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G1 to S phase cell cycle transition in somatic and embryonic stem cells

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Abstract

It is well known that G1 to S phase transition is tightly regulated by the expression and phosphorylation of a number of well characterised Cyclins, Cyclin Dependent kinases and members of the retinoblastoma gene family. In this review we discuss the role of these components in regulation of G1 to S phase transition in somatic cells and human embryonic stem cells. Most importantly, we discuss some new tenable links between maintenance of pluripotency and cell cycle regulation in ESC by describing the role that master transcription factors play in this process. Finally, the differences in cell cycle regulation between murine and human ESC are highlighted raising interesting question on their biology and stages of embryonic development from which they have been derived.

1. Focus of the review

A large body of literature suggests that the cell's decision to differentiate, proliferate, apoptose, become quiescent or enter into senescent arrest is often made in the G1 phase of the cell cycle. One key difference between somatic and embryonic stem cells is the length of G1 phase during which cells prepare for S phase entry. Recent studies have shown that the length of G1 phase in embryonic stem cells (ESC) is much shorter as compared to somatic cells (Becker et al. 2006, Savatier et al., 1994; Fluckiger et al., 2006). A few studies have shown that lengthening of G1 phase of the cell cycle results in loss of undifferentiated phenotype and induction of differentiation in neural stem cells, thus suggesting some close links between cell cycle regulation and stem cell potency (Calegari and Huttner, 2003). It is therefore very important to understand the mechanisms that govern the G1 to S phase progression in detail in both somatic and embryonic stem cells.

2. Summary of pathways that govern G1 to S transition in somatic cells

In differentiated mammalian cells, G1 to S progression is regulated by hypophosphorylated Rb gene or its related proteins, p107 and p130 which inhibit the expression of genes required for entry into S phase by sequestering the E2F family of transcription factors. During G1 phase the Rb/HDAC repressor complex binds to the E2F-DP1 transcription factors inhibiting downstream transcription (for a schematic view see **Figure 1A**). Eukaryotic cell cycle progression is dependent, in part, on the tightly regulated activity of Cyclin dependent kinases (Cdks). Cdk4/Cdk6 and Cdk2 whose regulatory partners are the D type Cyclins (D1, D2 and D3) and Cyclin E respectively represent two different classes of G1 specific Cdks whose activation is required for entry into S phase. Cyclin D/CDdk4-Cdk6 activity occurs in mid-late G1 phase, upstream of Cdk2/Cyclin E activity. Under conditions favouring proliferation, the inactivating phosphorylation of Rb by the Cdks in mid to late G1 results in liberation of E2F and other Rb-bound transcription factors that then activate transcription of S-phase genes (**Figure 2A**). This induces a partial release of the E2F gene which is sufficient to activate transcription of Cyclin E and Cdc25A. This point in the cell cycle is known as the restriction point (Pardee, 1974). Cyclin protein levels oscillate throughout the cell cycle, and their availability is a mean of controlling Cdk activity and cell proliferation.

A second related pathway is thought to involve the c-Myc gene which stimulates directly the expression of Cyclin E and Cdc25a gene (**Figure 1**). Working against the mitogenic activity of CDKs are cell cycle inhibitory proteins which include $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$, $p21^{CIP}$, $p27^{Kip1}$ and $p57^{Kip2}$. It is thought that Cyclin D/Cdk complexes also play a second non-catalytic role in G1 progression by sequestering proteins of the Cip/Kip family, including p27 Kip1 and p21Cip1, two potent inhibitors of Cdk2 (Sherr and Roberts, 1995). Binding of Cip/Kip proteins to CyclinD1/Cdk4 stabilizes the complex and facilitates its nuclear import (Cheng et al., 1999). Mitogen withdrawal results in the disassembly of the Cyclin D–Cdks and in addition mobilizes the latent pool of p27Kip1, which blocks the activity of CyclinE/Cdk2 and facilitates cell cycle exit (Sherr and Roberts, 1995). Murine embryonic fibroblasts (MEFs) lacking p27 and p21 do not express D-type Cyclins and have a significant reduction in Cdk activity, but nevertheless proliferate normally (Cheng et al., 1999). This suggests that D-type Cyclins might not be essential for cell cycle progression, at least in a setting where Cip/Kip proteins are absent. Further studies showed that activation of Cyclin D1/Cdk4 complex occurs when quiescent p21/p27– null MEFs are stimulated to re-enter the cell cycle (Sugimoto et al., 2002). In addition the ectopic expression of p34 SEI-1, a mitogen- induced Cdk4 activator increased the levels of active Cyclin D1/Cdk4 complex in the absence of p21 and p27, suggesting that there are several independent ways to stimulate the assembly of Cyclin D1/Cdk4 complex (Sugimoto et al., 2002).

More recent studies have highlighted the role of an additional cell cycle regulatory mechanism at the G1 to S transition that is able to govern the initiation of histone gene expression needed for packaging of newly replicated DNA (reviewed by Stein et al. 2006**; Figure 2A**). This is commonly referred as S point and is initiated by Cyclin E/Cdk2 dependent phosphorylation of $p220^{NPAT}$ and the formation of a functional HiNF-$p220^{NPAT}$ complex that controls H4 gene transcription (see **Figure 2A**). Although both pathways have been extensively characterised in some detail in somatic cells, less information is available in embryonic stem cells. In this review we will focus on the role of these two pathways in somatic cells and draw comparisons to embryonic stem cells.

3. Roles of D-type cyclins in somatic cell proliferation

In 1995, the phenotype of mice lacking *Cyclin D1* gene was reported (Fantl et al., 1995; Sicinski et al., 1995). These studies showed that mice lacking *Cyclin D1* were viable and exhibited only focal developmental anomalies confined to the retina and a predisposition to develop breast cancer during pregnancy. Later, mice lacking one or more D type Cyclins were obtained (Ciemerych et al., 2002; Kozar et al., 2004) and like cyclin D1 deficient mice, animals lacking

Cyclin D2 or Cyclin D3 were viable and exhibited only very specific deficits. The loss of Cyclin D2 compromised female fertility, postnatal cerebellar development and impaired the mitogen-dependent expansion of peripheral B-lymphocytes (Sicinski et al., 1996). Cyclin D3 inactivation affected the early steps of lymphocytes maturation in the thymus that depends on the pre-T cell receptor (Sicinska et al., 2003). These studies revealed that the tissues most affected by disruption of the Cyclin D genes were those that principally express only one D-type Cyclin. Hence, tissue – specific expression patterns of the different D-type Cyclins seems to determine the pathologic manifestations that arise from their individual elimination. However, during early embryonic development animals retaining only one D-type Cyclin lose the tissue specific expression characteristic of that gene and up-regulate the remaining D-Cyclin in most tissues in order to compensate for loss of the other D type Cyclins (Ciemerych et al., 2002).

It has been suggested that the apparently normal organogenesis (at least until midgestation) observed in single D-Cyclin knock out mice can be explained by the ubiquitous expression of the remaining D-type Cyclins. A second possibility is that normal development in some lineages is likely to proceed independently of the D type Cyclins. The generation of mice lacking all three D-type cyclins has now shown that the latter is most likely, and those D-Cyclin independent cell cycles may be much more widespread than initially suggested (Kozar et al., 2004). The Cyclin D1-/-D2-/-D3-/- mice develop until late gestation (day E17.5) and die due to heart abnormalities combined with severe anaemia that has been linked to requirement for D Cyclins in the expansion of haematopoietic cells. In contrast, Cyclin-deficient fibroblasts proliferate nearly normally but show an increased requirement for mitogenic stimulation in cell cycle re-entry. Expression studies have shown that the level of other cell cycle regulators, including Cyclins E and A, Cdk4, Cdk6 and Cdk2 are unaffected by Cyclin D loss. In addition, these cells are resistant to Cyclin D–dependent kinase inhibitor p16Ink4a; however they are critically dependent on Cdk2 (Kozar et al., 2004). Downregulation of *Cdk2* strongly inhibits proliferation of MEFs lacking all three D-type Cyclins (Kozar et al., 2004), suggesting that Cdk2 might substitute for the lack of cyclin D/Cdk-dependent kinase activity during mouse development (Sherr and Roberts, 2004). This is however true for differentiated cells such as MEFs but not for stem cells, since *in vitro* studies have indicated that the loss of all D-type Cyclins abolishes the ability for long-term reconstitution of hematopoietic stem cells and multipotential progenitors raising the possibility that D type Cyclins may be essential for self-renewing stem and precursor cell populations to progress through the cell cycle. It is also possible that the requirement for D-type cyclins may be evident only after these factors have declined below a critical level or after inhibitors have accumulated above a threshold (Sherr and Roberts, 2004).

