iPS treated with ascorbic acid the complex CERC2 is present but in increased levels.

4.2.15 E2F/RB COMPLEX INTERACTIONS WITH SOME GENES THAT CONTROL THE CELL CYCLE IN IPS CELLS TREATED WITH LIF AND RA

The balance between the levels of functionally active Rb protein levels and E2F may, in part, define a critical point for cell cycle progression in G1 / S. However, the complex formation E2F/Rb not only blocks the transcriptional activation mediated by E2F, but can also actively repress transcription. In this condition, the Rb promoter recruits several proteins that modify chromatin structure, such as histone deacetylase complex and the SWI / SNF. In particular, in differentiated cells, several studies show that Rb represses the E2F transcriptional activity more efficiently in the presence of brm (Trouche et al., 1997) and that cooperation between Rb and brm or BRG-1 can induce the arrest cell proliferation (Muchardt and Yaniv, 2001). The dynamic changes of chromatin, mediated by these proteins may be a mechanism by which iPS regulate gene expression during differentiation and development.

In this regard, in the present original work we have analyzed the interactions of E2F complexes with Rb and CYC E in iPS treated with LIF and RA. The total protein extracts prepared from these cells were analyzed by EMSA (Fig. 20). The oligonucleotide used is the wt DHFR, containing a binding site for E2F. Surprisingly these experiments have shown that, undifferentiated ESCs form E2F complexes containing Rb high molecular weight and are present mainly in free form. While in iPS treated with RA these complexes are very weak. In agreement with these results, immunoprecipitation analysis found the same result. In RA samples, however, is abolished the ability of E2F to form complexes

with Rb. For this reason, these samples were tested for the presence of CYC E. Reaction with specific antibodies indicate that cyclin E (Fig. 20, column 5 and 10) is present in this high molecular weight complex and the surprisingly the antibody performs a specific supershift. The effects of this antibodies are specific, in fact, no interaction was observed after the addition of NRS, used as a negative control. It has been done another experiment using the DHFR probe studing the presence of fact or Rb. In figure 20 B is shown that this factor is present bound to E2F complexes in iPS LIF but less in iPS induced to differentiate with RA.

4.2.16 CRE-DNA BINDING COMPLEXES IN RA AND EPA+RA TREATED ES CELLS.

Retinoic acid (RA) induction of ES cells may be used as a cellular model for studying commitment and neural specification (Guan et al., 2001, Cell Tissue Res.2001:305: 175176). Spontaneous neural commitment of mouse ES cells require induction of RA signaling to suppress Nodal signaling (Engberg et al., Stem Cell 2010). After RA treatment of ES cells for 24 h (RA 24h), the CRE-DNA binding -pattern change very rapidly and is composed of different protein complex very similar to the CRE-DNA binding complexes (data not shown) already observed in pup brain tissues. This RA effect on the three CREB-1 like complexes in ES cells, is similar to the results obtained by EPA treatment alone. In more detail, in the ES cells induced to differentiate with RA for 24h, the complex I and II and the CREB-1.1 band were up-modulated compared to the pattern of CRE binding complexes observed in the LIF treated ES cells. By contrast, the CREB-1.3 complex was down-modulated by 24h RA treatment compared to the LIF treated ES cells With our surprice, in the EPA treated ES cells induced with RA for 24h, all the five CRE-DNA complexes (particularly, complex I complex II and CREB1.1, CREB1.2) were up-modulated according to the length of the EPA treatment (1h, 3h and 6h), reaching the higher intensity at 6h. The complexes CREB of iPS cells is similar but non identical than the CES. In fact in iPS treated with LIF the complexes CREB I and II are not visible and the CREB-1.1 is up modulated than CES cells.We have also evaluated by western blot the expression of total CREB and its activated form (p-CREB) in RA treated ES cells. We observed a significant increase of p-CREB from 1 h to 3h and at 9 hr. Moreover a decrease at 6h and 24h later time point was observed (data not shown). On the other hand, total CREB show s more costant expression from 1h to 24 h RA treatment (data not shown). Our

CREB data are in agreement with published data (Canon et al., 2004. Molecular Biology of the Cell 15,5583-5592). The p-CREB/tot-CREB ratio show a significant upmodulation at 3h after ES cells RA treatment (data not shown). Instead in the iPS cells (figure 14C) we observed that after 1h RA treatment 0,5 μM there is an increasing expression of CREB P of about 20% (than LIF)($p < 0,05$). Instead at 2μM for 1h is shown a decrease of about 20% ($p< 0,01$) than control LIF and more at 5 μM of about 60% ($p< 0,01$). In iPS at 24 h RA treatment (0,5 μM; 2μM;5 μM) we observed only slightly decreasing amount of CREB P expression statistically not significative.

5 .DISCUSSION

The ability to reprogram human somatic cells to a pluripotent state offers the possibility to produce large numbers of cell types with a patient's own genetic background, which raises exciting new prospects for biomedical research and autologous cell replacement therapies. The plasticity of ES and iPS cells renders them difficult to control and is considered a major obstacle on their route to clinical applications, because they possess the risk of teratoma formation. To this end, the accessibility of purified lineage-specific progenitors may represent a significant advantage for safer future clinical and translational applications. In addition, in organs such as the heart where multiple cell types have to be replaced, multipotent progenitors could ideally contribute to both remuscularization and revascularization, because they should be able to proliferate and differentiate into diverse mature cells in response to different microenvironmental cues. In the current study, we generated mouse iPS cell lines that allow irreversible genetic marking of Isl1-expressing cells and demonstrated that iPS cells can serve as a source of multipotent Isl1_ cardiovascular progenitors similar to the ones derived from ES cells. The use of retroviruses to deliver the reprogramming factors may trigger cancer formation and disrupt endogenous gene expression. The absence of microscopic evidence of tumor in all injected hearts suggests that in both iPS cell lines used there was no reactivation of retroviral oncogenes or "undesired" viral genomic integration.

AA has long been studied by groups involved in nutrition and has been considered a food supplement (Belin et al, 2010). Through its antioxidant property it promote extracellular

matri formation by stabilising collagen structure. Recent progress allows us to consider additional functions, among which embryonic/cell differentiation is probably the most promising. Probably AA act on cell division , because the division rate could be affected by the local AA concentration. As the progression and inhibition of cell division are crucial during embryogenesis and cell differentiation, we could hypothesize that the local AA concentration influences these processes. This heterogeneity in AA concentration is probably linked to the concentration of the transporter SVCT2, which may act as both a transporter and a receptor. The local AA concentration could modulate the expression of a battery of genes and influence cell division. An alternative mechanism may be direct action of AA on the expression of genes involved in mammalian development. Regarding the signalling pathway that could be involved, Belin et al 2010 suggest cAMP pathways (probably through CREB synthesis, asseems to be the case for *PMP22*). Some recent work suggests, AA is a competitor of adenylate cyclase activity, suggesting that it is a global regulator of the intracellular cAMP pool. In conclusion, AA could modulate the expression of a battery of genes expressed under the control of cAMP-dependent pathways.

A purpose of this research was to study the role of Ascorbic Acid during the differentiation of embryonic bodies derived from iPS. My studies on the induction of differentiation induced by ascorbic acid have shown that this compound is able to increase the morphological differentiation of iPS cells into cardiomyocytes. This result indicates that this compound acts in the same way in both the IPS as in ES, as evidenced by the bibliography. The preliminary molecular studies through WB have shown that Ascorbic Acid induces the decreasing of the protein expression of CYC A, CDK2 and Oct4 but also

a increasing of the protein quantity of BRM and HDAC1. This results shows that BRM is very expressed in iPS cells induced to differentiate with Ascorbic Acid as in iPS treated with RA. Naturally the level of Oct4 protein is less expressed because the ascorbic acid induce the differentiation of the iPS in cardiomyocites. Also the level of CDK2 and CYC A are decreasing in iPS induced to differentiate with Ascorbic Acid. All this surprisingly results indicate that Ascorbic Acid induce the differentiation very soon by the induction of this chemical.

The group of Casalino (2011) successfully used a novel robotic platform, the Cellmaker, on phenotype-based screenings (i.e. ESC proliferation/CPAs) and identified two metabolically related amino acids, namely L-Pro and L-Orn, as ESC regulators. Worth noting, a third metabolite was found, namely L-Lysine, which acts as a potent inhibitor of ESC proliferation. Interestingly, a similar inhibitor effect of L-lysine on HeLa cell proliferation was early reported (Eagle, 1955). In line with our findings, Washington et al. (2010) very recently reported that L-Pro induces differentiation of ESCs, even if, conversely to the Cellmaker strategy, this finding was obtained through conventional multi-step, timeconsuming, biochemical processes implying: chromatographic fractionation of HepG2-conditioned medium, evaluation of the biological activity of all obtained fractions, and resolution of the chemical composition of each active fraction. The first response of ESCs to L-Pro or L-Orn is a significant induction of proliferation; later on, proliferating ESCs undergo a phenotypic transition that results in the generation of EpiSC-like cells. Worth nothing, our data suggest that both generation (ESC_ PiC transition) and self-renewal of PICs depend on L-Pro oxidation, catalyzed by a mitochondrial enzyme, i.e. POX. In

correlation, it has been recently reported that self-renewing divisions of ESCs depend on another amino acid, i.e. threonine, and particularly on its oxidation, catalyzed by a mitochondrial enzyme, i.e. threonine dehydrogenase (Dejosez et al., 2010). Hence, our data in colloaboration with Laura Casalino Group of CNR in Naples support the intriguing idea that metabolism represent an almost unexplored level of stem cell pluripotency control (Dejosez et al., 2010). Thus, our data suggest that L-Pro may be used to improve protocols for deriving induced pluripotent stem (iPS) cells from fibroblasts, by selectively promoting ESC/iPS rather than fibroblast proliferation.Interestingly, the in vitro ESC_EpiSC-like transition induced by L-Pro is reminiscent of the in vivo blastocyst_epiblast transition occurring during mouse embryo implantation.All together, these findings argue against the involvement of ROS in the induction of the ESC_ PiC and iPS_PIC transition and suggest that antioxidants, particularly Vc, impair L-Pro activity likely influencing other biological functions. The ultimate goal was to observe what are the differences in the behaviour of the genes of the cell cycle in EPISC derived from exposure of iPS to the proline. In this study, I found that proline induces the same effects on the morphology of iPS after induction than ES. In fact these cells after exposure to this agent and in absence of LIF assume a more irregular shape and the nuclei seem smaller and heterochromatic, whereas in EPISC in presence of LIF the nuclei are larger and heterochromatic (figure 10). Regarding the behavior of genes in the cell cycle EPISC I found that the CYC A and CYC E , Oct4, decreasing during the induction with L-Prolyn. The fact that Oct4 decrease means that very probably the iPS treated with this chemical have been induct in EPISC. Instead the level of

CDK2, mSin3A and BRM increase.This very interesting results means that the EPISC have done a sort of differentiation during the induction with L-Prolyn.

Regarding the molecular analysis we were study the cell cycle during the reprogramming of iPS cells because it is still unclear whether the cell cycle of the iPS cells presents unique characteristics compared to the cell cycle of ES cells. This study is of considerable importance for understanding the molecular mechanisms that control the unlimited capacity of self-renewal and differentiation potential of iPS. One of the purpose of this original work is to study the behavior of the cell cycle genes during differentiation induced by withdrawals of LIF and by RA in iPS.

The results indicate that cell cycle genes are modulated as expected from exposure to Retinoic Acid and by withdrawals of LIF in the medium culture.It have been found that the Oct4 gene expression is modulated in a very interesting manner. In fact at day 10 after withdrawals of LIF we found an increment of this gene expression than the control LIF. Is difficult to explain this results but maybe at day 10 there is an induction by through cellular contact that has stimulated the expression of Oct4. This results means that the staminality is not only manteined by LIF but also by other factors maybe by cellular contacts. Other genes are modulated during the LIF withdrawal, as Cyclin E and Cyclin A. Infact the cyclin E increase at day 2 significantly but at day 6 decrease and at day 10 increase again. Also the behaviour of Cyclin A is similar to the cyclin E. There is an oscillation of these protein expression during the withdrawal of LIF. Instead HDAC1 expression is not modulated. We only observe an increasing of the expression at day 10. These results confirm that exist other controller to us unknown that modulate the proliferation; I think that also the density

of cell growing is an aspect very important that control the proliferation. I was able to show that the majority of genes that regulate the cell cycle of iPS are modulated by the Retinoic Acid differently to ES. In short, the behavior of iPS during differentiation seems to be different than the differentiation induced in the ES. These results reflect the different nature of self renewal of the iPS compared to the ES; in fact it is highly likely that iPS cells as derived from murine fibroblasts did not behave during the cell cycle and differentiation as ES cells.

In particular, the murine iPS are maintained in the undifferentiated stage in the culture medium with the addition of leukemia inhibitory factor (LIF). The iPS induced to differentiate into specific cell types by treatment with certain chemicals such as retinoic acid, is a useful experimental model to study the pluripotency of iPS. The rapid proliferation of cES seems to be driven by an unusually high activity due to the cyclin E and cyclin A, which show no periodicity in the cell cycle (Stead et al., 2002). This study shows that CYC A, CYC E, E2F1, E2F2, E2F4 are present in greater quantities in undifferentiated iPS and that their expression is modulated when the iPS differentiate. These results suggest that each factor, in combination with different DP, has a specific time and a different mode of action. Individual members of the E2F family can be involved in the regulation of differentiation, activating or repressing different genes at different stages of development (figure 11C). Alternatively, individuals could control the expression of E2F genes themselves differently in different cell types, perhaps in combination with specific factors. The characteristics of the cell cycle of ESCs are often associated with uncontrolled proliferation of cancer cells. For this reason, E2F1 may be an important regulator of CES

auto-replication extensive and early stages of differentiation. In addition, the results shown in this study support the idea, advanced in several works (Savatier et al., 1994 and 1995, Stead et al., 2002), that in the CES activity of E2F is independent of the route of regulation Rb, in fact, no complex E2F/Rb was observed in undifferentiated cells and of E2F target genes do not appear to be regulated during the cell cycle. In agreement with this, cyclin E, the gene is subject to repression complexes E2F/Rb in differentiated cells, is expressed in undifferentiated ESCs in quantity kinase activity associated with it seems to be constitutive. The Rb-dependent regulation (figure 11D) is restored when the cells begin to differentiate. This could explain why the cES express Rb protein: its presence makes the cells ready to establish the regulation of G1 phase immediately after the removal of the stimulus of LIF, which maintains the "self-renewal" of CES. Instead, treatment of ESCs for 1 hours with retinoic acid appears to have only a weak effect on complex formation E2F/Rb. It is likely that these complexes are E2F/Rb recovered completely only after a long treatment (two or three weeks) with RA.You can assume that many changes of enzyme complexes related to cell cycle regulation occur when cells begin to differentiate pluripotent. First, the activities associated with cdk of G1 / S cell cycle become regulated and / or dependent mitogenic signals. Secondly, it sets the cell cycle regulation for E2F-dependent transcription. Finally, the cell cycle phases G1 and G2 acquires full with a consequent decrease in proliferative rate. Data reported in the literature indicate that undifferentiated murine embryonic stem cells in the expression of brm is low or absent, its level gradually increases during development until reaching a maximum in the post-mitotic adult tissues, the protein BRG-1, instead is constitutively expressed during the various stages of embryonic development

(figure 11 A) (Muchardt and Yaniv, 2001). It is possible, therefore, suggest that the induction of the expression of brm allows the formation of new complex SWI / SNF that can regulate specific target genes to modulate the proliferation and differentiation of iPS. In addition, the high kinase activity associated with cyclin E and the direct interaction between brm / BRG-1 and cyclin E suggests a controlled adjustment of the activities of the complex SWI / SNF in modulating cell proliferation during differentiation. Analysis of cyclin E-containing complexes in the cell demonstrated that cyclin E associated with several cellular proteins. These include its kinase partner, cdk2, and molecules that affect the activity of cyclin E-cdk2 complexes both positively (cdc25A) and negatively (p27). In addition, this approach has previously yielded the identification of novel substrates, including both p107 and p130, suggesting that cdks canform stable enzyme-substrate complexes in vivo. This stable interaction may provide a mechanism by which increased specificity and selectivity can be achieved. In this study, we have characterized a novel cyclin E-associated proteins as components of the mammalian SWI-SNF complex. BRG1 contain cdk consensus phosphorylation sites, and both could be phosphorylated by cyclin E-cdk2-associated kinase activity in vitro. Furthermore, BRG1 and BAF155 are in a phosphorylated form in the cyclin E complex. Another component of the SWI-SNF apparatus, the Ini1-hSNF5 protein, is also present in cyclin E immunoprecipitations, which suggests that the entire SWI-SNF complex may be recognized by cyclin E. Our experiments further demonstrate an intriguing requirement for the presence of BRG1 in the SWI-SNF complex to promote the recruitment of cyclin E. This observation suggests that either BRG1 recruits some essential

factor to the complex, or the SWI-SNF apparatus is somehow modified in the presence of BRG1 so that it is recognized by cyclin E.

