Cell cycle regulation in human embryonic stem cells: links to adaptation to cell culture

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Abstract

Cell cycle represents not only a tightly orchestrated mechanism of cell replication and cell division but it also plays an important role in regulation of cell fate decision. Particularly in the context of pluripotent stem cells or multipotent progenitor cells, regulation of cell fate decision is of paramount importance. It has been shown that human embryonic stem cells (hESCs) show unique cell cycle characteristics, such as short doubling time due to abbreviated G1 phase; these properties change with the onset of differentiation. This review summarizes the current understanding of cell cycle regulation in hESCs. We discuss cell cycle properties as well as regulatory machinery governing cell cycle progression of undifferentiated hESCs. Additionally, we provide evidence that long-term culture of hESCs is accompanied by changes in cell cycle properties as well as configuration of several cell cycle regulatory molecules.

Keywords: human embryonic stem cells, cell cycle, culture adaptation, long-term culture

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Introduction

Embryonic stem cells (ES) are characterized by unlimited self-renewal and pluripotency – that is the ability to differentiate into all somatic cell types. They have become an essential tool for investigation of developmental processes *in vitro* and recently also in other areas of basic biology. Moreover, since derivation of hESCs in 1998, these cells hold a great promise for use in regenerative medicine or drug discovery.[1] What precedes their safe use in clinic is the precise understanding of molecular pathways which regulate prolonged self-renewal *in vitro*, differentiation as well as maintenance of genomic integrity. Despite considerable effort and interest in resolving these pathways, many questions remain to be addressed.

Pluripotent cell cycle

The understanding of stem cell biology has been recently largely influenced by the observation that cell cycle regulation is closely connected to maintenance of stemness and pluripotency.[2–4] In comparison to proliferating somatic cells, ES cells show unique cell cycle properties, such as short doubling time (~15–16 h in hESCs) due to abbreviated G1 phase (~3–4 h), absent G0 phase and differentially regulated cell cycle checkpoints.[5–7] Importantly, these characteristics rapidly change with the onset of differentiation, suggesting that abbreviated G1 phase is one of the key components of maintaining undifferentiated phenotype.[3,5,8] Indeed, it is understandable that highly proliferative self-renewing population of ES cells is propelled to pass quickly through G1 phase, as this phase represents the time with increased sensitivity to differentiation signals.[2,6,8–10] However, how exactly this fast progression through G1 – and possibly also other phases – is achieved at molecular level is still only partially understood.

Initial insight into the unique regulation of pluripotent cell cycle was gained by data from mouse ES cells (mESCs). It has been shown that cyclin-dependent kinase (CDK2)-cyclin E complex is constitutively active throughout the cell cycle, keeping pRb protein in hyperphosphorylated and thus inactive state. This allows transition of mESCs from M phase directly to the late G1 phase, resulting in absence of D-type cyclins and therefore abbreviated G1 phase (reviewed in[11,12]). However, in contrast to mESCs, most of the cell cycle regulators in hESCs show

cell cycle-dependent expression, thus revealing important differences in the expression of cell cycle regulatory components between these two embryonic cell types.[3]