Cyclin D1 is overexpressed in many human cancers as a result of gene amplification or translocations targeting this locus on human chromosome 11q13 (Sherr, 1996). A direct role for Cyclin D1 involvement in oncogenesis is supported by studies with transgenic mice, in which targeted overexpression of Cyclin D1 in mammary epithelial cells leads to tumour formation (Sherr, 1996). Mice lacking Cyclin D1 expression show profound defects in mammary lobuloalveolar development during pregnancy, indicating that Cyclin D1 plays a critical role in the maturation of this tissue. Although none of the components of the Cyclin D/Cdk complexes are required in early embryogenesis, specific inhibition of Cyclin D1 in cancer cell lines results in cell-cycle arrest (Lee et al., 2000). Most importantly, Cyclin D1 or Cdk4 null mice are resistant to breast cancers triggered by the ErbB2 oncogene (Landis et al., 2006) or c-myc overexpression (de Marval et al., 2004), thus suggesting an important role for Cyclin D1/Cdk activity in neoplastic transformation and highlighting important differences between somatic cell proliferation during early embryogenesis and cancer initiation.

4. The role of Cdk4, Cdk6 and Cdk2 in somatic cell proliferation

Mice lacking Cdk6 or Cdk4 are viable (Malumbres et al., 2004), although most Cdk4 null males and females are sterile because of low sperm counts and defects in the hypothalamic-pituitary axis that affects the female oestrus cycle. Cdk4 is also essential for the development of β-islet cells in the pancreas and these results in insulin-dependent diabetes in these mice. Cdk4-null MEFs proliferate normally in serum containing media, however, only a fraction of these cells are able to respond to serum stimulation and to re-enter the cell cycle from a quiescent state induced by serum starvation (Tsutsui et al., 1999). Mice lacking both Cdk4 and Cdk6 survive to midgestation (E14.5) and exhibit megaloblastic anaemia and failure of haematopoiesis, similarly to Cyclin D null mice. MEFs deficient in both Cdks are partially able to respond to mitogenic stimulation and to exit quiescence. Interestingly, these cells continue to synthesize D-type Cyclins, which can directly bind to and activate Cdk2 *in vitro* (Matsushime et al., 1992). This is supported by experimental observations showing that complexes between Cyclin D2 and Cdk2 are more abundant in MEFs lacking Cdk4 and Cdk6 than in wild-type MEFs (Malumbres et al., 2004). Knockdown of *Cdk2* inhibits the proliferation of Cdk4/6 –deficient MEFs, but not their wild-type counterparts, indicating that Cdk2 can compensate for the loss of Cdk4 and Cdk6. The earlier embryonic lethality of mice lacking three D-type cyclins as compared to those with double deficient in Cdk4/6 reflects the ability of the D-type Cyclins to activate Cdk2 in a latter setting (Sherr and Roberts, 2004). Thus, both models (double knock out for Cdk4 and Cdk6 and triple knockout for Cyclin Ds) indicate that D-type Cyclins/Cdk4 and Cdk6 complexes are

involved in development of haematopoietic lineage, but that, overall, they have little effect on cell proliferation and organogenesis. Analysis of MEFs confirmed that inactivation of Cdk4/6 or D-type Cyclins delays S-phase entry (or re-entry after serum starvation), but does not prevent proliferation (Kozar et al., 2004; Malumbres et al., 2004).

Viability of Cdk2 null mice was reported in 2003 by Berther (Berther et al., 2003) and Ortega (Ortega et al., 2003). Cdk2-null MEFs proliferate normally, with a decreased ability to exit quiescence and/or enter S phase at the normal rate. Cyclin A–associated kinase activity is reduced in Cdk2 null MEFs from young mice but was not detected in Cdk2 –deficient adult spleen cells and in immortalized MEFs (Berthet et al., 2003). However, when Cdk2-/-Cdk6-/- mice were generated (Malumbres et al., 2004), it was reported that MEFs from these mice demonstrated a significantly lower proliferation rate compared to Cdk2 null MEFs, indicating that at least one of these Cdks is required for G1/S phase transition. It is of interest that in contrast to Cdk2-/- Cdk6-/- mice which are viable, Cdk2-/-Cdk4-/- and Cdk4-/-Cdk6-/- mutants are embryonic lethal at E15 (Berthet et al., 2006). This indicates intrinsic differences between Cdk4 and Cdk6 and points to a role for Cdk4 or Cdk6 on cell viability (Malumbres et al., 2004). It is likely that Cdk4 can phosphorylate Rb more efficiency than Cdk6, or may be Cdk4 has other substrates as compared to Cdk6 (Berthet et al., 2006). Also it was shown that loss of Cdk2 and Cdk4 causes hypophosphorylation of Rb, which leads to repression of E2F-target gene expression. For example, Cdc2 and Cyclin A2 cease to be expressed, resulting in cell cycle defect. This shows that Cdk2 and Cdk4 are important for the regulation of Rb *in vivo* and for Cdc2 (Cdk1) expression (Berthet at al., 2006).

In addition to Cdk2, Cdk4 and Cdk6, mammalian cells express many additional cyclins and Cdks that have not been fully studied, and these may provide compensating mechanism(s) in G1 phase. One such example is Cdk1 whose knock-down causes lethality at the very early stages of development (Martin et al., 2005). Although this suggests that Cdk2 and other Cdks are unable to compensate for Cdk1, the opposite can be true. Cdk1 can compensate for Cdk2 in Cdk2-/-p27-/- mice and can bind to Cyclin E, thus allowing G1/S transition (Aleem et al., 2005). Cdk1 and Cdk2 have the same cyclin binding partners, A-, B-, and E-type cyclins, but published data suggests that Cdk1 shows much higher kinase activity during M phase than Cdk2; however a direct comparison in kinase assays has not been published yet (Bashir and Pagano, 2005). Nevertheless, Cdk2 is essential for meiosis, indicating that Cdk1 cannot compensate for Cdk2 in germ cells (Berthet et al., 2003; Ortega et al., 2003). A recent publication has shown that mouse embryos lacking all interphase Cdks (Cdk2, Cdk3, Cdk4 and Cdk6) undergo organogenesis and

develop to midgestation. In these embryos, Cdk1 binds to all Cyclins, resulting in the phosphorylation of the retinoblastoma protein pRb and the expression of genes that are regulated by E2F transcription factors. However, these embryos fail to develop to the morula and blastocyst stages in the absence of Cdk1. These results indicate that Cdk1 is the only essential cell cycle Cdk. Moreover, they show that in the absence of interphase Cdks, Cdk1 can execute all the events that are required to drive cell division (Santamaria et al. 2007).

Specific polypeptide inhibitors of CDK4 and CDK6 –the so –called INK4-proteins –can directly block CyclinD-dependent kinase activity and cause G1 phase arrest (**Figure 1A**; Sherr, 1996). The four known 15-to 19-kD INK4 proteins (p16INK4a, p15INK4b, p18INK4c and p19INK4d) bind and inhibit Cdk4 and Cdk6, but not other Cdks. Thus, INK4 proteins are able to inhibit Cyclin D –dependent kinases and further phosphorylation of Rb.