The imposition of a control of the Rb-dependent G1 phase could therefore provide an opportunity for the remodeling of chromatin and for the establishment of a new transcriptional program, both needed to guide cells to specific lines-(Burdon et al ., 2002). Clarify the transcription factors and target gene activated by RA acid may be useful to direct ESCs to differentiate into a particular cell line.However, recent studies (Martinez-Balbas et al., 2000) show that acetylation represents a new mechanism to regulate the activity of E2F1, increasing the stability of the protein, its ability to bind DNA and the ability transcriptional activation. This suggests that acetylation may regulate the functions of the form of E2F1 complexes not linked to Rb and Rb / HDAC may deacetylated E2F1 itself. Given that E2F1 activity is very high in the CES, future experiments should clarify whether the acetylation of this protein is important in supporting the rapid proliferation of these cells. This is in agreement with recent studies on mice "knockout" for various cyclins (cyclins D and E) in which it was found that in vivo during the early stages of embryonic development, signs of growth are provided in constitutively by the placenta and via control mediated by D-type cyclins, which leads to inactivation of Rb function is not required for cell proliferation. In this way the embryo is provided a series of very rapid cell division until it reached the number of cells is sufficient to initiate gastrulation (Pagano and Jackson, 2004).

This study also suggests that the complex E2F/Rb have an important role during differentiation of iPs. Further studies, involving genes specifically activated or repressed by

different E2F/Rb complex, will be useful both for understanding the cell cycle control of the iPS and for the ability to direct these cells toward a specific differentiation path.

To determine the role played by other complex cyclin / CDK in undifferentiated iPS we turned our attention to the study of the expression of cyclin A and cyclin E. We observed that in both iPS cyclins are expressed in the cell cycle of iPS. This allowed us to speculate that cyclin A and ciclyn E are involved in cell cycle regulation of iPS. The cell in response to extra cellular stimuli activate signal transduction SOS / RAS / MEKK / MAPK, which increases levels of cyclin D in association with CDK4-6. Rb is then phosphorylated and releases HDACs. This allows the elimination of the block in G1 phase. The subsequent entry into S phase is due to additional phosphorylation of Rb, which mediates the release of the complex SWI / SNF and the synthesis of cyclin A which advances the cell cycle in G2 phase. (Roberts and Orkin C.W.M. S.H., 2004). We observed that RA treatment of iPS affect transcriptional repressors such as mSin3A and HDAC-2. Their expression is very high and decreases after RA treatment. The expression of BRG-1-1 and HDAC-1 is not altered by treatment with RA. E 'therefore likely that BRG-1 and HDAC-1 are both involved in differentiation and proliferation of iPS , while mSIN3A HDAC-2 and RbAp46/48 seem more involved in the mechanisms that regulate the proliferation of iPS because more able to suppress differentiation.

Through immunoprecipitation analysis has been possible to obtain a characterization of the biochemical components of these complexes in iPS. Analysis of the results of immunoprecipitation showed that the interactions between HDAC-1, Rb, mSIn3A, Brg-1, Id2 are more present in into iPS induced to differentiate with RA (HDAC interaction E2F-1

is present only in the LIF). It can be assumed that the complex HDAC-1/Rb, HDAC-1/mSIn3A, HDAC-1/BRG-1 Rb/BRG-1 and are important to suppress the proliferation and activate differentiation. Instead the complex BRG-1/mSIn3A seems important in iPS to recruit HDACs later. In conclusion from our study shows that the regulation of the proliferation of iPS is mainly based on cyclin A and E, the E2F transcription factors and genes brm or BRG-1 remodeling chromatin in association with other factors that influence the transcription as HDAC-1, mSIn3A. Hence the proliferation of iPS and maintaining self-renewal depends on the coupling between membrane receptors and signal transduction pathway that allows iPS to respond to extracellular stimuli it receives from the outer cell influencing gene expression. A model for the iPS cell cycle progression is in figure 3.

Molecular analysis of these mechanisms is of paramount importance to discover the incredible potential of iPS and to facilitate their use in new and promising therapies.

Over the last few years, the identification of new regulators of stem cell proliferation and/or differentiation, which are essential to realize the potential of regenerative medicine, has been greatly facilitated by the application of automation technologies. Briefly, my original studies on the behavior of the cell cycle genes during differentiation induced by various chemicals in the iPS cells showed that these cells resemble in the ES phenotype but some genes behave differently than the ES and many interactions between the proteins that regulate the cell cycle in iPS are different from those in the ES.Given the importance of the transition G1 / S in the control of cell proliferation, one of the purposes of my project was to study the regulation of gene expression of cyclin E1 at the "self-renewal" of mouse iPS. In differentiated cells such as K562, has been seen that the activity of the cyclin E1 gene

promoter is controlled by a DNA sequence defined as "cyclin E repressor module" (CERM) through association with a complex high molecular weight E2F G1-specific molecular called "cyclin E repressor complex 2" (CERC2) (figure 11B). In proliferating cells in early G1 phase sequence is bound by the CERM CERC2 complex and cyclin E1 gene is not transcribed. In proliferating cells during S, G2, M the dissociation of the complex sequence CERM CERC2 by the end of G1 phase, is correlated with the induction of the transcription of cyclin E1.The analysis of the repressor complex CERC2 on the self renewal and differentiation of iPS began with EMSA experiments performed in undifferentiated iPS treated with LIF and RA. As a control we have used the line C6 glioblastome of rat representing a more advanced stage of differentiation. The comparison showed that the intensity of the band corresponding to the complex in iPS treated with LIF CERC2 is lower than that found in K562 cells to stress in reduced functionality.Although several antibodies have been used, only the addition of the antibody specific for p130 determines the capacity reduction of the complex to bind the oligonucleotide and therefore a decrease in the intensity of the band in the samples LIF stressing that p130 might be one of the components CERC2. To test whether the imposition of a G1 phase-dependent control of Rb could alter the activity of CERC2 EMSA experiments we have used iPS cells treated with LIF and treated with RA 0,5 μM for 1h and incubated with antibodies specific for transcription factors belonging to the family E2F (E2F4), the pocket proteins (p130), Rb and cyclin E1.Analysis has revealed that even in ESCs treated with RA 0,5 μM for 1 hour the complex CERC2binds to the sequence of the gene promoter CERM CCNE1 preventing the expression and that its composition does not differ much from that found in K562 cells

blocked in G1.There was a sligthly increase in the intensity of the band corresponding to CERC2 in the samples RA compared to that found in samples LIF. Variation of this band was detected following the addition of antibodies specific for E2F4, Rb, CYCE, p130. These original results means that the comples CERC2 in iPS treated with RA is more functional than iPS LIF and this complex is made by a interaction between E2F4, Rb, CYCE, p130 proteins (a model is in figure 3B). Using another probe of a sequence of promoter DHFR that bind specifically E2F proteins I have surprinsinly discovered and confirmed that E2F are present and bind Rb and CYC E proteins in a stronger manner in iPS LIF than iPS differentiating with RA (a model is in figure 3C)..In conclusion, by molecular point of view, the iPS cells closely resemble ES, but are not exactly equal, because these original studies indicate that these iPS cells and their differentiation into particular cell fates are similar but for other aspects are different to those observed in ES . In fact, it will need to make improvements so that the genetic behavior of iPS stem cell and them differentiation can be just equal to that of ES cells to be able to use iPS cells for the treatment of major diseases and not cause damage to the human health.

6. FIGURE

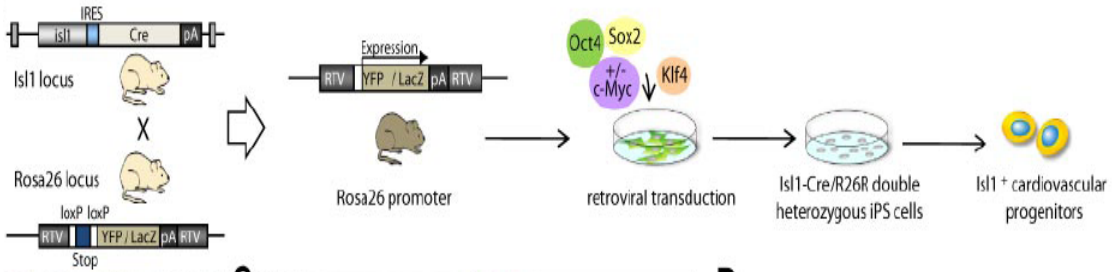

Figure 1 Generation of iPS cells from Isl1-Cre/R26R-indicator double-heterozygous mice. *Scheme for in vitro reprogramming* of skin fibroblasts with 3 or 4 defined transcription factors to generate genetically marked Isl1 cardiovascular progenitors. Isl1-Cre mice were crossed into the conditional Cre reporter strains R26R-YFP or R26R-LacZ, in which Cre-mediated removal of a stop sequence results in the ubiquitous expression of YFP or -galactosidase under the control of the endogenous Rosa26promoter (A. Moretti, 2010).

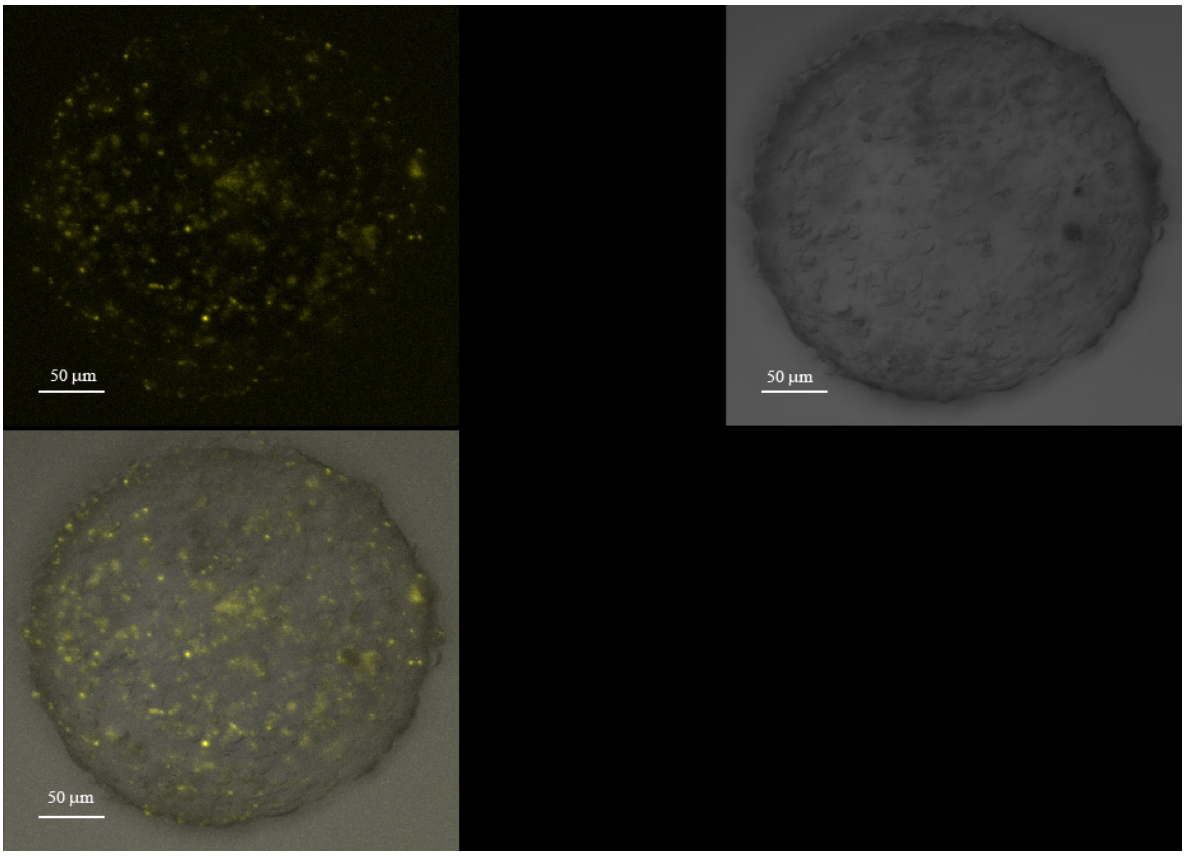

Figure 2. Embryonic Bodies generated by Hanging Drop methods. The iPS differentiated in cardiomyocytes express the YFP protein (in yellow).The bar indicates 50 µm.

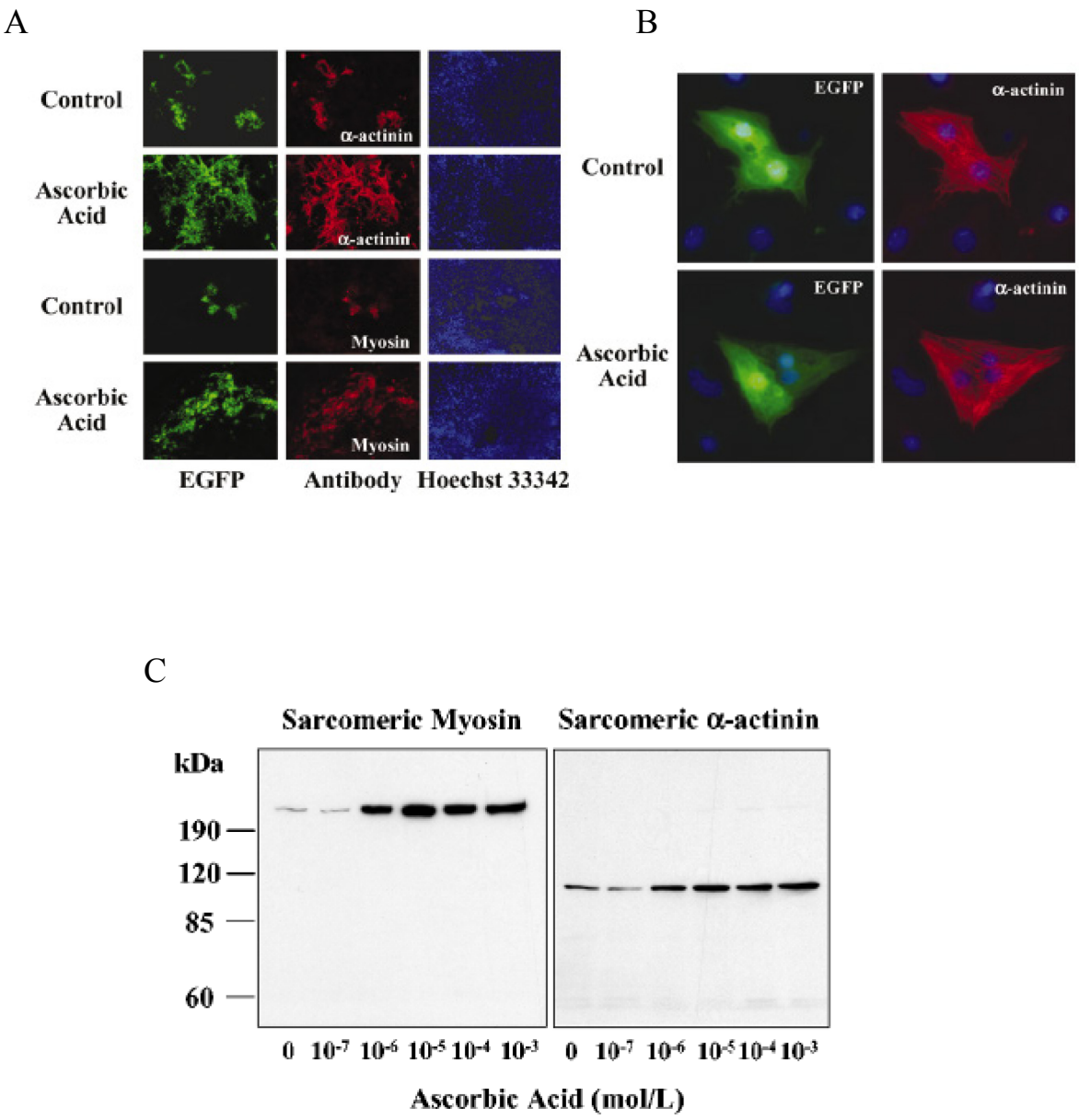

Figure 3A Immunostaining of ES Embrionyc Bodies with antibodies against sarcomeric myosin and a-actinin. A, Cells were treated with or without 10-4 mol/L ascorbic acid for 12 days and stained with the indicated antibodies(Takahashi T, 2003).