Cell cycle regulation in hESCs: CDKs, cyclins and CKIs

Detailed studies of cell cycle regulation in hESCs were initiated by Becker *et al.* in 2006.[6] Results of molecular profiling of cell cycle regulators of the mRNA level revealed that hESCs show high expression of CDK4 mRNA and its partner cyclin D2 mRNA. They concluded that CDK4-cyclin D2 complex is responsible for the unique regulation of G1 progression in hESCs. Indeed, they supported these data recently by showing that depletion of cyclin D2 protein in hESCs causes cell cycle defects in G1 phase.[5] In addition, they show that in comparison to cyclin D2, mRNA as well as protein expression of cyclin D1 is very low and mRNA of cyclin D3 and cyclin E1 is almost undetectable[5,6] (Figure 1). In contrast to both studies by Becker (2006, 2010)[5,6], results of Filipczyk (2007)[8] demonstrated by immunocytochemistry that hESCs did not express any of the D-type cyclins. Moreover they showed that 100% of hESCs are positive for cyclin E1 protein, thus concluding that cyclin E1 is constitutively expressed in undifferentiated hESCs similarly to mESCs[11,13] (Figure 1). Notably, Neganova *et.al.*[3] compared the expression of all major cyclins and CDKs in undifferentiated hESCs with hESCs differentiating in embryoid bodies (EBs) and thus introduced a third scenario. They showed that cyclins E1, A2 and B1 fluctuated during the cell cycle and that undifferentiated hESCs expressed only cyclins D1 and D3, while the level of cyclin D2 was very low or undetectable. Importantly, they also measured the activity of partner CDKs of D-type cyclins (CDK4 and CDK6) and showed that both CDK4 and CDK6 exhibit increased activity during G1 phase of the cell cycle[3] (Figure 1). Unpublished data obtained in our laboratory largely support this scenario by showing that all analyzed hESC lines express low, but detectable, levels of all three D-type cyclins and that their partner CDKs (CDK4 and CDK6) are also present and active as kinases. Collectively, although some studies found significant levels of D-type cyclins and also active CDK4/6 in undifferentiated hESCs, the major driving force of G1/S transition in such cell is still ascribed to CDK2 by majority of investigators. Both CDK2-activating partners (cyclins A2 and E1) as well as CDK2-activating phosphatase Cdc25A are highly expressed in undifferentiated hESCs,[3,7] and our own data). Importantly, functional experiments show that down-regulation of CDK2 results in arrest of hESCs in G1 phase and induces differentiation to extraembryonic lineages.[2,3] It is plausible that unrestricted activity of CDKs leading to rapid proliferation of undifferentiated hESCs is further ensured by generally low levels of inhibitors of cyclin-dependent kinases. Zhang *et al.*[14] showed that members of INK family of inhibitors (p15, p16, p18 and p19), which regulate activity of CDK4 and CDK6, show no or very low expression at the protein level. Similarly, members of Cip/Kip family (p21, p27 and p57) are expressed in hESCs in very low quantities or are not detectable at all.[3,7,14–17] Expression of all CDK inhibitors increases only upon induction of differentiation that is in consonance with the activation of classical cell cycle regulatory pathways that are typical for somatic cells.

Functioning of checkpoint pathways in hESCs

Undoubtedly, fast progression of ES cells via their cell cycle may also be linked to functioning of checkpoints that are positioned throughout cell cycle. Capability of hESCs to activate key G1/S checkpoint has been experimentally addressed by several groups with somewhat dissimilar conclusions.[7,18,19] Still, all studies accomplished on mESCs and hESCs commonly describe non-functionality of the p53–p21 axis of the G1/S checkpoint pathway.[7,18,19] The inability of p53 regulator to transactivate, upon DNA damage, its downstream genes other than p21 was also reported in hESCs.[20] In our experiments, activation of G1/S checkpoint in hESCs was achieved by low-dose ultraviolet C irradiation.[7] Instead of the slow-acting pathway involving

	CKIs	CDK2	CDK4	CDK6	Cyclin D1	Cyclin D2	Cyclin D3	Cyclin E	Cyclin A	Cyclin B	Cdc25A	pRb	References
Scenario 1		–	~	–	–	~	–				N/A	Not operational	(5,6)
Scenario 2		N/A	N/A	N/A				–	~	N/A	N/A	Operational	(8)
Scenario 3		~	–	–	–	–	–	~	~	~	~	Operational	(3,7,32,33)

High expression
Intermediate expression
Low or undetectable expression
– constant expression throughout cell cycle
~ fluctuating expression throughout cell cycle
N/A not available

Figure 1 Summary of functional and molecular characteristics of cell cycle of human embryonic stem cells, which define three different scenarios of regulation based on the data determined by several investigators.

p53 and p21, however, execution of G1/S checkpoint was mediated by fast-acting degradation of Cdc25A phosphatase resulting in an inability to activate CDK2. Still, DNA damage in hESCs is always accompanied by accumulation and phosphorylation of p53 and also by increased production of mRNA coding for p21, which is not followed by accumulation of the p21 protein.[7,17] Expression of the p21 protein becomes measurable and further inducible by DNA-damaging insults only upon entry of hESCs into differentiation pathway, along with establishment of classical cell cycle regulation.[17] We have recently addressed mechanism by which production of p21 protein is prevented in undifferentiated hESCs. We found that hESCs have functional p53 protein but utilize miR-302s and the microRNA pathway to prevent p21 mRNA from being translated.[17] In full consonance with this finding, overexpression of miR-302s[21] as well as inactivation of p53–p21 pathway[9,22,23] increase the efficiency of re-programming of somatic cells to pluripotency. Interestingly, under certain conditions, microRNA-mediated inhibition of p21 in undifferentiated hESCs may be abolished, since stabilization of p53 by chemical compound Nutlin results in accumulation of p21 protein leading to hESCs differentiation.[2] This is, however, an artificial situation that possibly involves molecular targets other than p53 and thus may not reflect actions of molecular circuitries typical for DNA damage reaction. In this context, a link between pluripotency gene Oct4 and p21 is of notice. It has been shown that down-regulation of Oct4 results in higher levels of p21 protein while up-regulation of the same drives expression of CDK4 and Cdc25A thus facilitating faster cell cycle progression.[24,25] Similarly, another pluripotency gene Nanog promotes transcription of Cdc25A and CDK6 and thus accelerates S-phase entry of undifferentiated hESCs.[14]

It can be deduced from the above described experimental data that both features of ES cells, sustained fast proliferation and pluripotency, are tightly linked together by more than one molecular pathway/principle, thus further underlining their key ES cell-defining character.