Deregulated activity of Cdk4 and Cdk6 can lead to inappropriate cellular proliferation and tumorigenesis. Certain tumor types preferentially activate either Cdk4 or Cdk6, suggesting that these kinases may not be equivalently oncogenic in all cell types (Grossel et al., 1999). Overexpression of Cdk6 in certain cancer cell line causes an accelerated progression through G1 phase that is dependent on kinase activity and that does not correlate with p27 binding. In addition to the regulation by these inhibitors, Cyclin/Cdk complex are regulated by reversible phosphorylation.

Cyclin/Cdk activity is also controlled by the CDK-activating kinase (CAK).This complex composed of Cyclin H1, a kinase subunit Cdk7 and a assembly protein Mat1, catalyses activating phosphorylation of Cyclin/Cdk complexes at a threonine residue of the Cdks. Cyclin H/Cdk7/Mat1 can phosphorylate and activate Cdc2, Cdk2, Cdk3, Cdk4 and Cdk6 complexes *in vitro* (Kaldis, 1999). Mouse Mat1 deficient embryos do not display any proliferative defects at the beginning of preimplantation stages, while maternally inherited stock of Mat1 are still available. However, they become lethal at E3.5, and this probably reflects the depletion of maternal Mat1 protein below threshold levels. *In vitro*, Mat1-/- blastocysts give rise to viable post-mitotic trophoblast giant cells, while mitotic lineages fail to proliferate and survive (Rossi et al., 2001). One of the targets of Cyclin H/Cdk7/Mat1 kinase is the Cyclin E/Cdk2 complex. It was suggested that in trophoblast cells, Cyclin E/Cdk2 might represent a targets of Cyclin H/Cdk7/Mat1, highlighting a critical role of Mat1 in the control of cell proliferation. Also, mice null for Mat1 gene represented the earliest embryonic lethality associated with the disruption of

genes important for cell cycle regulation. Interestingly the development of the inner cell mass in these embryos was dependent on the presence of Mat1, while post mitotic trophoblasts cell did not, suggesting that Mat1 is essential for the mitotic cell cycle.

5. The role of Cdc25 proteins in G1 to S progression

Three CDC25 genes have been identified in humans, all of which are subject to alternative splicing, suggesting the existence of a potentially wide array of CDC25 phosphatase activity. Recent evidence has demonstrated that all three CDC25 isoforms play essential roles during the G1-S and G2-M transitions, and in mitosis, where all three isoforms show specific activity towards Cdk1/cyclin B1. Cdc25A belongs to a family of phosphatases that are important regulators of Cdk activity in different phases of the cell cycle. Activation of Cdc25A proteins occurs upon phosphorylation by Cyclin/Cdk complexes (Hoffmann et al., 1994). Cdc25A has been shown to promote entry into S phase by dephosphorylating and activating Cyclin E/Cdk2 and cyclin A/Cdk2 complexes (**Figure 1A**; Blomberg and Hoffmann, 1999). However, antisense and /or siRNA molecules that specifically target either Cdc25B or Cdc25C can inhibit S-phase progression in human cells, even in a presence of the functional Cdc25A (Turowski et al., 2003), suggesting that all three isoforms cooperate to promote S phase entry. Also, the activity of Cdc25A is high from the G1/S boundary to mitosis, which suggests a role in the completion of the cell cycle. Cdc25A has been proposed to be a target of c-Myc (Galaktionov et al., 1996). However, Cdc25A gene expression was unaffected in myc-/- cells (Bush et al., 1998). Therefore, it is not clear whether Cdc25A is a target of c-Myc or part of the mechanism by which CyclinE/Cdk2 activation is induced by c-Myc (**Figure 1A)**.

6. The role of c-Myc on cell proliferation and cell cycle regulation

c-Myc is a cellular proto-oncogene associated with a variety of human cancers and it is strongly implicated in the control of cellular proliferation, programmed cell death, and differentiation (Grandori et al., 2000; Patel et al., 2004). c-Myc protein is a transcriptional factor with basic, helix-loop-helix and leucine zipper domains (Mateyak et al., 1999). In addition to its role as a transcriptional activator, c-Myc has been also shown to participate in repression of transcription (Li et al., 1994; Penn et al., 1990). The expression of the c-Myc gene is closely correlated with cell growth, and removal of growth factor at any point of cell cycle results in its downregulation. c-Myc expression is absent in quiescent cells but is rapidly induced upon the addition of growth factors (**Figure 1A**; Spencer et al., 1991). Overexpression of c-Myc in growing cells leads to

reduced growth factor requirements and a shortened G1 phase (Karn et al., 1989), whilst reduced c-Myc expression causes lengthening of the cell cycle (Shichiri et al., 1993). Various studies have a shown a role for c-Myc in increasing the expression of Cyclin E and A, repression of Cyclin D1 expression (Philipp et al., 1994; Solomon et al., 1995) as well as maintenance of Cyclin E/Cdk2 kinase activity by inducing the expression of a hitherto-unidentified p27 – sequestering protein which allows Cyclin E/Cdk2 complex to remain active (Vlach et al., 1996). Further systematic analysis of key regulatory components in c-Myc -/- cells indicated that the absence of c-Myc reduces the activity of all Cyclin/Cdk complexes. In particular, 12-fold reduction was observed in the activation of Cyclin D1/Cdk4 and Cdk6 complexes. Also, examination of CKIs showed that p16 expression was unchanged, but p21 expression was decreased, and p27 expression was elevated in c-Myc -/- cells (Mateyak et al., 1999). c-Myc null fibroblasts isolated by target homologous recombination display a lengthening of both the G1 and G2 phases of the cell cycle, while duration of S phase remains unchanged (Mateyak et al., 1999), thus indicating an important role for c-Myc at multiple points during cell cycle regulation. Earlier it was reported that the level of c-Myc as a transcription factor oscillates as cells progress through the cell cycle and there is a marked increase in transactivation during the S-to G2/M transition. The regulation of transactivation potential can be accounted for, in part, by changes in the phosphorylation state of c-Myc, suggesting importance of its function at G2 stage of the cell cycle.

7. Interactions between positive and negative regulators of the cell cycle

Cyclin D/Cdk4-6 complexes are subject to negative regulation by both the Ink4 and Cip/Kip families of inhibitors, whilst the CyclinE/Cdk2 complexes are inhibited only by the Cip/Kip family. Cip/Kip inhibitors, such as p21Cip/Waf1, p27 Kip1 and p57Kip2 are balanced between Cyclin D/Cdk4,-6 and Cyclin E/Cdk2 complexes (**Figure 1A)**. When Cyclin D expression is increased by mitogens, more p27Kip1 is bound to Cyclin D1, resulting in the redistribution of p27Kip1 from the Cyclin E/Cdk2 complex to a Cyclin D/Cdk4,-6 complex, thereby releasing Cyclin E/Cdk2 from the negative control of p27. Therefore, apart from their kinase function in phosphorylating Rb, the Cyclin D/Cdk4-6 complexes indirectly stimulate Cyclin E/Cdk2 complexes by titrating out their inhibitors (Sherr and Roberts, 1999; Sherr and Roberts 1995). In addition, p21 and p27 have been shown to positively regulate CyclinD/Cdk4 complexes at low concentration by facilitating their assembly and possibly their nuclear translocation (Cheng et al., 1999). Consistent with this MEFs lacking p21 and p27 showed low Cyclin D1/Cdk4 assembly and activity as well as lack of nuclear localization (Cheng et al., 1999). However, p21 and p27 deficient MEFs do not exhibit cell cycle defects**.** At the same time, null mutations in p27 or

Cyclin D1 result in tissues global defects. As it was discuss above mice homozygous for null mutation in the Cyclin D1 gene are small indicating that all organs are affected. In contrast, p27Kip1 -/- mice exhibit multiorgan hyperplasia (Fero et al., 1996). Female p27-/- mice are infertile due to abnormal oestrous cycle, poor ovulation rate and a failure to produce estrogen (Tong et al., 1998). However, cyclin D1 null mice appear fertile (Sicinski et al., 1995). Taken together, these data suggest opposite action for Cyclin D1 and p27 in regulating rates of cell proliferation *in vivo*.