3B, After treatment of ES Embrionyc Bodies with or without 10-4 mol/L ascorbic acid for 12 days cells were dissociated, re-plated on glass coverslips, and stained with anti-αactinin antibody (Takahashi T, 2003).

3C, Immunoblot analysis of sarcomeric myosin and α-actinin. Cells were treated with the indicated concentration of ascorbic acid for 12 days, and immunoblot analysis was carried out with the indicated antibody(Takahashi T, 2003).

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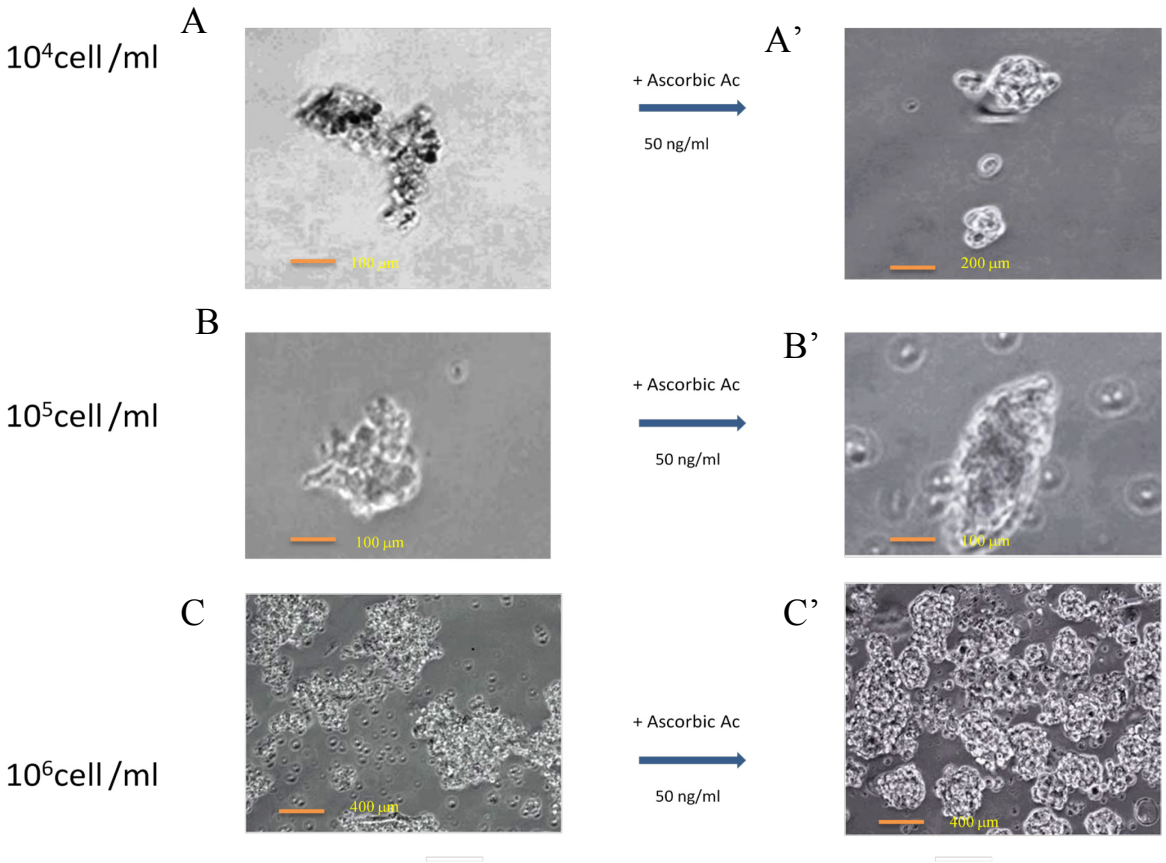
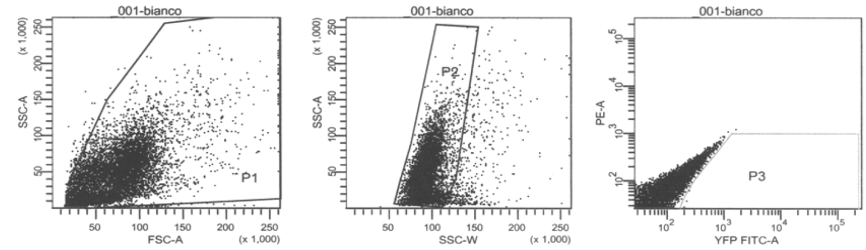


Figure 4. Acid Ascorbic Effect. The Embryonic Bodies was stimulated to differentiate with the add of Ascorbic Acid at a concentration of 50 µg/ml. The induction of differentiation was done at 3 different density of seeder: 10^4 cell/ml (figure A), 10^5 cells/ml /figure B) , 10^6 cells/ml (figure C).



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Tube Name:	bianco
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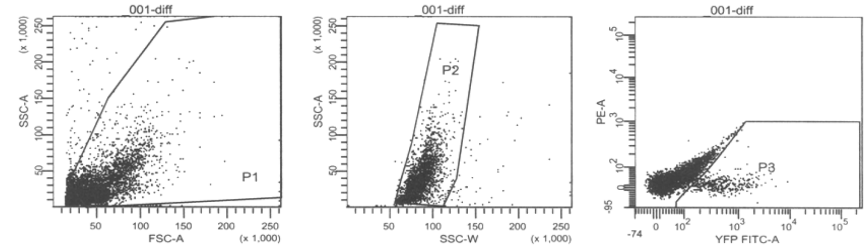
$OP:	Administrator

Population	#Events	%Parent	FSC-A Mean	SSC-A Mean	YFP FITC-A Mean	PE-A Mean
P1	8,461	84.6	78,006	48,045	145	97
P2	7,809	92.3	75,608	47,812	144	96
P3	11	0.1	108,550	75,868	317	104



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Tube Name:	diff
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$OP:	Administrator

Population	#Events	%Parent	FSC-A Mean	SSC-A Mean	YFP FITC-A Mean	PE-A Mean
P1	5,452	48.7	54,183	26,409	114	35
P2	5,144	94.4	53,996	26,882	118	36
P3	396	7.7	55,557	21,686	722	25

Figure 5. FACS analysis of Embrionic Bodies (EB) derived by iPS. The iPS that differentiate in cardiomyocytes express the YFP and are fluorescent yellow.This analysis indicate that the cardiomyocytes are about 8% of the total EB cells.

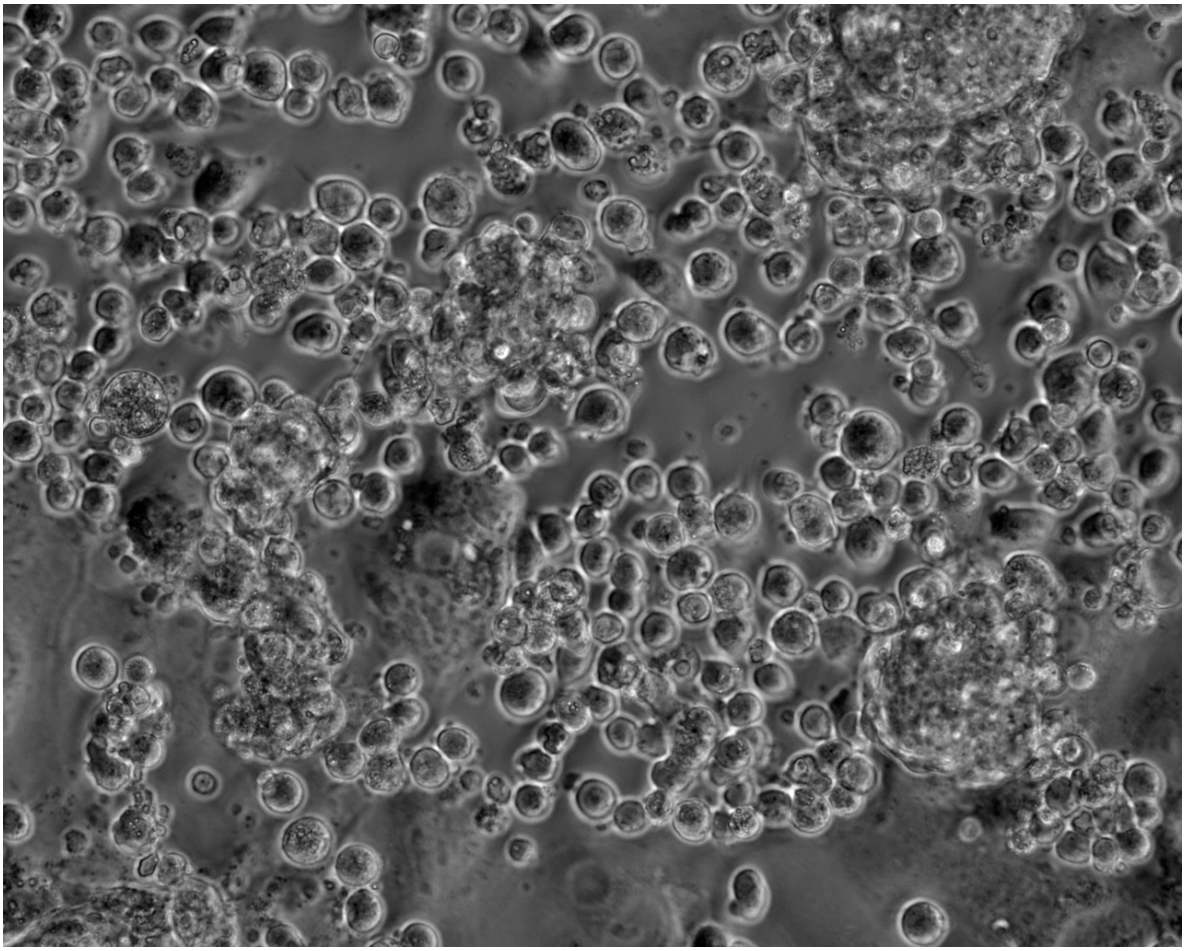


Figure 6 iPS treated with LIF that permit the establishment of undifferentiated state and the self-renewal.

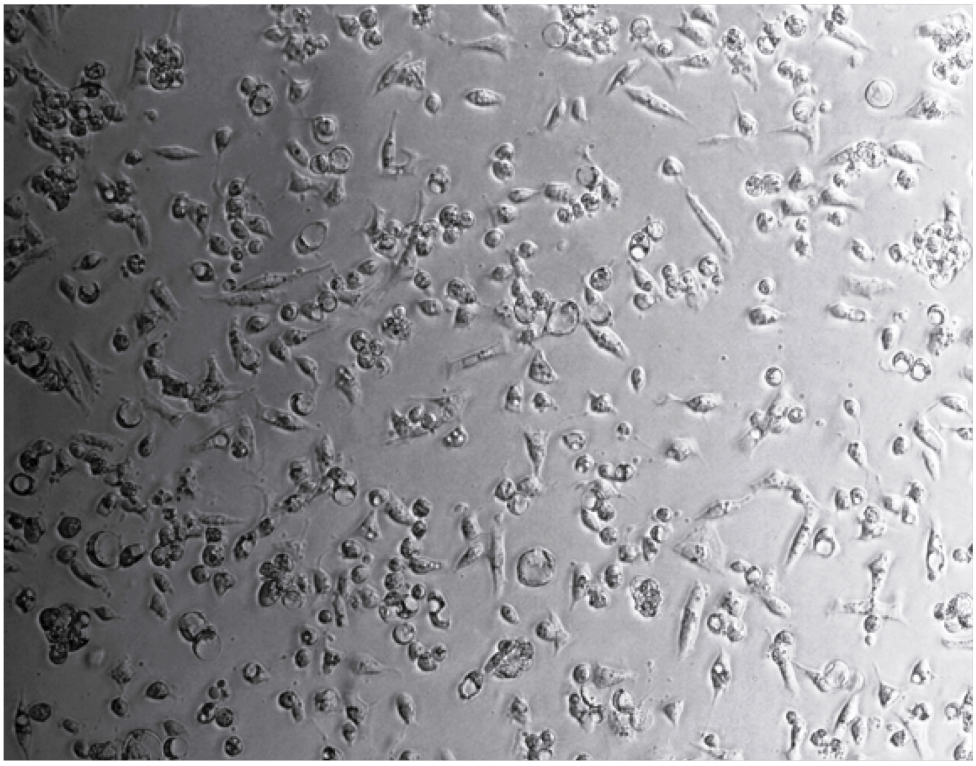


Figure 7. iPS treated with RA 5uM for about 2 weeks. Begin of Differentiation is evident .

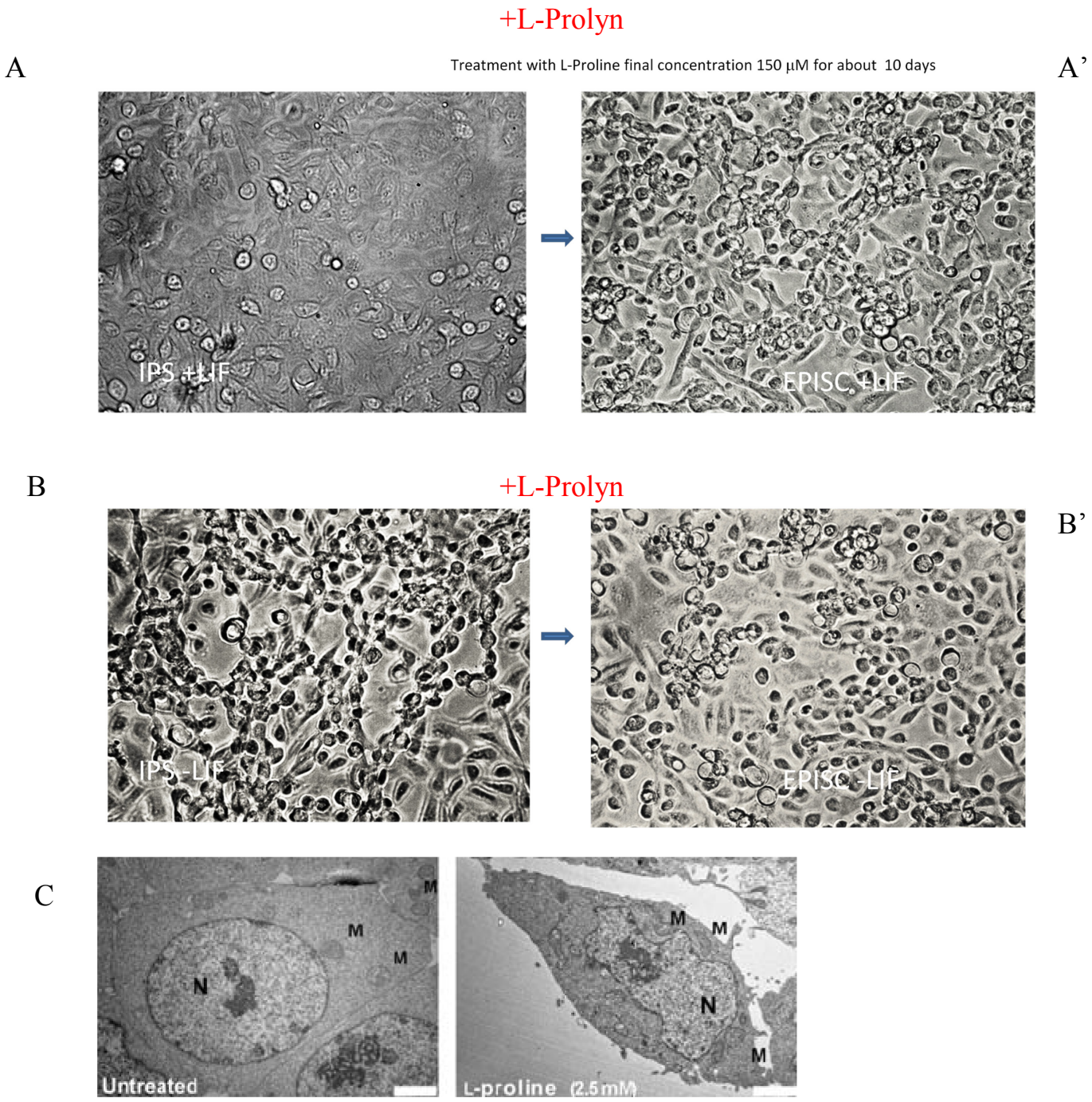


Figure 8. L Prolyn Effect for the Induction of EPISC. The EPISC was stimulated to differentiate with the add of L-Pro at a final concentration of 150 µM for 10 days. The induction of differentiation was done starting by iPS grown with LIF (A) and iPS grown without LIF (B) (Rossi M., 2011). TEM photomicrographs of ESCs untreated or treated with L-Pro (C). N, nucleus; M, mitochondrion. Scale bar, 200 nm (Casalino L 2011).