Long-term cultured hESCs undergo changes to cell cycle properties

Importantly, sustained proliferation in culture is vital for the capability of hESCs to be expanded by timely unlimited propagation to great numbers required for their potential practical use. Despite a general appreciation for such unlimited growth capacity, it is well recognized that prolonged culture under non-differentiating conditions is accompanied by occurrence of profound alterations to hESC properties; however, molecular basis as well as outcomes are still unclear. Such alterations to pristine hESC phenotype are commonly attributed to progressive adaptation to *in vitro* conditions.[26–29] Still, the outcome of such adaptation is not inherently negative since long-term propagated hESCs exhibit normal (or even increased) expression of pluripotency markers, such as SSEA3, Tra-1–60 or Tra-1–81 and retain their ability to *in vitro* differentiate into all three germ layers and to effectively form teratomas in nude mice.[26,27] On the other hand, culture adaptation of hESCs very often involves various karyotypic changes,[26,28] though it seems that individual hESC lines have different predisposition to genomic instability and may even retain normal karyotype over 215 passages[29] (and our unpublished data). In the context of this minireview, it is important that such long-term propagated cells significantly increase their proliferation rate and plating efficiency with no obvious correlation to their putative karyotypic differences (see Refs.[26,27] and our observation). Intriguingly, the relationship between prolonged culture of hESCs, their increased capacity to proliferate and cell cycle regulation has not yet been addressed. We envision such data to be interesting as they may help to: (i) better understand molecular mechanisms underlying culture adaptation of hESCs, (ii) unravel relationship between key properties of hESCs and the machinery that regulates cell cycle, (iii) evaluate risks potentially associated with adapted phenotype, and (iv) possibly identify molecular factors that underlie deregulated proliferation of cancer stem cells.

Long-term cultured hESCs have shorter interval of passage accompanied by faster cell cycle progression

Similarly to the findings of others, shortening of passaging interval in hESCs growing in culture for longer periods of time has been repeatedly observed in our laboratory. We began to investigate molecular grounds of such change on two independent lines of hESCs, CCLT12 and CCTL14.[30] Cells in passage lower than 50 are further referred to as short-term cultured, while cells maintained in culture for over 240 passages are referred to as long-term cultured. In short-term cultured hESCs, the average passage interval was ~7.3 days while in long-term cultured hESCs it was abbreviated to only ~3.4 days (Figure 2a). Since this finding might or might not reflect the difference in cell cycle length, we directly measured this parameter in both short- and long-term cultured cells by cumulative BrdU incorporation strategy, as described in Kunova *et al.*[31] This experiment has unraveled that long-term cultured hESCs have shorter cell cycle, by about 25%, compared with their short-term cultured counterparts (Figure 2b). In absolute numbers, this represents 16.2 h (+/− 0.65) for short-term

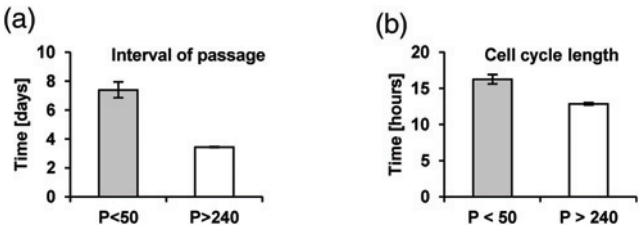

Figure 2 Compared with short-term cultured human embryonic stem cells (hESCs), long-term cultured hESCs have abbreviated interval of passage associated with shorter length of cell cycle. Graphs represent: (a) interval between passages in days; (b) length in hours of the cell cycle of short- and long-term cultured hESCs. The data represent means and standard deviations of three independent experiments