In addition, loss of Cyclin D1 corrected the implantation defect of p27-/- females and also almost completely rescued the disorganization of the retinal structures, while loss of p27 restored the cellularity of the Cyclin D1-/- retinas. This was achieved with only a partial restoration of phosphorylation of Rb protein and Cdk4 activity, but with dramatic elevation of Cdk2 activity, suggesting that p27 acts as a negative regulator of CyclinE/Cdk2 activity and that it can be titrated away by Cyclin D/Cdk4 complexes (Tong and Pollard, 2001). However, failure of corpus luteum cells to appropriately exit the cell cycle, the abnormal oestrus cyclicity, the incidence of pituitary adenomas associated with p27 loss argues for independent action of p27 in some tissues.

8. The roles of retinoblastoma gene family

Retinoblastoma (Rb) is quite often regarded as a negative regulator of the cell cycle progression and a positive regulator of cellular differentiation. In the absence of growth stimuli, unphosphorylated Rb binds proteins such as E2F family members, as well as co-repressors that enable chromatin remodelling activities. Phosphorylation of Rb is specific for cycling cells and is proposed to inactivate its activity (Khidr and Chen, 2006).

It is proposed that Rb1 (also known as p105), and its related family members (Rb2/p107 and Rb3/p130), maintain the critical G1 juncture. Cells in G0/G1 express Rb in un- or hypophosphorylated form. D-type cyclins trigger phosphorylation of Rb resulting in its inactivation and liberation of E2F factors, whose activity is required for entry in S phase (**Figure 1A**; Sherr and Roberts, 1999). Accumulation of Cyclin E together with Cdk2 orchestrates the continued phosphorylation on Rb protein and leads to the release of E2F, thereby facilitating the activation of genes critical for S-phase progression.

The Rb/EE2F complex consists of three Rb like proteins as outlined above and six E2F family members (E2F –1, -2, -3, -4, -5, -6) each of which can heterodimerize with two DNA-binding

protein partners (DP1 and DP2) to form 12 different DNA-binding transcriptional regulators (Cam and Dynlacht, 2003). E2F family members can be divided into two classes: activators (E2F1, E2F2, E2F3) and repressors (E2F4 and E2F5) of transcription. E2F6 is considered to be an independent member that lacks several functional domains, including the Cyclin – and pRb – binding domains and the trans-activation domain (Trimarchi et al., 1998). Activator –E2Fs utilize Rb as their major binding partner, while repressor –E2Fs bind p130 with the exception of E2F4 which is shown to preferentially bind to p107 (Frolov and Dyson, 2004). The Rb/E2F complex can be detected in G1 phase and seems to play a critical role in G1 to S transition. Activator-E2Fs exist in free form during S-phase. The p130/E2F complex is most evident in quiescent cells and p107/E2F complex is present in both G1 and S-phases.

Phosphorylation cascades in coordination with growth stimuli gradually mould Rb to release E2F in preparation for cell division. When cells are committed to eliciting the differentiation program, Rb needs to be removed or counteract differentiation inhibitors. Rb deficiency then leads to either apoptosis for cells committed to a specific differentiation program, or uncontrolled cell growth for cycling cells. To date, it is unclear how many functional Rb complexes exist. In addition, it is unclear how the Rb interacting proteins find Rb and how they execute their function. Does an Rb core complex exist while transiently associated members are recruited under specific growth and differentiation conditions in the cell? Or there are various Rb complexes, each participating in distinct processes (Khidr and Chen, 2006)?

The expression of each Rb family protein differs according to cell status. Rb2/p130 is highly expressed in quiescent and differentiated cells and its levels drop rapidly when quiescent cells are stimulated to enter the cell cycle (Classon and Dyson, 2001). Moderate levels of Rb1/p105 can be found in most cell types as well as in both quiescent and cycling cells. *In vivo* studies have demonstrated that Rb gene family members differ in their binding to the E2F family of transcription factors and fluctuation of this binding are seen throughout the cell cycle. Thus Rb2/p130 binds to E2F4 and 5 in G0 and Rb3/p107 is associated with E2F4 in G1, whereas Rb1/p105 binds to E2F4 in S phase (Howard et al., 2000). Like Rb1/p105, Rb3/p107 can inhibit proliferation of certain cell types, arresting cells in G1. It has been observed that “pocket proteins” share many functions, sometimes overlapping with one another in the cell cycle regulation; nevertheless, *in vivo* studies have proven that each of them also play very distinctive roles (Genovese et al., 2006).

Both Rb3/p107 and Rb2/p130 can stably interact *in vitro* and *in vivo* with cyclin A/Cdk2 and Cyclin E/Cdk2 complexes. Induction of pRb2/p130 inhibits Cyclin A- and Cyclin E-associated kinase activity. Through this inhibition, pRb2/p130 also induces p27 levels by impeding Cyclin E/Cdk2 phosphorylation of p27, which in turn generates the proteolytic degradation of p27 (Howard, 2000). Moreover, Rb2/p130 possesses a kinase inhibitory domain in its spacer region that preferentially inhibits Cdk2 kinase activity (De Luca et al., 1997). Interestingly, both Cyclins A and E have been found to rescue Rb2/p130 induced cell cycle arrest, indicating the importance of this interaction in cell cycle progression (Claudio et al., 1996).

Rb is also recruited to certain replication initiation sites after DNA damage, presumably to suppress abnormal replication activity (Avni et al., 2003). Several tumor suppressor proteins play key roles in the maintenance of an appropriate DNA damage response. The G1 checkpoint is disabled through a variety of mechanisms, such as loss of p16, loss of Rb, overexpression of Cyclin D1 or overexpression of Cdk4 (Sherr, 1996). Rb is crucial for the maintenance of the DNA damage checkpoint function because it elicits cell cycle arrest in response to a variety of genotoxic stresses. Cells lacking Rb are deficient for the G1 checkpoint response to DNA damage. DNA damage activates Rb by elevating its dephosphorylated form, potentially through the p53 pathway (Bosco and Knudsen, 2005). In addition, Rb has been localized to sites of DNA replication early in S phase. Rb plays a role in S-phase arrest in cells that have encountered a high dose of DNA damaging agents, such as mitomycin C (Bosco and Knudsen, 2005). In fact, the Rb protein is dephosphorylated in S-phase cells following DNA damage. Rb may also regulate the progression through S phase via its interaction with the SWI/SNF complexes (Genovese et al., 2006). The initiation of G2 checkpoint is an Rb –independent event, but Rb is required for maintenance of G2 arrest. Rb becomes activated in G2 arrested cells following a high dose of irradiation through p53 and p21. The extended G2 arrest in the Rb +/+ cells correlates with a gradual accumulation of hypophosphorylated Rb that it is believed to be initiated by radiation induced damage initiate signalling pathways that lead to Rb-dephosphorylation (Naderi et al., 2002).

pRb2/p130 and p107 are also critical regulators in the expression of G2/M required genes, e.g. Cdk1 and Cyclin B1 (Jackson et al., 2005). Thus, Rb is essential not only in the regulation of the proliferation-promoting factor E2F but also in preventing DNA damage accumulation (Genovese et al., 2006). Also, studies focusing on the overexpression effects of Rb3/p107 protein in fibroblasts demonstrated a subsequent inhibition of Cdk2 activation and a marked delay in S phase entry (Rodier et al., 2005). The inhibition of Cdk2 activity is correlated with the

accumulation of p27. It is speculated that p27, an inhibitor of Cdk2, indirectly triggers Rb hypophosphorylation in late G1. Association between p27, pRb2/p130 and Cyclin E have been shown by immunoprecipitation assays (Paggi and Ciordano, 2001). It is therefore likely that p27 binds the Cyclin E/Cdk2 complex causing a decrease in Cyclin E/Cdk2 activity which results in the hypophosphorylation of Rb1/p105 and Rb2/p130 in late G1. Induction of Rb2/p130 expression results in inhibition of Cyclin E –associated kinase activity that induces p27 levels by preventing its degradation (Howard et al., 2000). Also, Rb2/p130 and Rb3/p107 contain a motif similar to the one found in the p21/Cdk-inhibitor, which grants the ability to bind and inhibit Cdks , suggesting that pRb3/107 and pRb2/p130 may also be responsible for an interaction that affects Cdk activity (Woo et al., 1997). Rb2/p130 uniquely also possesses another distinct kinase inhibitory domain, found within its spacer region, which selectively inhibits Cdk2 kinase activity (De Luca et al., 1997; Howard et al., 1998).