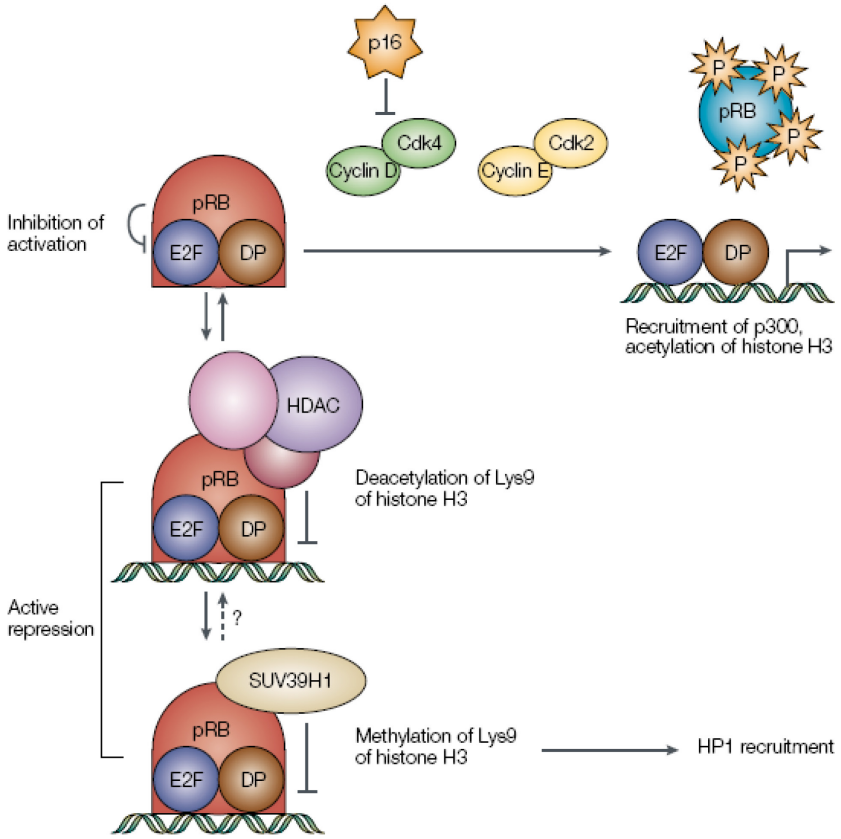


Figure 9. Mechanisms that control the activity of E2F repressors. The retinoblastoma protein is able to bind the complex E2F/DP cells found in G0/G1 phase, through two possible mechanisms. In the first mechanism, pRb inhibits E2F transcriptional activation by binding to its transactivation domain and preventing interaction with transcriptional machinery. But in the second mechanism the complex reinforces its activities through the recruitment of HDAC repressors that remove acetyl groups from Lysine 9 of the tail of histone H3. This facilitates the assembly of the nucleosome or SUV39H1 that methylates the same lysine residue to create binding sites for HP1, which results in transcriptional repression. Entry into the cell cycle depends on the activation of complex sequential cyclin and cyclin E/CDK2 D/CDK4-6 that phosphorylate pRb and causing the release of E2F. The consequent activation of genes that respond at of E2F seems to depend partly on the ability E2F to recruit the protein p300, which acetylates histone H3 lysine 9 (Trimarchi JM and Lee, JA, 2002)

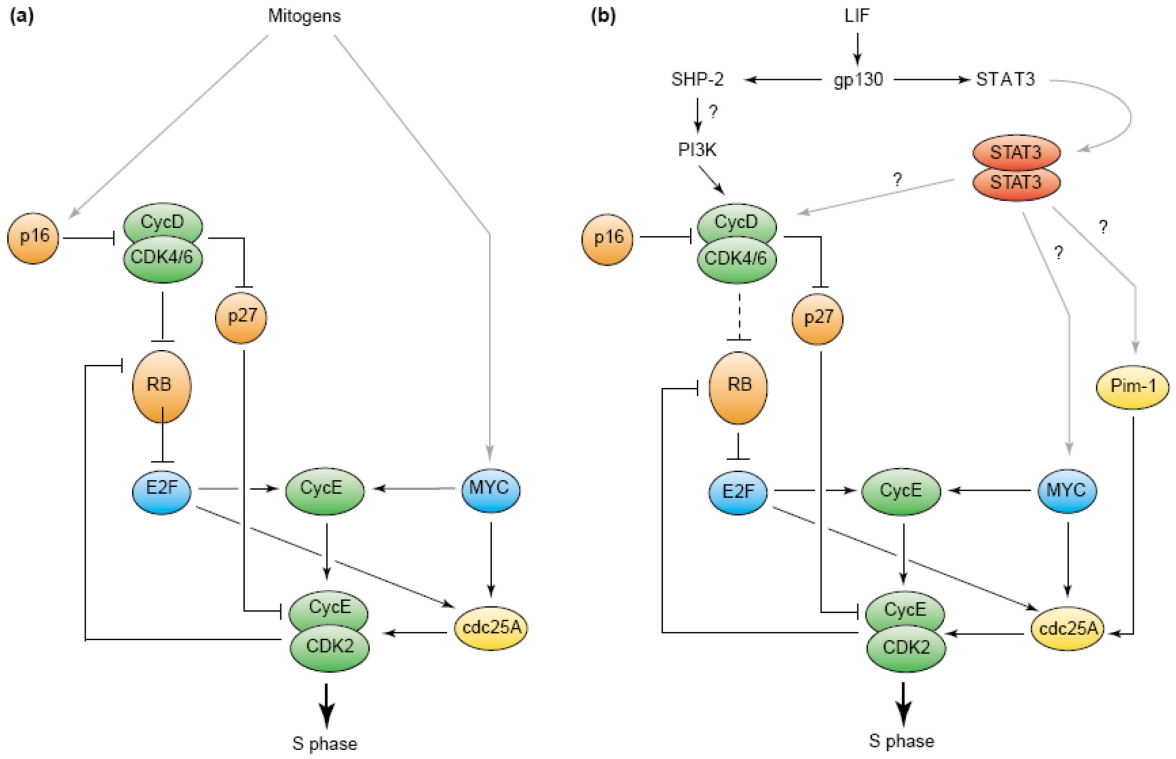
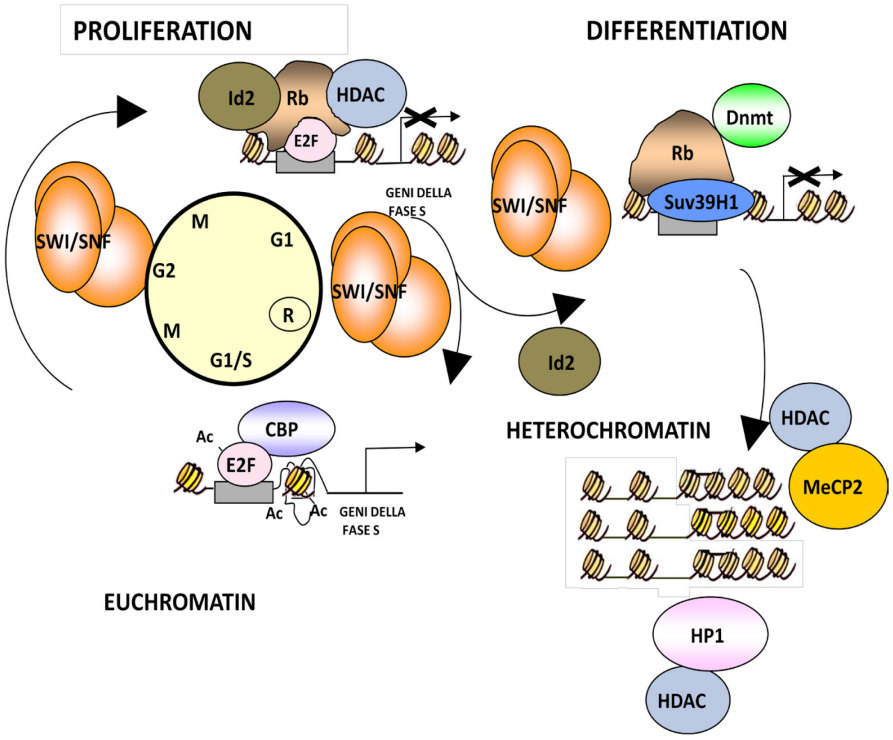
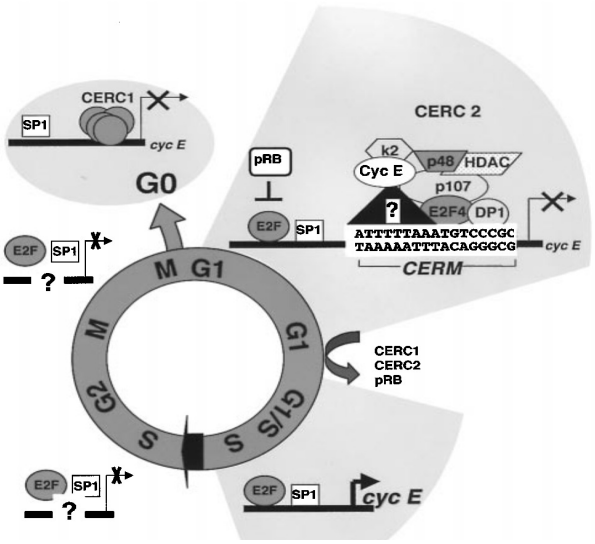


Figure . 10. Models of cell-cycle control in differentiated cells and embryonic stem (ES) cells. (a) The RB/E2F and Myc pathways function in parallel to control accumulation of cyclin E/CDK2 and entry into S phase in differentiated cells. (b) Hypothetical pathways for regulating the accumulation of cyclin E/CDK2 in ES cells. The broken line indicates that cyclin D/CDK4 or cyclin D/CDK6 kinase activity seems to be dispensable in activating RB (Burdon T. et al., 2002).

A


PROLIFERATION
DIFFERENTIATION
Id2
Rb
HDAC
E2F
GENI DELLA FASE S
SWI/SNF
M
G1
G2
R
M
G1/S
SWI/SNF
SWI/SNF
Dnmt
Rb
Suv39H1
Id2
HETEROCHROMATIN
HDAC
MeCP2
Ac
CBP
E2F
Ac
Ac
GENI DELLA FASE S
EUCHROMATIN
HP1
HDAC

B


CERC1
SP1
cyc E
G0
CERC 2
pRB
k2
Cyc E
p48
HDAC
p107
E2F4
DP1
E2F
SP1
?
ATTTTTAAATGTCCCGC
TAAAAATTTACAGGGCG
cyc E
CERM
E2F
SP1
?
M G1
G1
G1/S
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S
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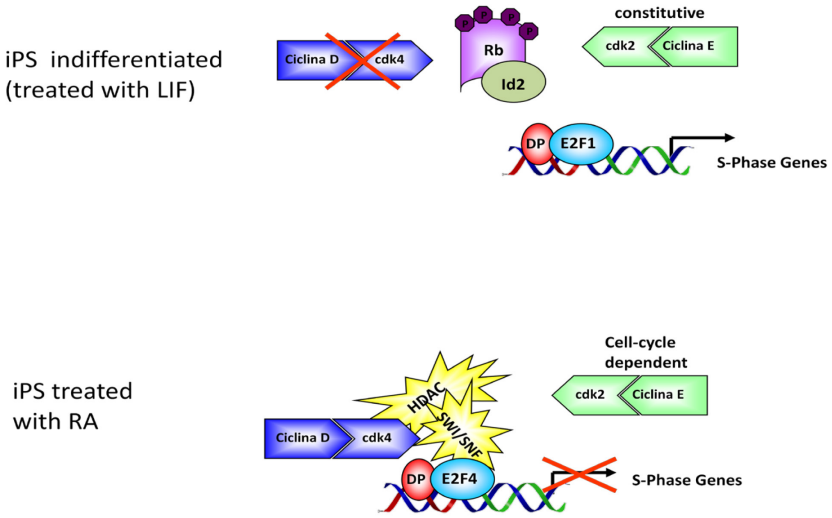


D

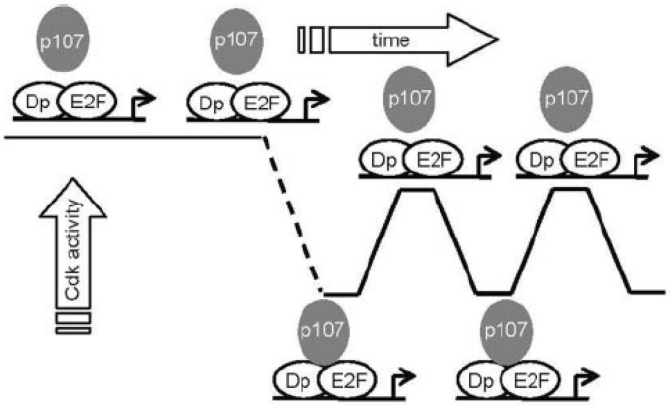


Figure 11A Model for regulation of E2F target genes during iPS differentiation. In cells in active proliferation of E2F target genes are regulated by the cyclic recruitment of HATs and HDACs but remain in the euchromatin. During the differentiation the E2F target genes are methylated at both the DNA and on histone H3, and this triggers the repositioning of genes in the heterochromatin and their silencing through HDAC (Ferreira et a., 2001; Rossi M et al 2011).Figure 11B. Model for the cyclin E promoter control by the System CERM / CERC in iPS cells.Figure 11C Model for E2F/Rb Multiprotein Complex in iPS. Figure 11 D. Establishment of G1 regulatory (R-point) controls through cell cycle-regulated Cdk2 activity during ES cell differentiation. Pluripotent ES cells exhibit elevated, constitutive Cdk activities, pRb family members are inactive and E2F-dependent transcription is cell cycle independent. During differentiation, Cdk activities collapse and become cell cycle regulated. As part of the establishment of cell cycle regulation, p107 and other pocket proteins (such as pRb and p130) become active and are able to repress E2F target genes (J. White 2005).