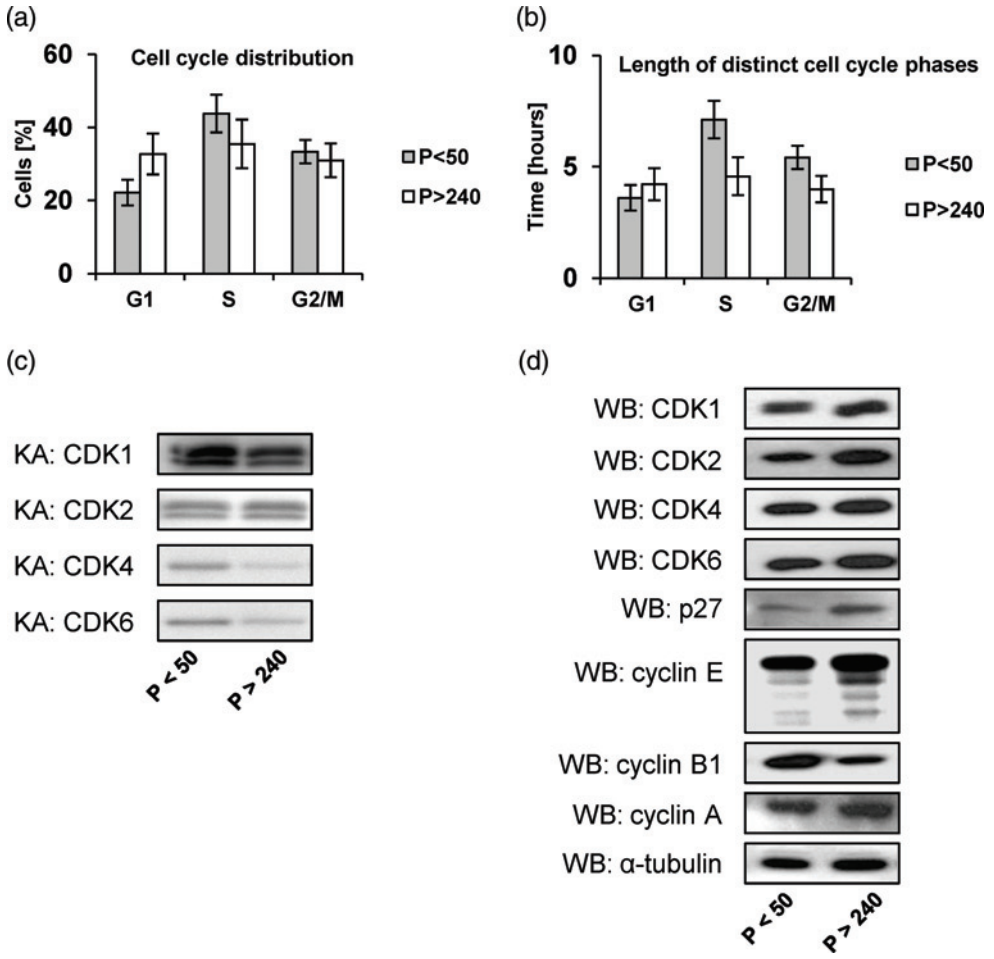

Figure 3 Short- and long-term cultured human embryonic stem cells (hESCs) differ in their cell cycle distribution and regulation. (a) Cell cycle profiles as determined by flow cytometry. (b) Length of individual cell cycle phases in hours. (c) Activities of CDK1, CDK2, CDK4 and CDK6 in short- and long-term cultured hESCs as determined by kinase assay. (d) Levels of CDKs and cyclins in short- and long-term cultured hESCs as determined by Western blot analysis

cultured hESCs and 12 h (+/− 0.15) for long-term cultured hESCs.

The most prominent changes to cell cycle characteristics that are associated with accelerated cycling in long-term cultured hESCs are (i) higher proportion of cells in G1 phase at the expense of cells in S phase (Figure 3a) and (ii) shortening of S phase to only about two-thirds of its length typical for short-term cultured hESCs (Figure 3b). In full consonance with such cell cycle behavior, long-term culture of hESCs is associated with slight elevation of quantities of CDK1, 2, 4 and 6, and, most importantly, with significantly increased levels of positive regulators of G1 and S phases, cyclins E and A (Figure 3d). Although these are all only associations and not causative relations, we envisage that they reflect changes, which may have some relevance to the applicability of stem cells in biomedicine and need to be further studied.

Summary

For years, regulation of cell cycle has been studied mainly because of its relevance to hyperproliferative diseases – cancers. Currently, the interest in cell cycle regulation expanded into the newly emerged arena of investigating properties of stem cells. The reasons for that are obvious. Proliferation of stem cells must be delicately controlled since these cells are crucial contributors to ontogenesis of every single individual, as well as they decide about the long-term maintenance and regeneration of tissues and organs during the adult age. Also, since stem cells are resource for all the functional cells in the body, multiplication of only those that are genetically pristine should be allowed. Here we summarize current knowledge on cell cycle regulation of ES cells. We postulate that although ES cells do not in any extent represent stem cells in general, the findings made on ES cells still set grounds for analyzing cell cycle control of all other natural and/or induced stem cell types, both in their original niches and in culture.

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