Mice lacking Rb show several defects in hematopoesis and myogenesis are greatly impaired (Lee et al., 1992, Lee et al. 1996). Murine embryos that lack both copies of the Rb gene have dividing neural precursor cells outside the normal neurogenic regions in both the central and peripheral nervous systems (Lee et al., 1992). Embryos lacking both Rb2/p130 and Rb3/p107 died in utero 2 days earlier than Rb1-deficient embryos (Lee et al., 1996). Disruption of Rb family members in MEFs induces loss of G1 control and immortalization (Sage, at al.,2000). MEFs that lack expression of all three Rbs are completely insensitive to senescence-inducing signals, show a strong increase in their proliferation rate, deregulate G1 phase and become immortal (Dannenberg et al., 2000). Many human cancer cells have mutation in one or more components of p53 and Rb pathways (Galderisi et al., 2006).

9. The role of HiNF-P/p220 ^{NPAT} complex in G1 to S phase transition

During S phase, newly synthesised DNA needs to be packaged into nucleosomes. It is therefore reasonable to expect that *de novo* synthesis of histone mRNA and protein occurs at the G1 to S phase transition. Recent data (summarised in the review by Stein et al. 2006) suggests that this process is functionally, temporally and spatially distinct to the transcriptional mechanisms that occur during R point**.**

The human histone H4 gene promoter has been extensively used as an exemplary model to illustrate the factors that regulate the synthesis of the four core histone proteins and it is for this reason that we will concentrate on its role during this review. One of the most critical factors that regulates histone H4 gene transcription is histone nuclear factor P (HiNF-P). HiNF-P is a Zn-

finger transcription factor containing an N-terminal binding domain that has been shown to recognise a large consensus sequence within histone H4 genes (Mitra et al. 2003; Aziz et al., 1998). The HiNF-P expression is constitutive during the cell cycle (Pauli et al. 1987), but its DNA binding activity and expression are downregulated during differentiation of HL60 cells, suggesting a role for this gene in proliferating cells **(**Howhannisyan et al. 2003).

The HiNF-P activation of H4 genes is dependent on the $p220^{NPAT}$ (nuclear protein mapped to the ATM locus) which has been shown to be a direct target of the Cyclin E/Cdk2 signalling (**Figure 2A;** Imai et al. 1997). Genetic characterisation of the HiNF-P promoter has shown that HiNF-P is activated by both HiNF-P and $p220^{NPAT}$ and this activation is further enhanced by Cyclin E and Cdk2 activity and inhibited by $p57^{Kip2}$, $p21^{CIP}$ and $p27^{Kip1}$ (Xie et al. 2007). These studies have also shown that $p220^{NPAT}$ can stimulate HiNF-P not only with HiNF-P itself but also with Sp1, p300 and CBP; however this stimulation is antagonised by E2F1 activity (Xie et al. 2007). The dependency of HiNF-P in transactivation of histone H4 genes on the Cyclin E/Cdk2 complex suggests that synthesis of H4 histone proteins is also cell cycle dependent.

11. Cell cycle regulation in murine and primate ESC

ESC are a population of pluripotent, self renewing cells that are derived from the epiblast of mammalian blastocysts (Hyslop et al. 2005). ESCs have the potential to differentiate to functional cell types of all three germ layers *in vitro* and upon integration into animals have the capacity to contribute to all cell types of the embryo (Stojkovic et al. 2004**)**. These cells can proliferate without apparent limit and can be readily propagated, ***however very little is known about these unusual proliferative properties, their cell cycle structure and how this affects the pluripotent phenotype.***

Studies carried out in murine and primate ESC have shown that the cell divisions are very short and their division is controlled through unusual mechanisms of Cdk regulation. Rapid division of these cells is associated with an unusual cell cycle structure composed of a rather large S phase and a truncated G1 phase (Savatier et al., 1994; Fluckiger et al., 2006). Murine ESC lack the cell cycle dependent Cdk4 and 6/Cyclin D as well as Cdk2/Cyclin E activity that is associated with G1 to S transition in normal somatic cells, however this is activated immediately upon differentiation (**Figure 2B)**. The only cell cycle regulators that show cell cycle dependent expression are Cdk1 and Cyclin B1 and this occurs at G2 phase (Stead et al. 2002). At the molecular level this is underpinned by high and constant levels of Cdk2/Cyclin E complex, low levels of p21Cip1 and p27Kip1 CDK inhibitors and almost undetectable levels of Cyclin D/Cdk4

activity (Stead et al. 2002; Savatier et al. 1996). Cyclin D3 and Cyclin D1 are expressed at modest levels in murine ESC; however Cdk6/Cyclin D3 activity is relatively high due to resistance of $p16^{INK4a}$ inhibition (Faast et al. 2004). While these cells express the cell cycle pocket proteins, Rb1/p105 and Rb3/p107, they are hypophosphorylated and held in a biochemically inactive state presumably due to the activity of cell cycle independent pRb kinase activity (Stead et al. 2002). In addition, pRb is not found in complexes with E2F transcription factors and E2F dependent transcription lacks cell cycle periodicity (Humbert et al. 2000). As these cells differentiate the length of G1 phase increases substantially and this is associated with the establishment of cell cycle dependent Cdk2 activity and activation of a functional Rb-E2F pathway (White et al. 2005). Similar studies have shown that primate ESC exhibit a non phasic expression of Cyclin E and do not growth arrest in G1 after gamma irradiation, reflecting the absence of G1 checkpoint signalling (Fluckiger et al., 2006).

In murine ESCs, the Rb1/p105 and Rb3/p107 proteins are hyperphosphorylated and inactive. Consequently, E2F responsive genes are transcribed independently of cell cycle progression (Stead et al., 2002; White et al., 2005). Loss of pluripotency and stemness is associated with decreased Cdk activity and activation of Rb pathway (White et al., 2005). However, little is known about the role of Rb3/p107 and Rb2/p130 in stem cell biology. It was shown that inactivating disruptions in Rb1/p105 together with Rb3/p107 and Rb2/p130 do not seem to compromise ESC proliferation, but they reduce differentiation in experimental teratocarcinomas. This indicates that Rb dependence is only acquired as ESC undergo differentiation (Dannenberg et al., 2000; Sage et al., 2000). In addition, it has been reported that ESC share some similarities in proliferative behaviour with MEFs which are triple knockout to three Rb family members (Rb1-/-, Rb2-/-, Rb3-/-). Both triple knockout MEFS and ESC fail to arrest in G1 at confluence and after DNA damage, while in normal cells, growth arrest in G1 is strictly dependent on a functional Rb pathway (Prost et al., 1998; Harrington et al., 1998; Brugarolas et al., 1999). Hence, lack of Rb control in G1 in ECS, suggested that c-Myc pathway could play a critical role in regulation of G1 to S phase transition.

Similar functional differences for Rb family members have also been reported in other stem cell types (Caputi et al., 2005). For example, regulation of cell cycle progression by both Rb1/p105 and Rb2/p130 was observed both in mesenchymal stem cells (MSCs) grown as undifferentiated cells and in those committed toward neural phenotype, suggesting that Rb1 and Rb2 have overlapping activities in cell cycle regulation (Galderisi et al., 2006). In addition, while the antiproliferative activity of Rb is mainly HDAC-independent the cell growth arrest induced by

Rb2/p130 relies at least in part on HDAC related pathway (Jori et al., 2005). In neural stem cells (NSCs) overexpression of Rb1/p105 and Rb2/p130 promotes significant variations of differentiation process towards neurons, astrocytes and oligodendrocytes (Jori et al., 2005). Also, it was shown that Rb3/p107 plays a role in the expansion of neural stem cells (NSCs) shown by higher numbers of secondary neurospheres generated from p107 null primary neurospheres, thus demonstrating a novel role for Rb3/p107 that is distinct from Rb1/p105 and Rb2/p130 (Vanderluit et al., 2004).