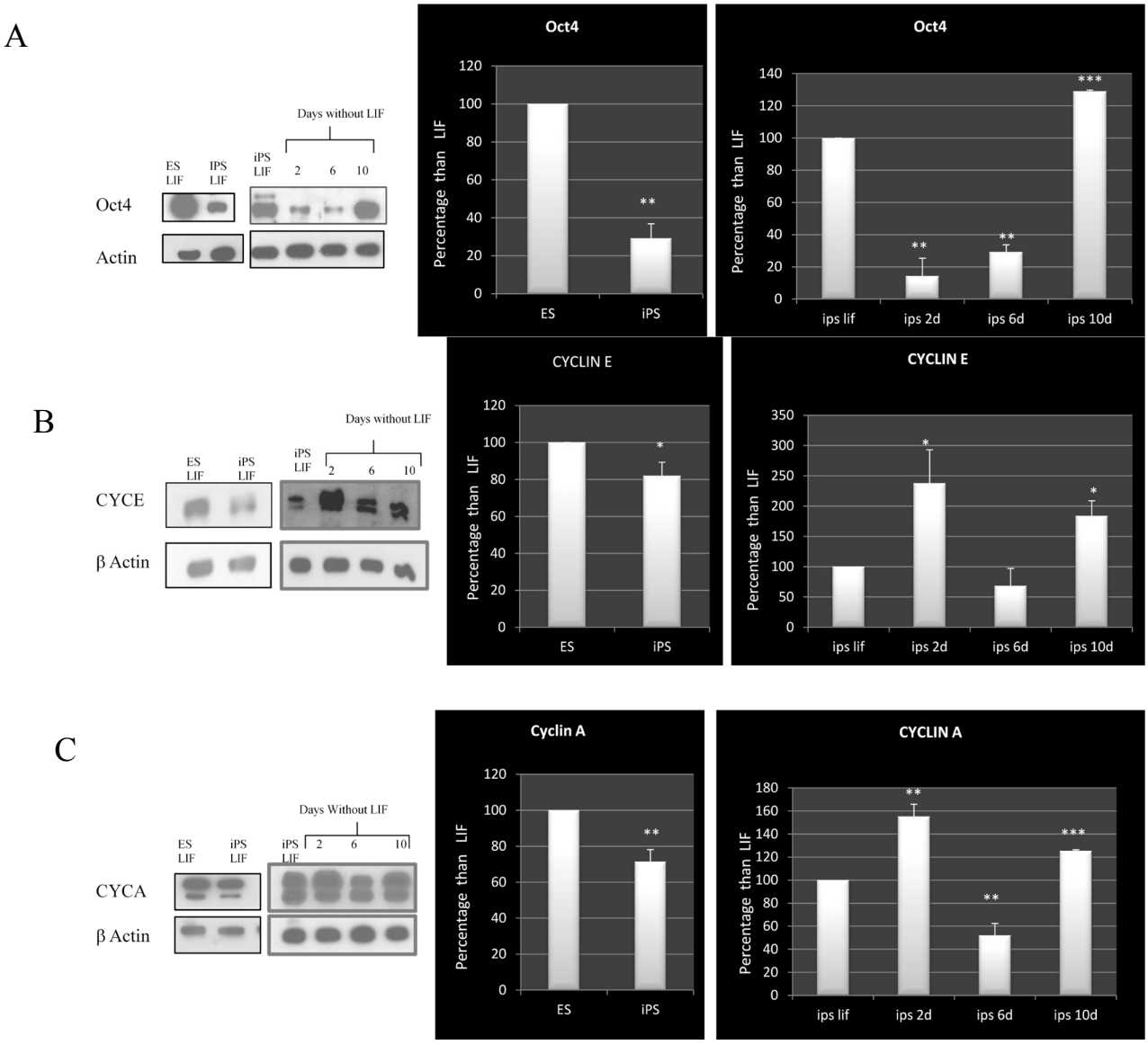


Figure 12. Analysis of expression of Oct4 (A), CYC E (B) and CYCA (C) by Western blot of nuclear protein extracts (15 μg / sample). After treatment without LIF (0,2,6,10 days) . LIF has been used as control. β-actin was used for normalization.

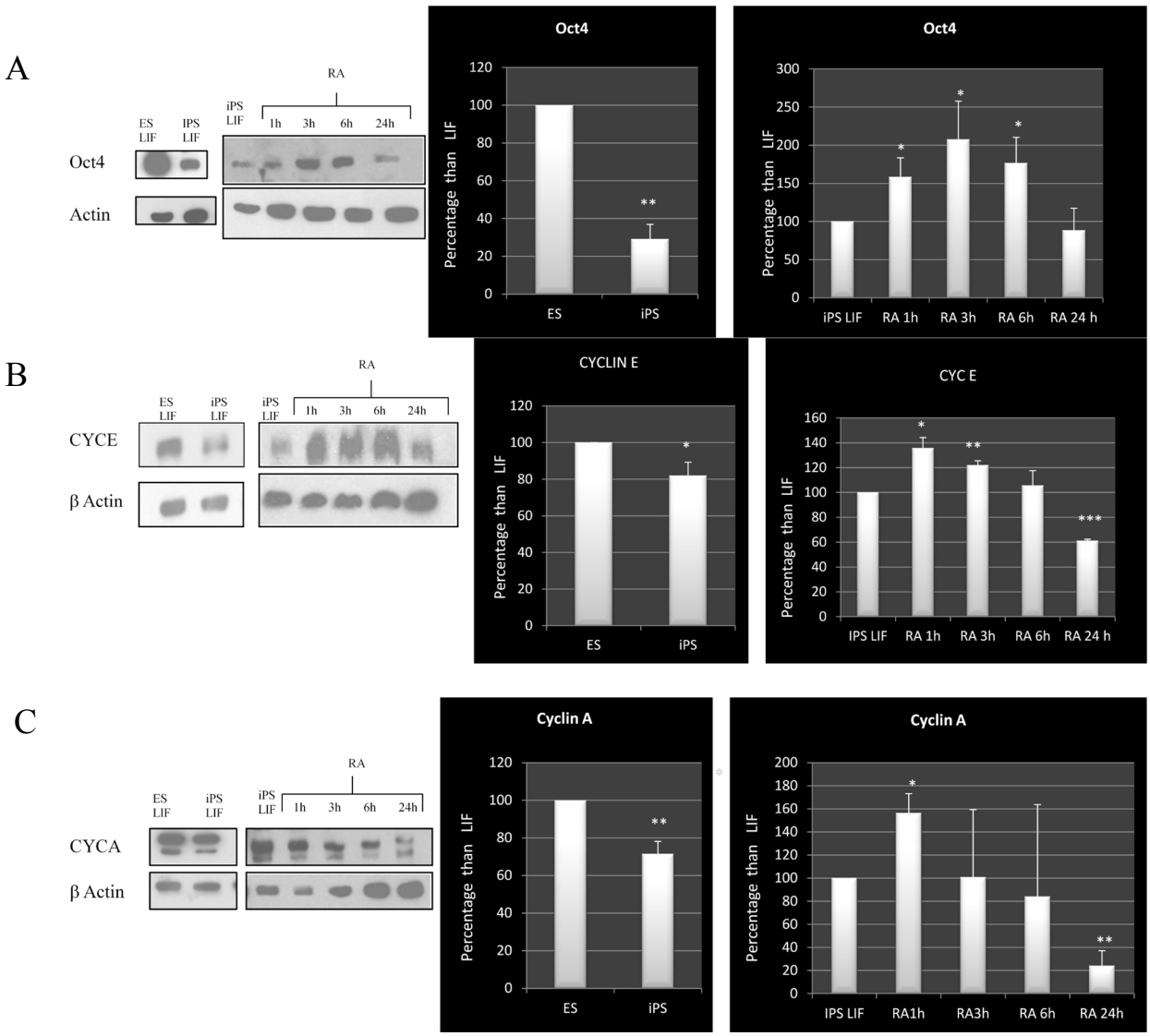


Figure 13. Analysis by Western blot of the expression of OCT4 (A), CYC E (B) and CYCA (C) in iPS cells treated with LIF and RA (final concentration 0.5 μM) for 1h, 3h, 6h and 24h. The nuclear protein extracts corresponding to 10^6 cells / sample (15μg/campione). β-actin was used for normalization

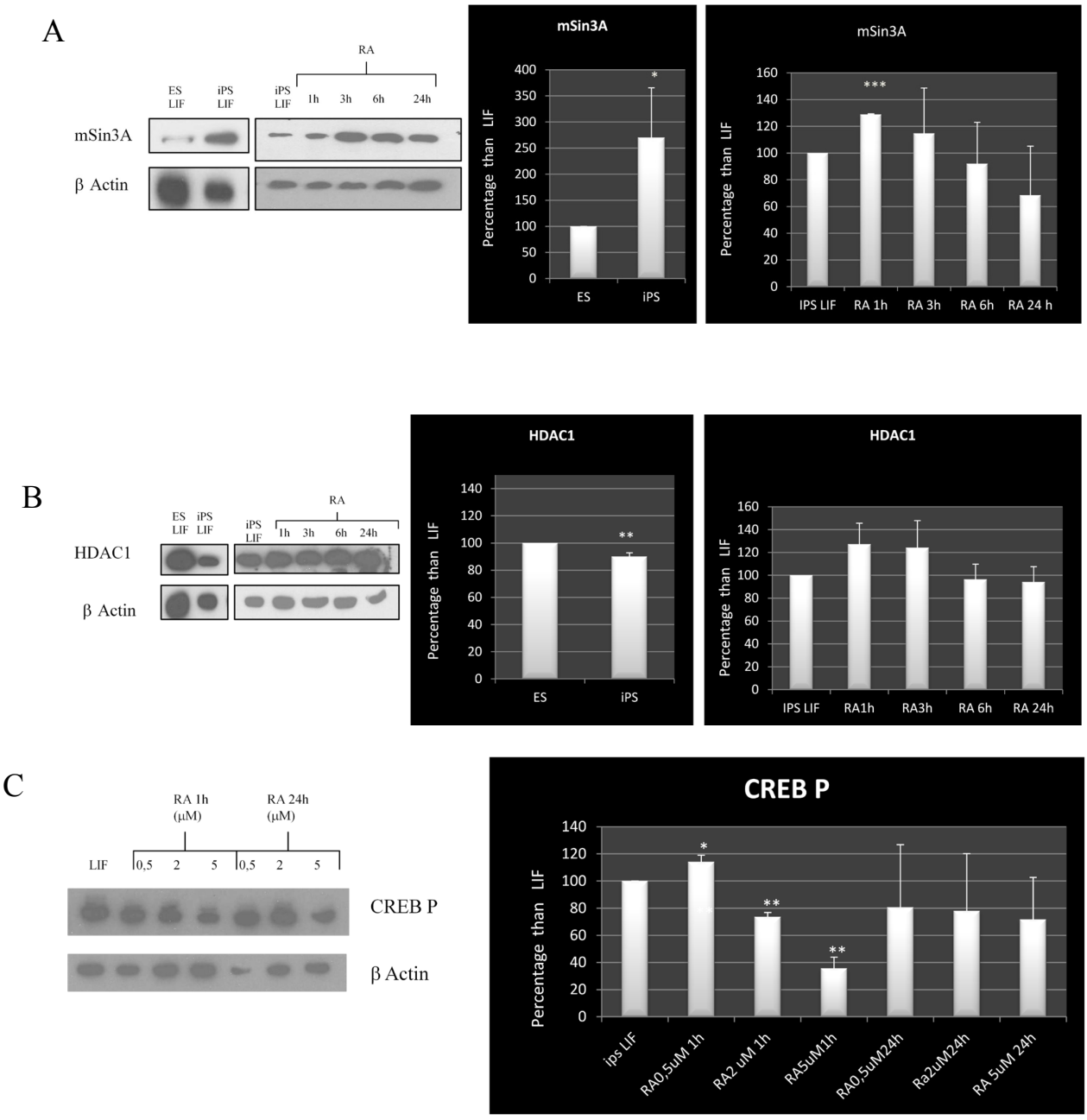


Figure 14. Analysis of expression of mSin3A (A) HDAC1 (B) and CREB P (C) by Western blot of nuclear protein extracts (15 μg / sample). After treatment with RA for 1h and 24 h at different concentration of RA (0,5 μM, 2 μM, 5μM). LIF has been used as control. b-actin was used for normalization.

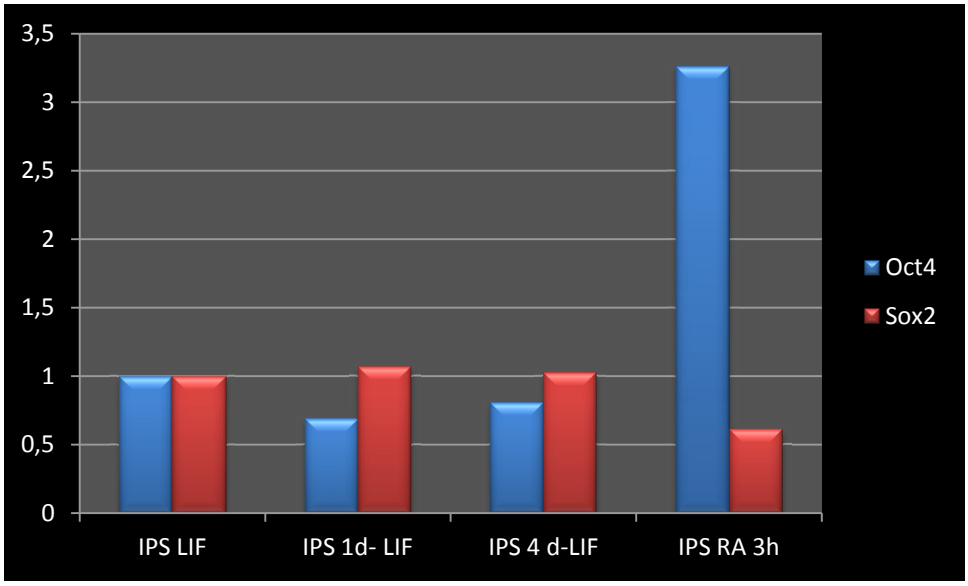


Figure 15. Real Time Experiment Assay. It has been studied the RNA expression of Oct4 and Sox2 genes in iPS induced to differentiate. iPS has been left 1day and 4 days withouth LIF or induced to differentiate after 3h of treatment with RA 0,5 μM. GAPDH was used as calibrator.

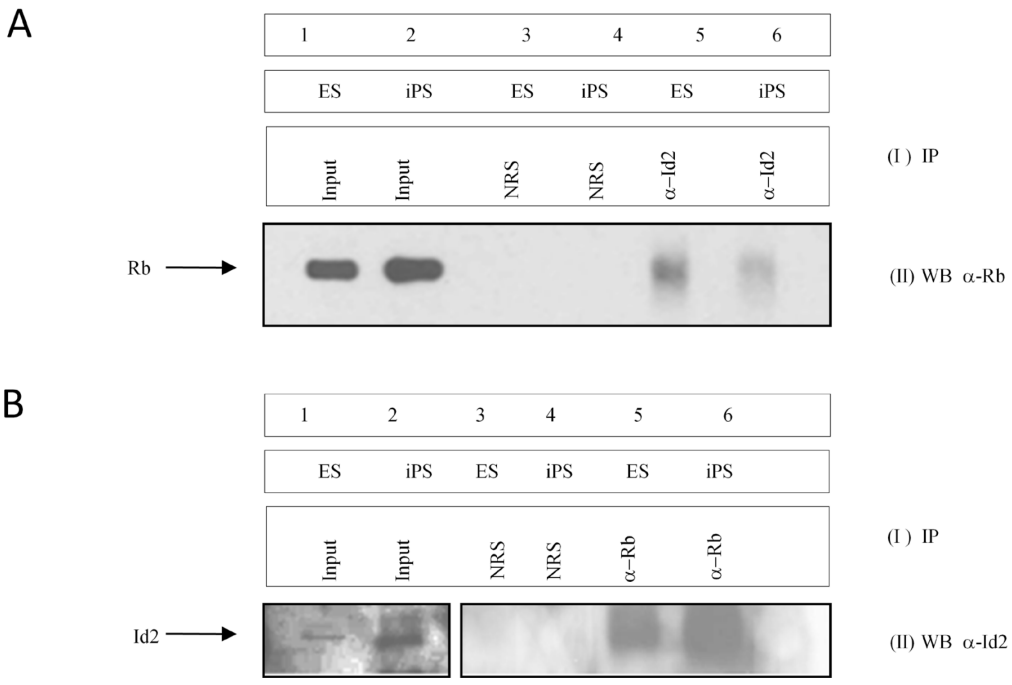


Figure 16.Id2 is complexed with Rb protein in ES and iPS cells. (A). Nuclear extracts from ES and iPS were immunoprecipitated by anti-Id2 serum (lanes 5, 6) or normal rabbit serum (lanes 3, 4). The resulting immunoprecipitates were resolved by SDS-PAGE and subjected to Western blot analysis using antibodies specific for Rb. An Rb protein was coprecipitated by the Id2 antibody (lanes 5, 6) but was absent in the normal rabbit serum used as negative control (lanes 3, 4). As input, nuclear extracts from ES or iPS was used as positive controls (lanes 1 and 2 respectively). (B). In the reciprocal experiments, nuclear extracts from ES and iPS were immunoprecipitated with Dynebeads by a specific polyclonal Rb antibody to coprecipitate a band corresponding to Id2 protein (lanes 5 and 6) but was absent in the normal rabbit serum (NRS) immunoprecipitates (lanes 3 and 4). As input, nuclear extracts from ES and iPS was used as positive controls (lanes 1 and 2 respectively).

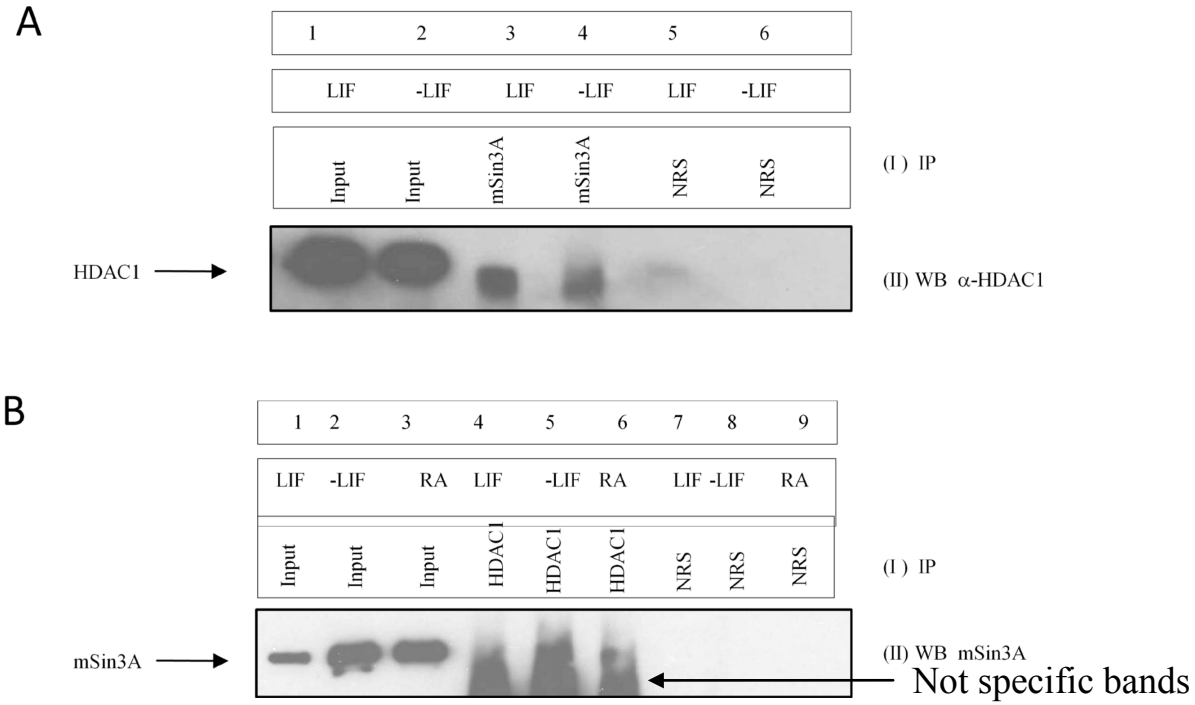


Figure 17.mSin3A is complexed with HDAC1 protein in iPS cells induced to differentiate. (A). Nuclear extracts from iPS treated and not treated with LIF were immunoprecipitated by anti-mSin3A serum (lanes 3, 4) or normal rabbit serum (lanes 5, 6). The resulting immunoprecipitates were resolved by SDS-PAGE and subjected to Western blot analysis using antibodies specific for HDAC1. An HDAC1 protein was coprecipitated by the mSin3A antibody (lanes 3, 4) but was absent in the normal rabbit serum used as negative control (lanes 5, 6). As input, nuclear extracts from iPS treated and not treated with LIF (10d –LIF) was used as positive controls (lanes 1 and 2 respectively). (B). In the reciprocal experiments, nuclear extracts from iPS treated and not treated with LIF and treated with RA 5μM for 1h were immunoprecipitated with Dynebeads by a specific polyclonal HDAC1 antibody to coprecipitate a band corresponding to mSin3A protein (lanes 4, 5 and 6) but was absent in the normal rabbit serum (NRS) immunoprecipitates (lanes 7,8 and 9). As input, nuclear extracts from iPS treated and not treated with LIF (10d) and treated with RA 5μM for 1h was used as positive controls (lanes 1 and 2 and 3 respectively).

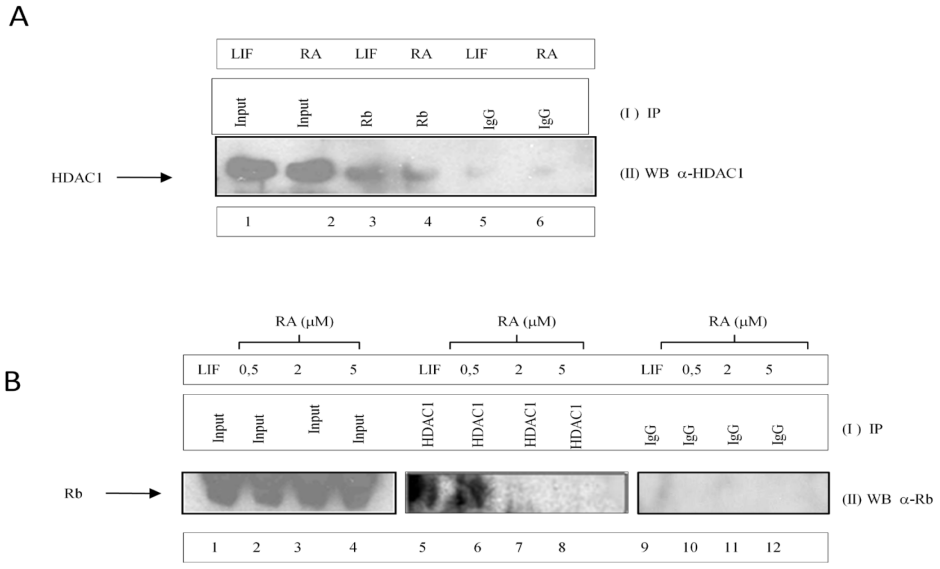
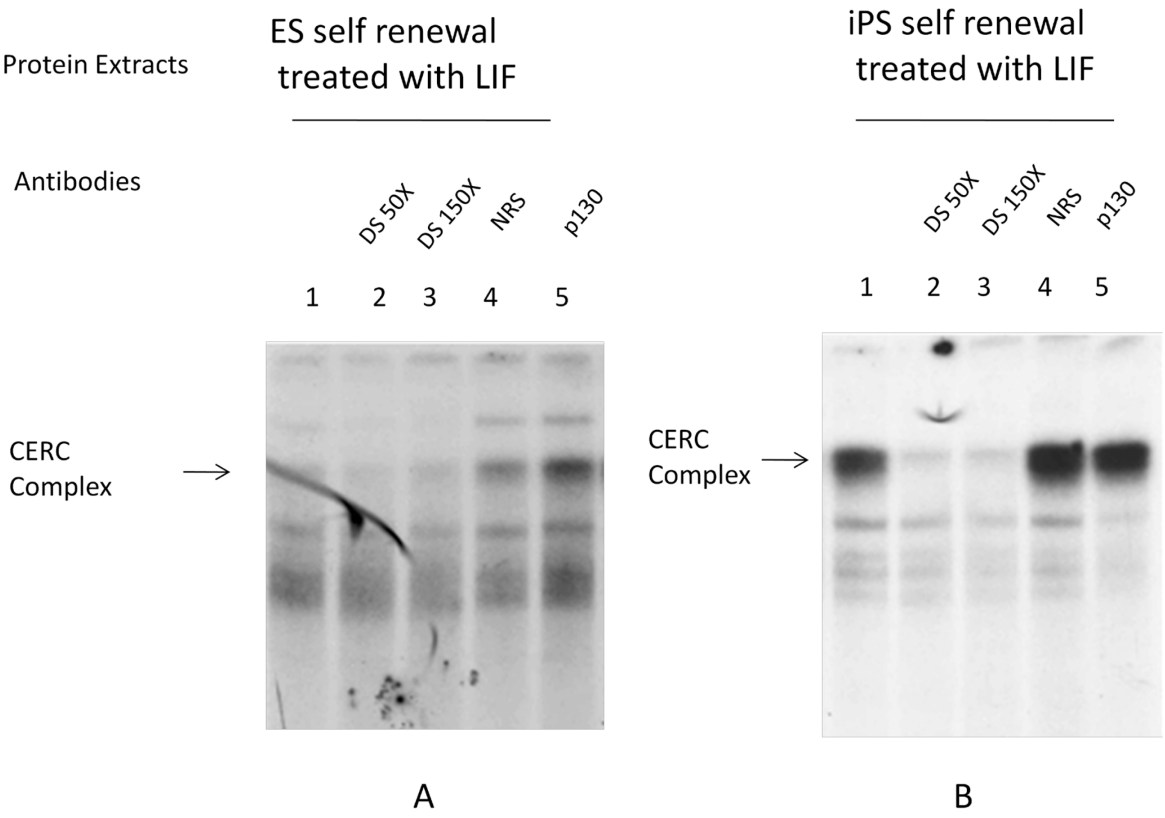
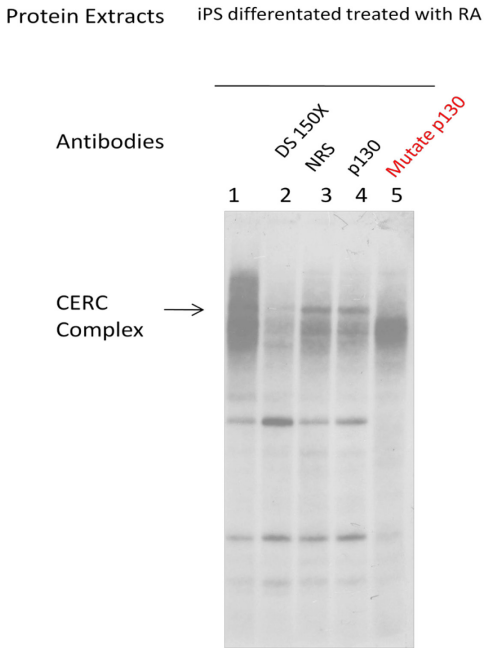


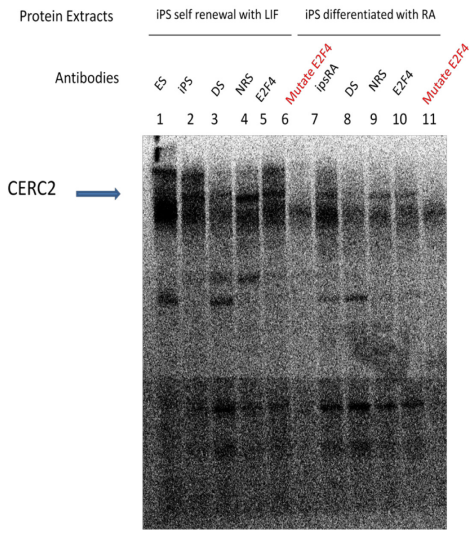
Figure 18.Rb is complexed with HDAC1 protein in iPS cells induced to differentiate. (A). Nuclear extracts from iPS not treated (LIF) and treated with RA 0,5μM for 1h were immunoprecipitated by anti-Rb serum (lanes 3, 4) or normal IgG(lanes 5, 6). The resulting immunoprecipitates were resolved by SDS-PAGE and subjected to Western blot analysis using antibodies specific for HDAC1. An HDAC1 protein was coprecipitated by the Rb antibody (lanes 3, 4) but was absent in the IgG used as negative control (lanes 5, 6). As input, nuclear extracts from iPS not treated (LIF) and treated with RA 0,5μM for 1h was used as positive controls (lanes 1 and 2 respectively). (B). In the reciprocal experiments, nuclear extracts from iPS not treated (LIF) and treated with RA 0,5μM, 2μM,5μM for 1h were immunoprecipitated with Dynebeads by a specific monoclonal HDAC1 antibody to coprecipitate a band corresponding to Rb protein (lanes , 5 and 6, 7 and 8) but was absent in the normal rabbit serum (IgG) immunoprecipitates (lanes 9,10,11, and 12). As input, nuclear extracts from iPS not treated (LIF) and treated with RA 0,5μM, 2μM,5μM for 1h was used as positive controls (lanes 1,2,3 and 4r espectively).