12. Cell cycle regulation in human ESC

Recent work carried out in our group has shown the presence of CDK2, CDK4, CDK6, CYCLIN D1, CYCLIN D3, CDC25A and c-MYC in human ESC (Neganova et al. in preparation, Zhang et al. in preparation). Most importantly, immunoprecipitation studies have shown the presence of CYCLIN D1 and D3/CDK6, CYCLIN D1 AND D3/CDK4 complexes as well as CDK2/CYCLIN E, CDK2/CDC25A and CDK2/c-MYC complexes (Neganova et al. in preparation). CYCLIN D3 and D2 expression increases upon differentiation, whilst CYCLIN A expression decreases during this process. We found that human ESC express CYCLIN E, but with the start of differentiation towards EBs, CYCLIN E expression is upregulated. Also, expression of CDK2 was significantly downregulated by day 10 of EB development. The level of CIK (p15, p16, p19, p18, p21, p27) is very low and in some cases absent (Zhang et al. in preparation; Neganova et al. in preparation) and both phosphorylated and unphosphorylated forms of RB1/p105 are present (**Figure 1B** and **2C)**. The low level of inhibitors is in some cases achieved by high expression of proteins that secure their ubiquitin dependent degradation. For example, p27 expression in human ESC is low; however the expression of the ubiquitin ligase, Skp2 is high (Egozi et al. 2007). Also, in contrast to somatic cells, which have high levels of Skp2 during S and G2 /M phases , in undifferentiated hESC Spk2 levels were also high at G1 phase of the cell cycle (Egozi et al., 2007). Similarly to murine ESC, the embryoid bodies (EBs) with low p27 expression and high Skp2/p27 ratio showed poorer differentiation than those with high p27 expression (Egozi et al. 2007**;** Bryja et al. 2005), suggesting an important role for p27 during differentiation process that is likely to be linked to its role in cell cycle regulation.

In contrast to murine ESC, we found that quite a few of the components involved in cell cycle regulation showed cell cycle dependent expression. For example, CDK2 showed highest expression in the S phase, CYCLIN A and c-MYC in S and G2 phase, CYCLIN B1 in G2 phase, CYCLIN E in G1 and S phase, whilst CDC25A in G1 phase of the cell cycle (**Figure 2C**). These results differ slightly to recent reports by Ghule et al. 2007 in which only CYCLIN A and E were

shown to be preferentially upregulated in early S phase. This could be due to the differences in the methods used to synchronise human ESC in particular stages of the cell cycle as well as omission of late S phase and G2 in the later study. Despite the discrepancies between the two studies, one important fact is emerging that regulation of these proteins is very different during the cell cycle of murine and human ESC.

Our own studies have also shown a functional role for CDK2, CDC25A and CDK6 in G1 to S transition in human ESC (Neganova et al. in preparation; Zhang et al. in preparation). For example downregulation of CDK2 results in accumulation of cells in G1 phase of the cell cycle, increase in p21 and p27 expression and induction of differentiation (Neganova et al. in preparation). Downregulation of CDK6 increases the timing needed for G1 to S transition, whilst downregulation of CDC25A interferes with human ESC progression along the S phase of the cell cycle (Zhang et al. 2007; in preparation). Upregulation of either CDK6 or CDC25A results in shortening of G1 to S transition and it is likely that this is dependent on Rb-E2F pathway, though more work is needed to prove this hypothesis (Zhang et al. in preparation).

Work done in other somatic stem cells has indicated that CDK4, CDK6 and CDK2 are expressed in neuronal progenitor cells and downregulated upon their differentiation (Ferguson et al., 2000). CDK4/CDK6 activity and phosphorylation of Rb have been shown to be essential for S phase entry in neuronal progenitor cells, thus suggesting that this cell type follows the normal canonical pathway for cell cycle regulation. Expression of Cdk6 in mouse astrocytes has also been associated with expression of progenitor cell markers, indicating that Cdk6 might have a more widespread role in cell fate differentiation than just in the cell cycle regulation as previously thought (Ericson et al., 2003; Grossel and Hinds, 2006). This hypothesis is supported by a number of further studies that show down-regulation of Cdk2 and Cdk6 activity upon differentiation of murine erythroid leukaemia cells (Hsieh et al, 2000). Taken together these data suggest some similarities between cell cycle regulation in embryonic and cancer cells.

The role of different RB/E2F complexes and the factors that determine which RB/E2F complex will bind to and regulate E2F response remains uncertain for human ESC. A recent report has shown that p105 responsive E2F members (E2F1, E2F2 and E2F3) are expressed at lower levels than E2F4, E2F5 and E2F6. E2F1, E2F2 and E2F3 together account for 17-24% of total E2F activity in hESC, thus indicating that inhibitory E2Fs are more prominently expressed in hESC than in somatic cells. Especially, E2F5 and its cognate pocket protein RB2/p130 are the most abundant members of their class in human ESC (Becker et al. 2007). These data have led to

suggestions that human ESC show a decreased reliance on E2F-RB1 pathway that characterises the R point in somatic cells. It has to be emphasised that these data are only carried out at the transcriptional level and it is unclear whether this is true at the protein level. In addition, abundance or low expression of a particular gene cannot tell much about function; thus functional studies to highlight the role of each RB and E2F factor in human ESC are desperately needed.

Recent data suggest that the "S point pathway" is also operative in human ESC (**Figure 2C**; Ghule et al. 2007). Cell synchronisation experiments coupled with immunofluorescence microscopy showed a duplication of $p220^{NPAT}$ foci prior to onset of DNA synthesis unlike normal somatic cells where such event is evident only in S phase. In addition, it has been shown that histone H4 gene, HiNF-P and $p220^{NPAT}$ expression in human ESC is DNA replication dependent unlike somatic cells where HiNF-P and $p220^{NPAT}$ expression is constitutive. It is likely than that the availability of mRNAs for these important components of S point pathway forms one of the mechanisms that control cell cycle dependent gene expression in human ESC (Becker et al. 2007). Functional assays remain to be carried out to prove the functionality of S point pathway in these cells.

An important aspect of cell cycle regulation is the presence of check point – apoptosis signalling that prevents the accumulation of genomic damage in these cells. DNA damaging agents, including UV irradiation in human ESC has shown to result in apoptosis. p53 protein accumulates in the apoptotic cells, however it fails to activate the transcription of its target genes (Qin et al. 2007). Results from this paper show that the p53 induced apoptosis pathway is mediated via the mitochondrial pathway. Downregulation of p53 is also shown to result in reduction of spontaneous differentiation of human ESC; whilst its accumulation can induce differentiation by suppressing the transcription factor *OCT4* and *NANOG* (Qin et al. 2007). Another report has suggested that gamma irradiation results in p53 dependent transactivation of the CDK inhibitor $p21^{CIP1}$ resulting in cell cycle arrest in human ESC (Becker et al. 2007). This indicates that at least the p53 checkpoint signalling is closely linked to the maintenance of pluripotent phenotype. This mechanism provides a protective role for human ESC by inducing differentiation, cell cycle arrest or apoptosis of those ESC that have suffered DNA damage in order to eliminate them from the undifferentiated stem cell pool. In spite of the presence of such protective mechanisms, human ESC have been shown to accumulate chromosomal abnormalities (Draper et al. 2004**).** New studies have revealed that the mitotic spindle checkpoint which helps to maintain chromosomal integrity during cell divisions functions in human ESC but does not

initiate apoptosis as it does in somatic cells. This allows an unusual tolerance to polyploidy resulting from failed mitosis which is also characteristic to human cancers (Mantel et al. 2007). The uncoupling therefore of spindle checkpoint from apoptosis is likely to provide one of the causes of karyotypic abnormalities in human ESC.

13. The role of key pluripotency factors in cell cycle regulation

Oct4, Sox2 and Nanog are key regulators shown to be essential for the formation and maintenance of the inner cell mass during mouse and human preimplantation development and for the self-renewal of pluripotent ESC (reviewed by Chambers and Smith, 2004). Recent large scale transcriptional and posttranscriptional studies have identified a number of interactions between these pluripotency factors. For example, it has been shown that Oct4 and Sox2 bind to the *Nanog* promoter *in vitro* and *in vivo* (Rodda et al., 2005). Further mutagenesis studies shows that Oct4/Sox2 motif is required for the activity of *Nanog* promoter in pluripotent cells (Kuroda et al., 2005), suggesting that Oct4 and Sox2 act to promote *Nanog* transcription in ESC cells. However, in Oct4 deficient embryos, Nanog expression is detected, suggesting that Nanog can be maintained without Oct4 (Chambers et al., 2003) and that other pluripotent factors may contribute to the regulation of Nanog expression. FoxD3, a forkhead family transcription factor, is highly expressed in mESC and in pluripotent cells of the early embryo (Sutton et al., 1996). FoxD3 null mouse embryos die shortly after implantation due to the loss of the epiblast, a phenotype which is quite close to the Nanog deficient embryos (Hanna et al., 2002). Interestingly, it has been shown that FoxD3 can activate the *Nanog* promoter (Pan et al., 2006).

Boyer and colleagues identified DNA regions bound individually by OCT4, NANOG and SOX2 in human ESC and showed the high frequency of co-occupancy within the same gene region by these three factors (Boyer et al. 2005). Among OCT4 bound genes, half of them are also bound by SOX2. Moreover, about 90% of the promoter region bound by OCT4 and SOX2 are also bound by NANOG. Recently a negative feedback loop formed by Nanog, Oct4 and FoxD3 was described (Pan et al., 2006). It was shown that Oct4 maintains Nanog expression by directly binding to the *Nanog* promoter when present at a sub-steady level, but represses it when *Oct4* is above the normal level. On the other hand, FoxD3 positively regulates Nanog to counter the repression effect of excess Oct4. Conversely, Nanog and FoxD3 function as activators for Oct4 expression. When the expression level of Oct4 rises above a steady level, it represses its own promoter as well as Nanog thus exerting a negative feedback regulation loop to limit its own expression (Pan et al., 2006). This negative feedback regulation loop keeps the expression of Oct4 at a steady level, thus maintaining the ESC properties. Overall, these data suggest that the

key pluripotency factors always work together, rather than individually, to control a whole set of target genes, as well as each other, to maintain the pluripotency of ESC (Pan and Thomson, 2007).

Results recently obtained in our group have shown that NANOG overexpression enhances the G1 to S transition. This results in shortening of the time needed for G1 to S transition and causes an increase in numbers of cells in S phase (Zhang et al., 2007, in preparation). This is achieved by direct binding of the C terminal domain of NANOG to the regulatory regions of CDK6 and CDC25A in human ESC (**Figure 1B)**. This example provides the first evidence that key pluripotency factors regulate the cell cycle transition in human ESC.

Oct4 has also been shown to regulate genes linked to cell cycle progression (Campbell et al., 2007). Oct4 knockdown results in downregulation of genes involved in proliferation and an increase in the cell cycle inhibitor p21 and expression of p63, which has been linked to differentiation (Keyes al., 2005). Knockdown of OCT4 in MSCs appears to shift cells from a cycling to a non-cycling state and what is of a great importance this paper showed that in human MSCs as well as in ESC, CDK4 and CDC25A transcription is regulated by OCT4 (**Figure 1B**; Greco et al., 2007).

A recent paper (Becker et al. 2006) has shown that human ESC have a short G1 phase compared to somatic cells. In addition, lengthening of G1 in neural stem cells by inhibition of Cdks results in differentiation (Calegari and Huttner, 2003), suggesting that the longer the stem cells stay in G1, the more likely they are to be subjected to differentiation signals. In support of this it was shown that murine embryonic carcinoma cells are particularly vulnerable to retinoic acid induced differentiation while they progressed from mitosis to the next S phase, but became refractory to this differentiation while in S phase (Mummery et al., 1986). These observations suggest that G1 phase corresponds to a window of increased sensitivity to differentiation signals. The regulation therefore of G1 to S transition of ESC by master pluripotency factors such as Oct4 and Nanog and perhaps the subsequent shortening of the G1 phase ensuing from this, shields the ESC from activities that will induce their differentiation (for a schematic representation see Figure 3).

Conclusions

During the resent years intensive investigation of mice lacking one or more cell cycle genes have increased our understanding of cell cycle machinery. At the same time, these mouse models have revealed that a large number of these proteins are dispensable for cell proliferation in a number

of cell types and in some cases for the embryonic development. An important lesson that can be drawn from these studies is that the cell cycle machinery operates differently in distinct cell types and that compensatory mechanisms exist to allow somatic cells to proliferate in the absence of a given cell cycle regulator. Whether this holds true for embryonic stem cells is as yet unknown since the cell cycle studies in human ESC are still in their infancy. Quite a few key differences have emerged between the cell cycle regulation in murine and human ESC. One speculative interpretation of these findings that takes into account the immediate upregulation of CDK/cyclin D activity in murine ESC upon their differentiation would be that human ESC are more similar to early differentiated murine ESC where the more sophisticated cell cycle dependent CDK/Cyclin D mechanisms are established. Indeed, new studies have shown that human ESC are more similar to the pluripotent cell lines derived from the post implantation epiblast (Brons et al. 2007; Tesar et al. 2007). In addition, new techniques have shown that human ESC contain two distinct cell populations that differ in their cell surface and transcription factor expression as well as their cell cycle regulation (Mantel et al. 2007**;** Stewart et al. 2006). Any differences therefore between human and murine ESC have to be explored with caution in these two reported populations before firm conclusions on their basic biology can be drawn up in the text books in years to come.

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Figure Legends

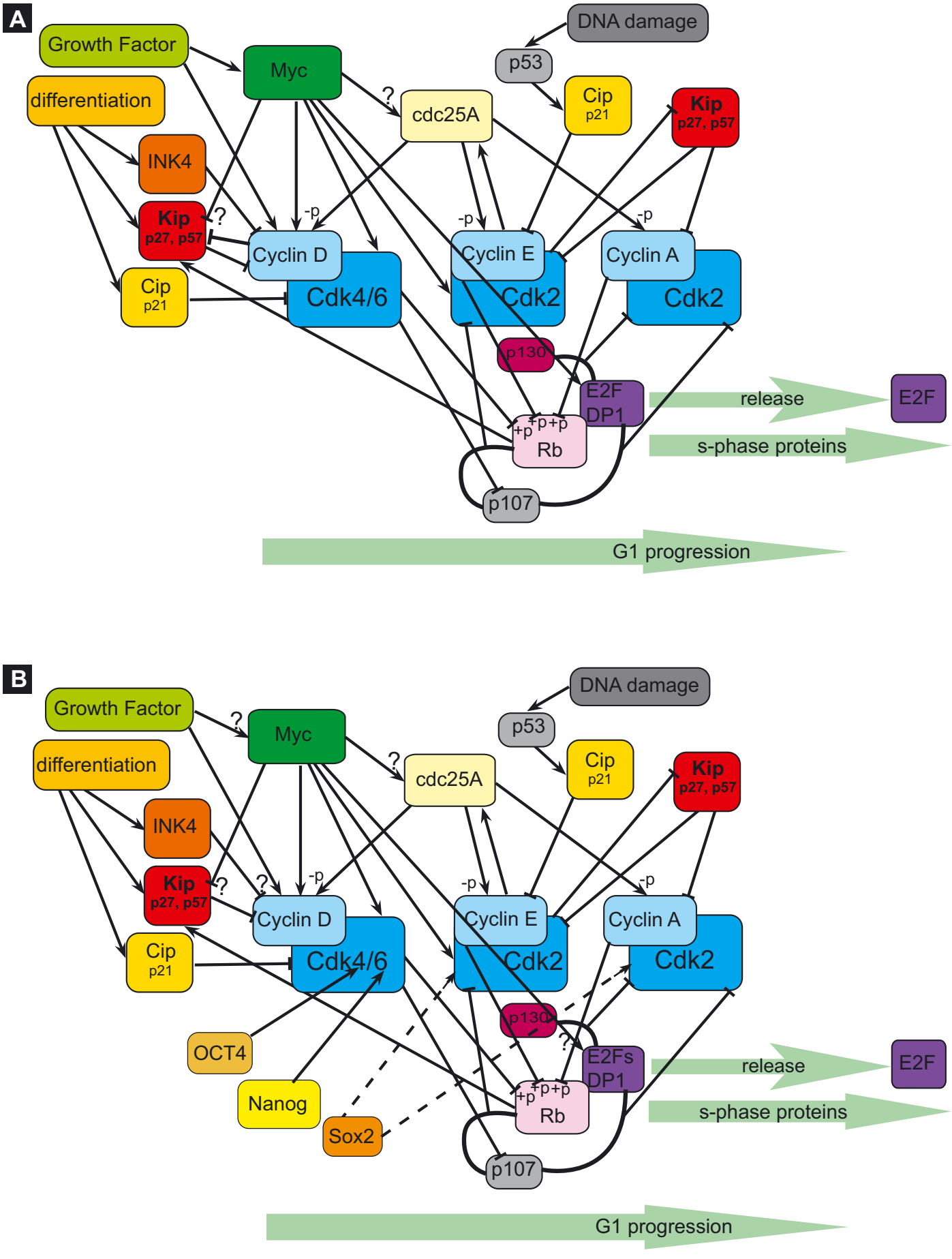
Figure 1. Simplified schematic representation of the key molecular pathways controlling G1 to S phase transition in somatic cells (A) and in human ESC (B). In proliferating cells, Rb phosphorylation by Cyclin/Cdk complexes releases E2F, which then induces genes that mediate S phase entry. Arrows indicated stimulatory modifications, blocked lines show inhibitory modifications. – p and + p indicate removal and addition of phosphorylation respectively. ? indicates scientific questions that have not been addressed as yet.