Protein Extracts
ES self renewal
treated with LIF
iPS self renewal
treated with LIF
Antibodies
DS 50X
DS 150X
NRS
p130
1
2
3
4
5
CERC
Complex
A
B

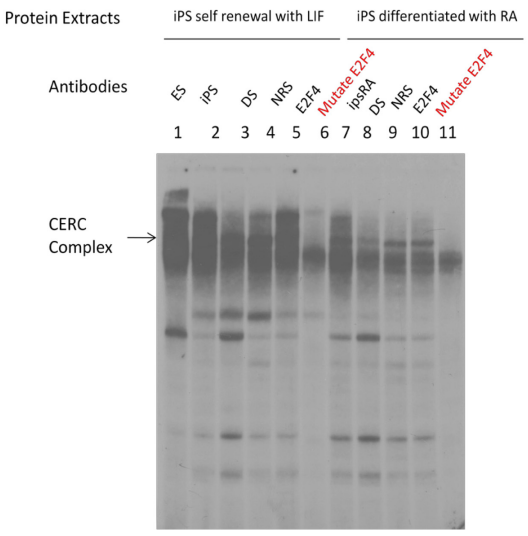
C


Protein Extracts
iPS differentated treated with RA 0,5 uM for 1h
Antibodies
DS 150X
NRS
p130
Mutate p130
1 2 3 4 5
CERC Complex

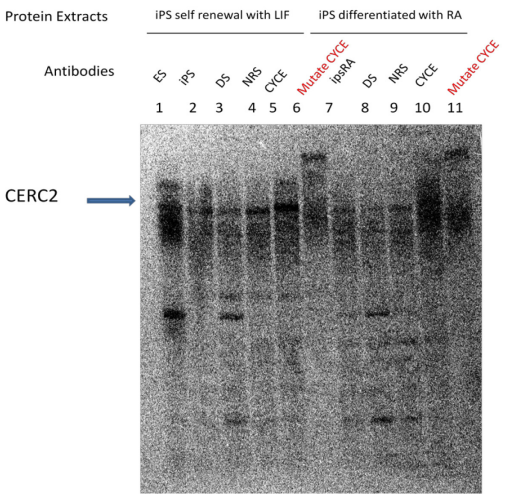
D


Protein Extracts
iPS self renewal with LIF
iPS differentiated with RA
Antibodies
ES
iPS
DS
NRS
E2F4
Mutate E2F4
ipsRA
DS
NRS
E2F4
Mutate E2F4
1 2 3 4 5 6 7 8 9 10 11
CERC2

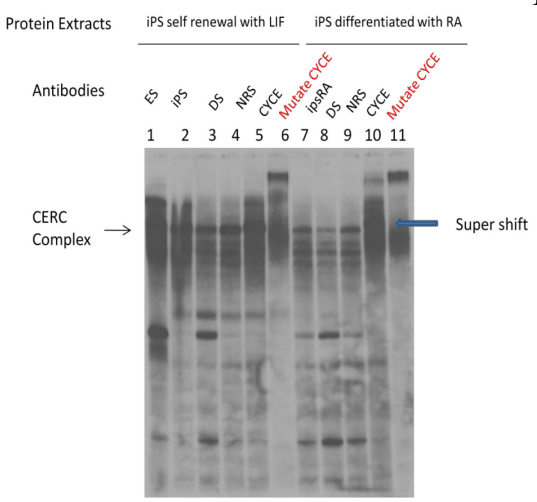
D'


Protein Extracts
iPS self renewal with LIF
iPS differentiated with RA
Antibodies
ES
iPS
DS
NRS
E2F4
Mutate E2F4
ipsRA
DS
NRS
E2F4
Mutate E2F4
1 2 3 4 5 6 7 8 9 10 11
CERC Complex

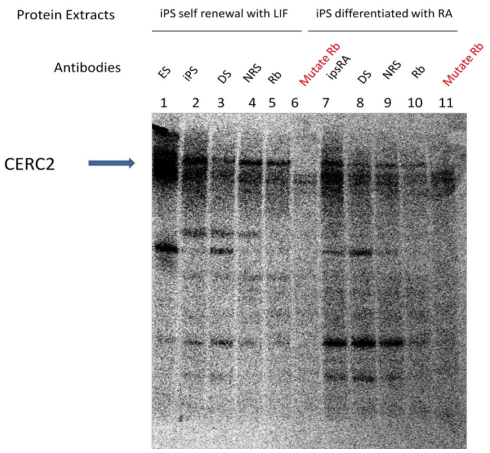
E


Protein Extracts
iPS self renewal with LIF
iPS differentiated with RA
Antibodies
ES
iPS
DS
NRS
CYCE
Mutate CYCE
ipsRA
DS
NRS
CYCE
Mutate CYCE
1 2 3 4 5 6 7 8 9 10 11
CERC2

E’


Protein Extracts
iPS self renewal with LIF
iPS differentiated with RA
Antibodies
ES
iPS
DS
NRS
CYCE
Mutate CYCE
ipsRA
DS
NRS
CYCE
Mutate CYCE
1 2 3 4 5 6 7 8 9 10 11
CERC Complex
Super shift

F


Protein Extracts
iPS self renewal with LIF
iPS differentiated with RA
Antibodies
ES
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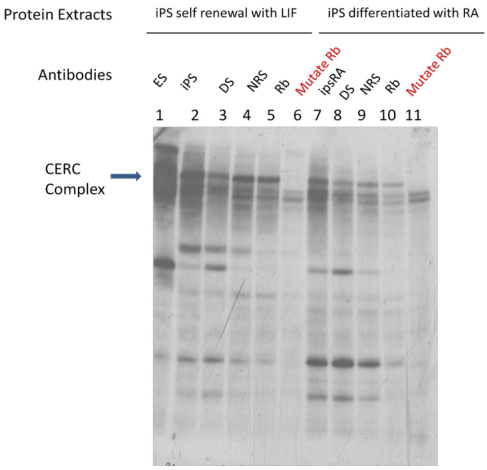
DS
NRS
Rb
Mutate Rb
ipsRA
DS
NRS
Rb
Mutate Rb
1 2 3 4 5 6 7 8 9 10 11
CERC2

F’


Protein Extracts
iPS self renewal with LIF
iPS differentiated with RA
Antibodies
ES
iPS
DS
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Rb
Mutate Rb
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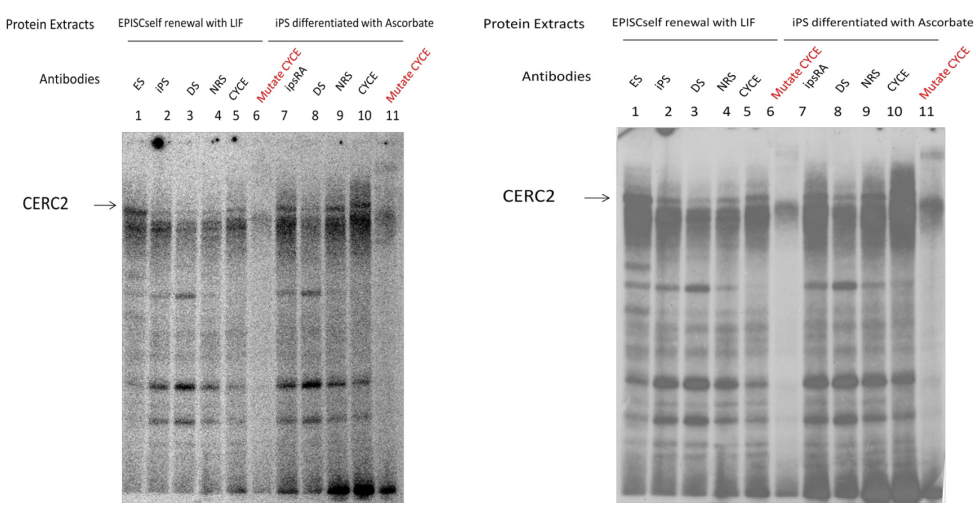
DS
NRS
Rb
Mutate Rb
1 2 3 4 5 6 7 8 9 10 11
CERC Complex

G



G’

Figure 19. EMSA Analysis for the composizion of CERC complex in cES (A) and iPS self renewal treated with LIF (B). Are indicated the specific antibodies (p130) A,B,C . In C is shown the EMSA experiment specific for p130 with iPS differentiated with RA. In D and D’ is shown the same EMSA experiment (in D showed with Typhon and D’ with slad) for studying the presence of protein E2F4 in the complex CERC2. In E and E’(in E showed with Typhon and E’ with slad) is shown the EMSA experiment with CYCE antibody. In F and F’(in F showed with Typhon and F’ with slad) is shown the EMSA experiment with Rb antibody. In G and G’ with slad is shown the EMSA experiment with CYCE antibody using EPISC cells and iPS treated with Retinoic Acid. NRS is used as negative control. DS (double-stranded DNA) is cold competitor 150X.

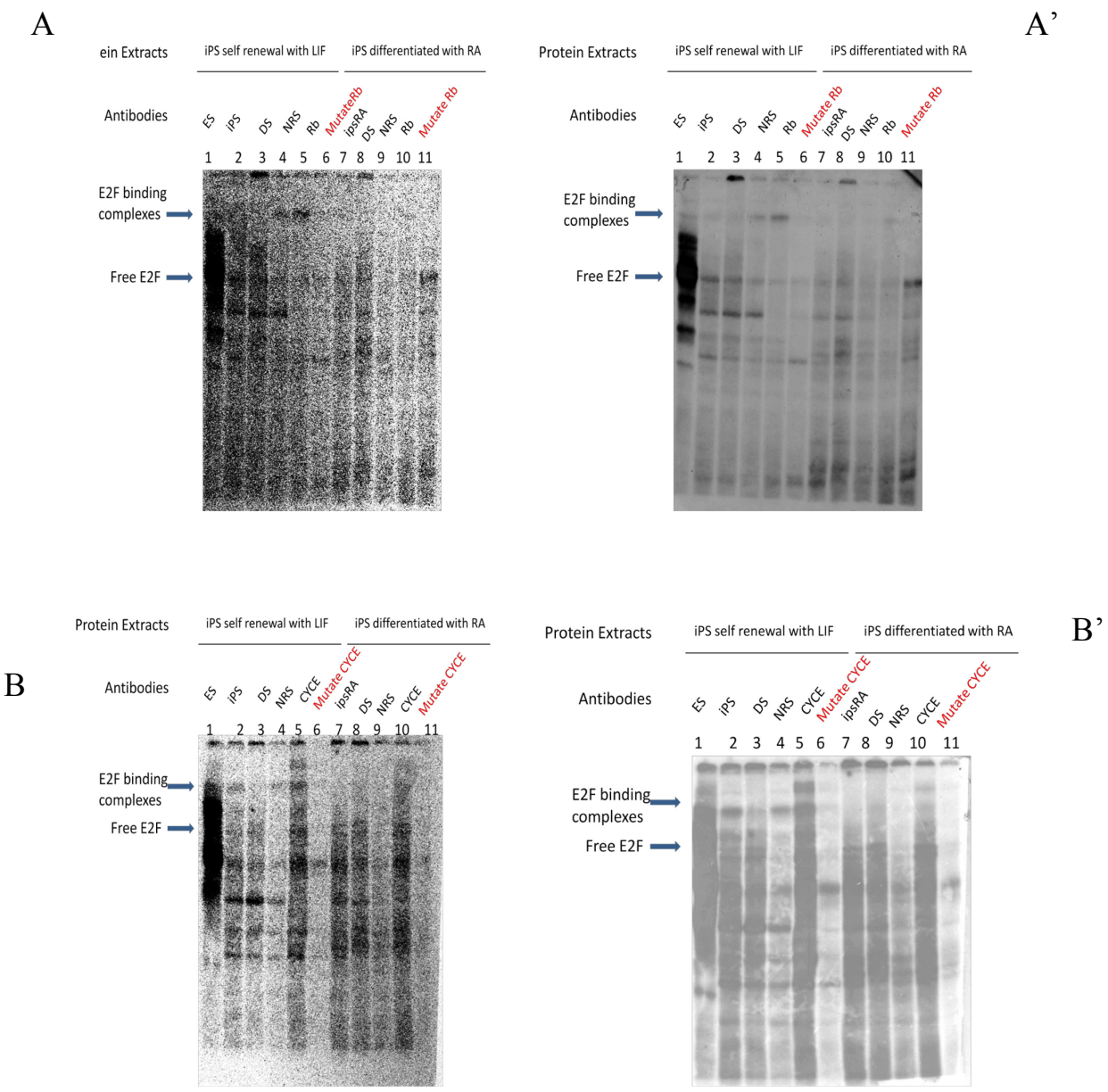
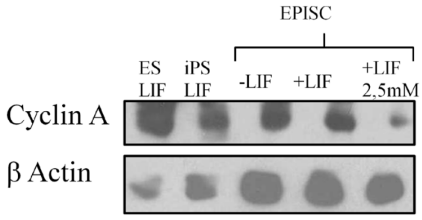
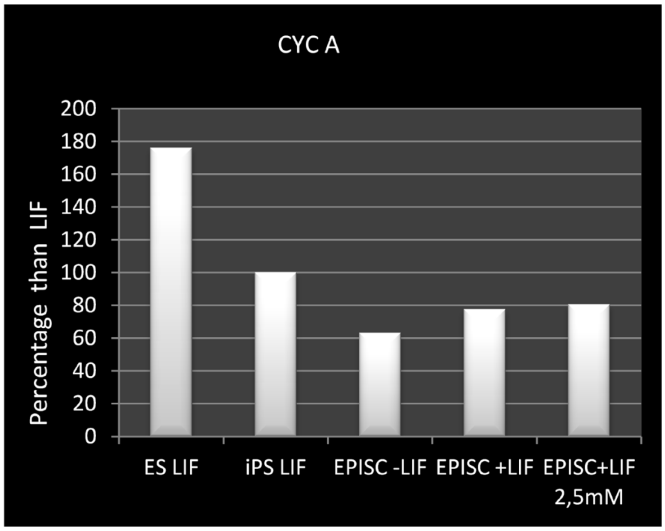


Figure 20. EMSA Analysis for the composition of E2F complex using the DHFR probe in iPS self renewal treated with LIF. Are indicated the specific antibodie Rb (A) and CYCE (B) (in A and B showed with Typhon instead A' and B' with slad). It is shown the EMSA experiment specific for CYCE with iPS differentiated with RA. It is shown the same EMSA experiment for studying the presence of protein CYCE in the complex E2F-CYCE. NRS is used as negative control. DS (double-stranded DNA) is cold competitor 150X.

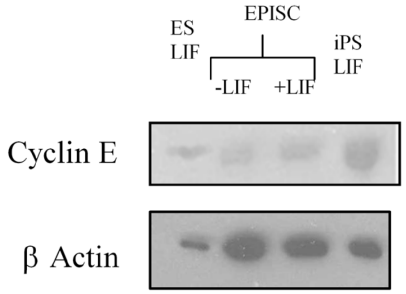
Preliminar Results on iPS treated with L-Prolyn (EPISC)