Figure 2. A simplified presentation of the fluctuation of expression of Cyclins and Cdks involved in G1 to S progression in somatic cells (A), mouse ESC (B), and human ESC (C). R and S point are shown in red.

(A) – Activity of Cyclin/Cdk complexes at different stages of cell cycle in somatic cells.

(B) –Expression of Cyclin D, Cyclin E and Cyclin A is not dependent on the cell cycle progression in murine ESC. Hyperphosphorylated Rb is present at all cell cycle stages and the only Cyclin that demonstrates cell cycle periodicity in mouse ESC is Cyclin B1 at G2 stage of the cell cycle (not shown). Although it is clear that R point does not operate in murine ESC, the existence of a functional S point and G2/M is not clear. Recent investigations suggest that although the mitotic-spindle checkpoint, which helps maintain chromosomal integrity during all cell divisions, functions in human and mouse ESCs, it does not initiate apoptosis as it does in somatic cells.

(C)- Expression of Cyclins in human ESC during cell cycle progression. **C**yclin D1 is constant throughout the cell cycle, but the level of Cyclin E is higher during G1-S phases and Cyclin A is high at S-G2 stages of the cell cycle. Cyclin B1 expression is also higher at the G2/M stage (data not shown). Recent investigations have indicated the existence of S point; however it remains to be determined whether the R and G2/M points are functional in these cells.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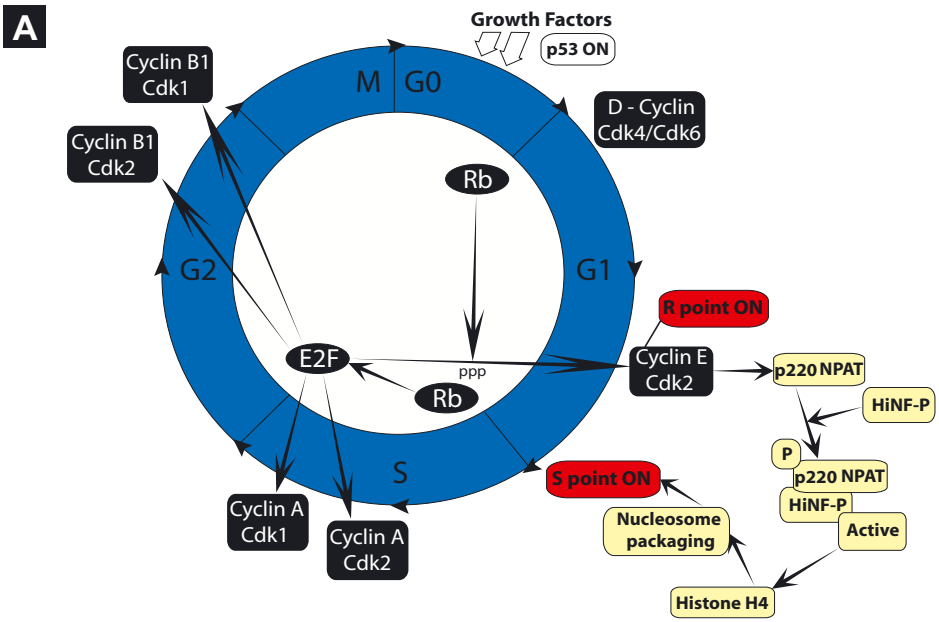
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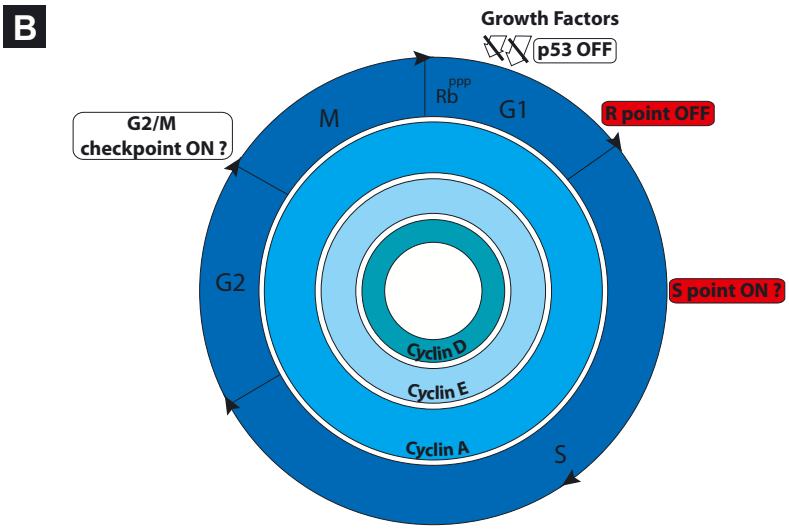
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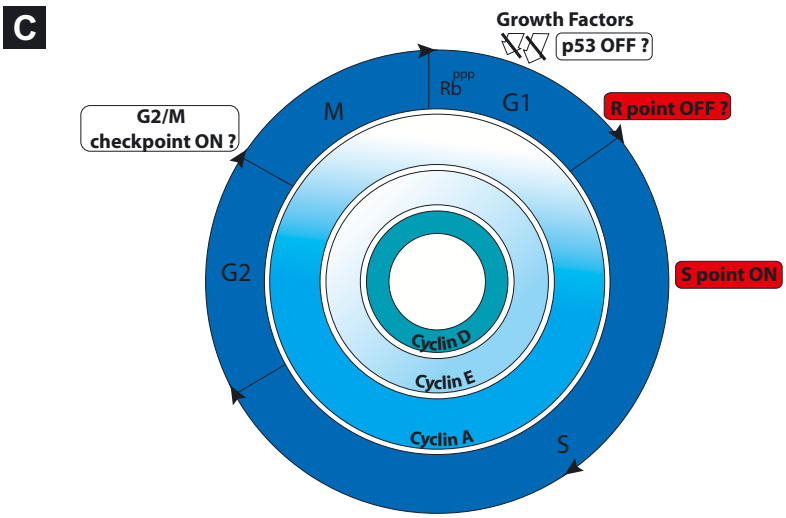
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