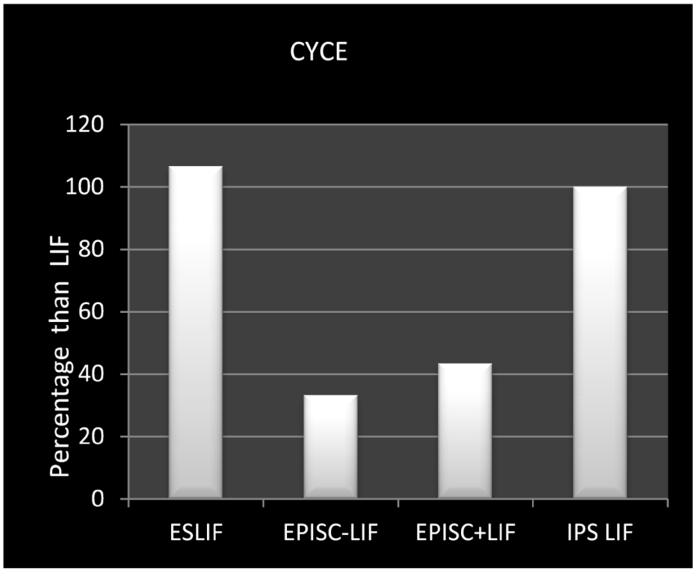
A



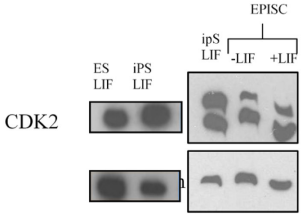


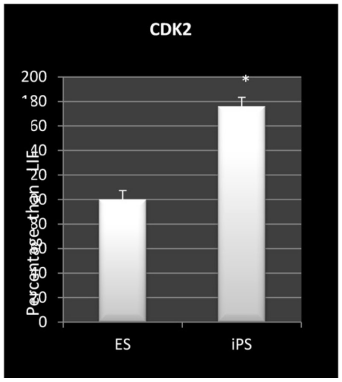
B

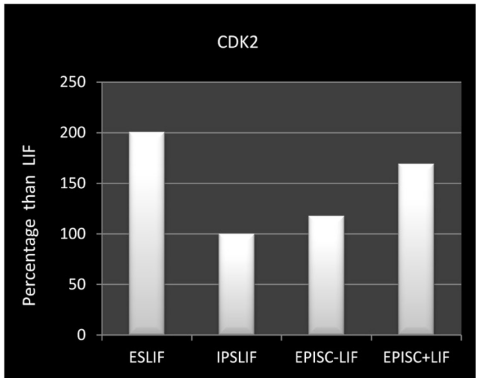




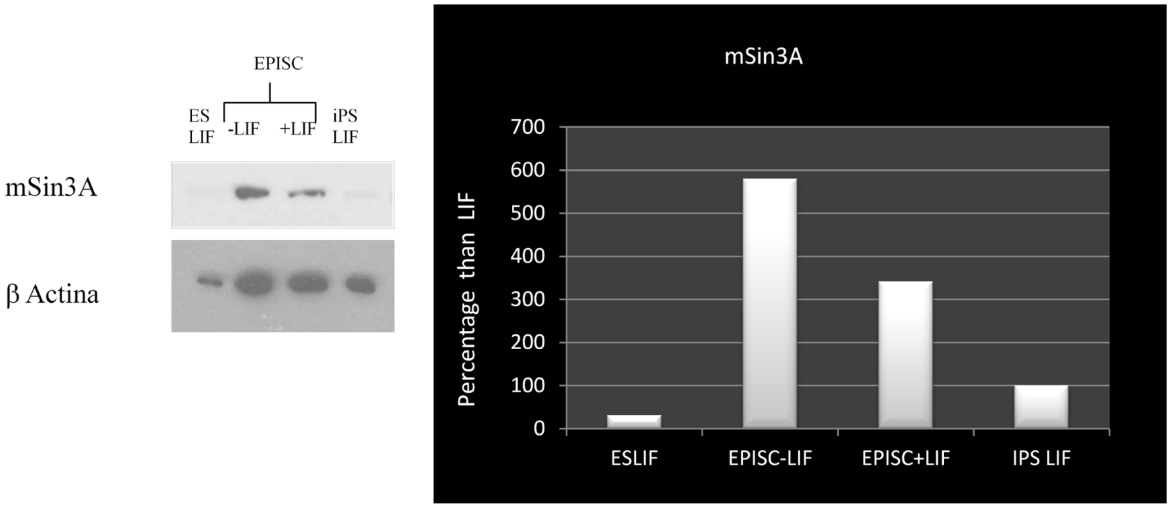
C







D


EPISC
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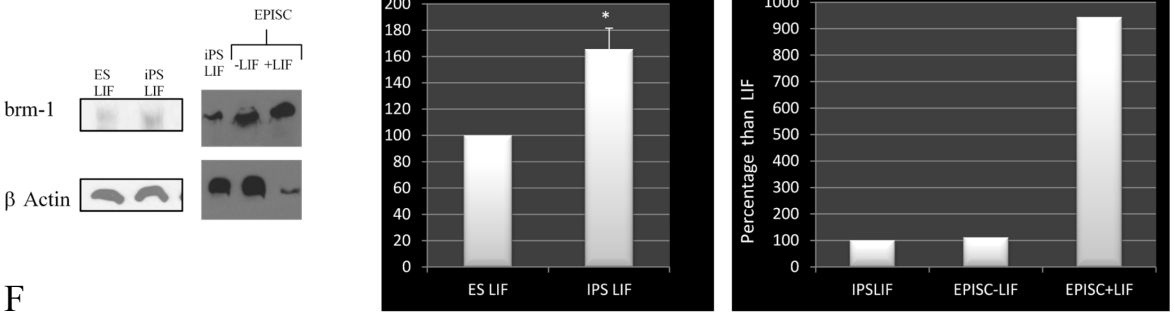
-LIF
+LIF
iPS LIF
mSin3A
β Actina
mSin3A
Percentage than LIF
700
600
500
400
300
200
100
0
ESLIF
EPISC-LIF
EPISC+LIF
IPS LIF

E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-LIF
+LIF
brm-1
β Actin
brm
*
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120
100
80
60
40
20
0
ES LIF
IPS LIF
brm
Percentage than LIF
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300
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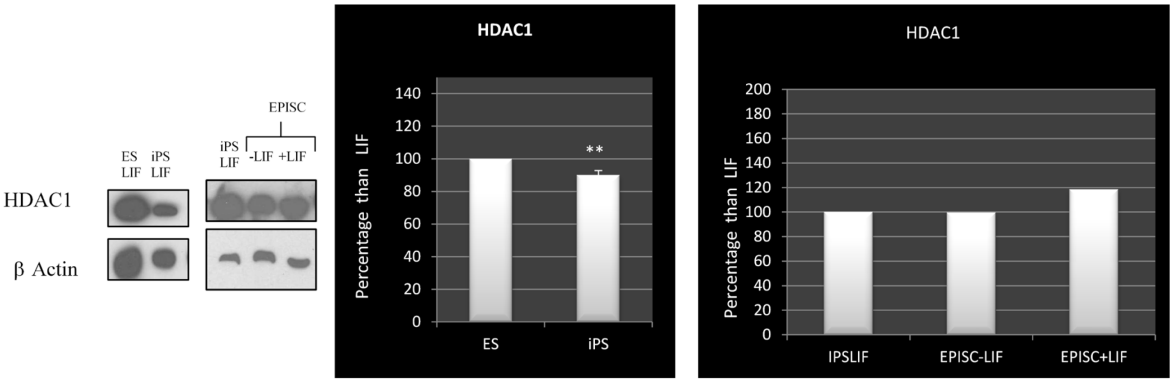
IPSLIF
EPISC-LIF
EPISC+LIF

F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HDAC1
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HDAC1
Percentage than LIF
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iPS
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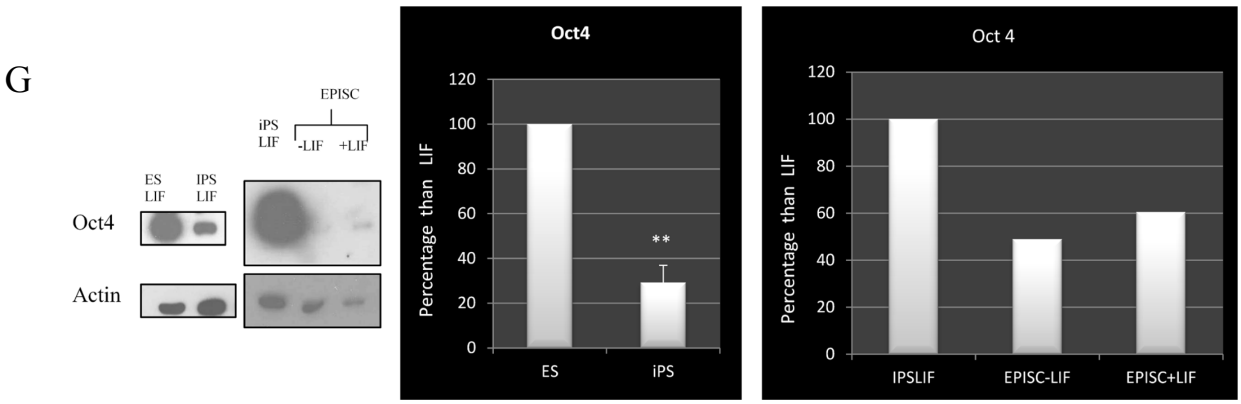
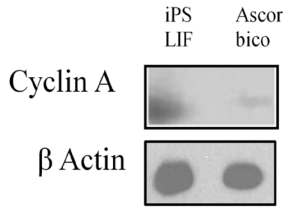
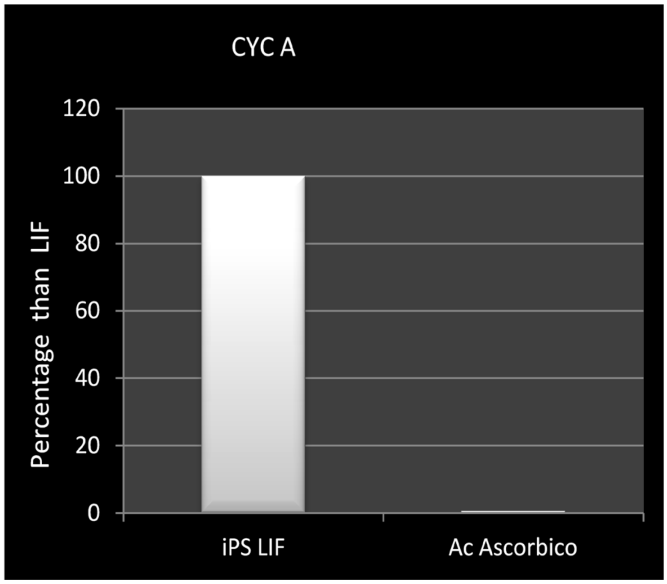
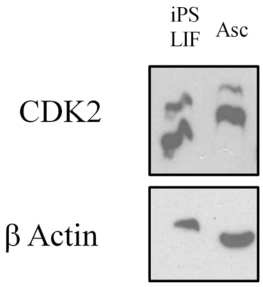


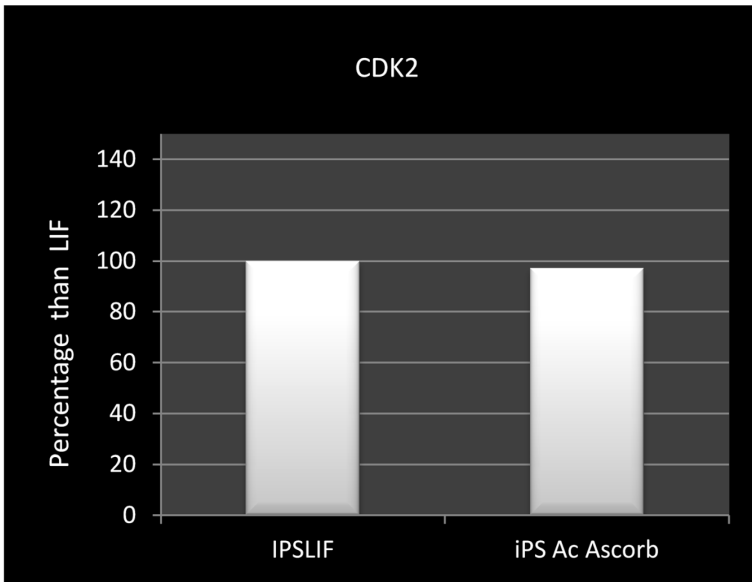
Figure 21. Each figure represents a WB of two independent experiments.(A) Western Blot of Cyclin A, (B) Western Blot of Cyclin E, (C) Western Blot of CDK2, (D) Western Blot of mSin3A, (E) Western Blot of brm, (F) Western Blot of HDAC1, (G) Western Blot of Oct4. Analysis of expression of different genes by Western blot of nuclear protein extracts (15 μg / sample) after treatment of iPS with L-Proline for 74h at final concentration of 150 μM. LIF has been used as control. β-actin was used for normalization.

Preliminar Results on iPS treated with Ascorbic Acid

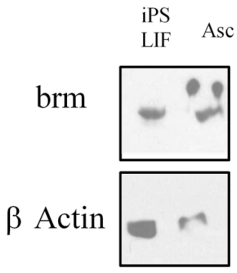


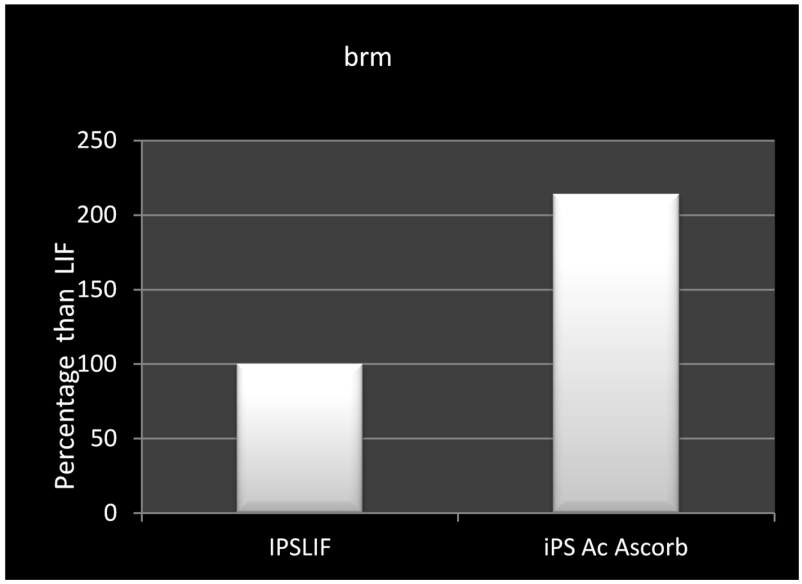




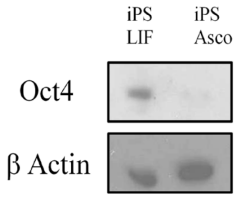


C


iPS
LIF
Asc
brm
β Actin


brm
250
200
150
100
50
0
Percentage than LIF
IPSLIF
iPS Ac Ascorb

D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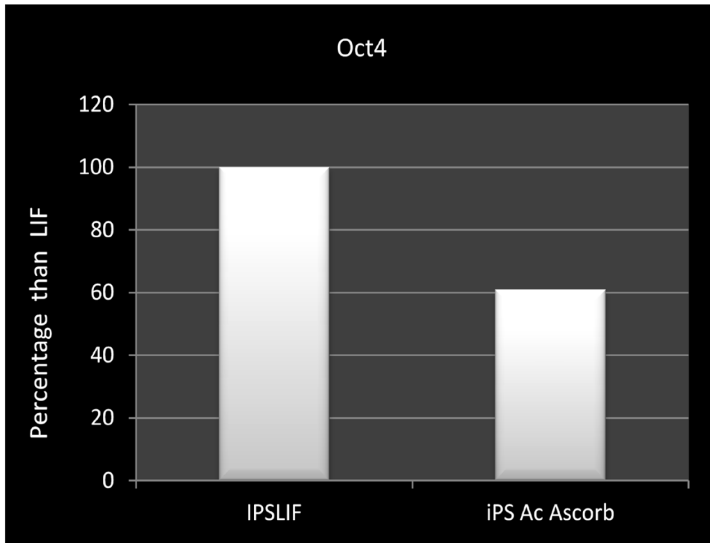
LIF
iPS
Asco
Oct4
β Actin


Oct4
120
100
80
60
40
20
0
Percentage than LIF
IPSLIF
iPS Ac Ascorb

E

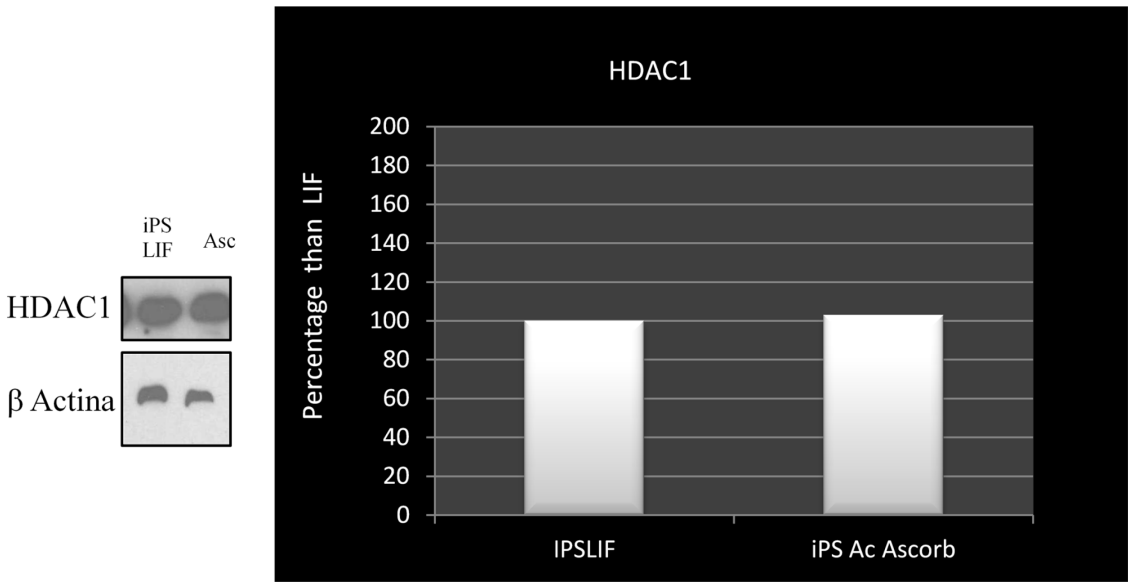


Figure 22. Each figure represents a WB of two independent experiments.(A) Western Blot of Cyclin A, (B) Western Blot of CDK2, (C) Western Blot of brm, (D) Western Blot of Oct4, (E) Western Blot of HDAC1. Analysis of expression of different genes by Western blot of nuclear protein extracts (15 μg / sample) after treatment of iPS with Ascorbi Acid for 1h at final concentration of 50 μg/ml. LIF has been used as control. β-actin was used for normalization.

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