

Cell cycle dependent phosphorylation and subnuclear organization of the histone gene regulator p22ONPAT in human embryonic stem cells

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Abstract

Human embryonic stem (ES) cells have an expedited cell cycle (~15 h) due to an abbreviated G1 phase (~2.5 h) relative to somatic cells. One principal regulatory event that controls cell cycle progression is the G1/S phase induction of histone biosynthesis to package newly replicated DNA. In somatic cells, histone H4 gene expression is controlled by CDK2 phosphorylation of p22ONPAT and localization of HINFP/p22ONPAT complexes with histone genes at Cajal body related subnuclear foci. Here we show that this 'S point' pathway is operative in situ in human ES cells (H9 cells, NIH-designated WA09). Immunofluorescence microscopy shows an increase in p22ONPAT foci in G1 reflecting the assembly of histone gene regulatory complexes in situ. In contrast to somatic cells where duplication of p22ONPAT foci is evident in S phase, the increase in the number of p22ONPAT foci in ES cells appears to precede the onset of DNA synthesis as measured by BrdU incorporation. Phosphorylation of p22ONPAT at CDK dependent epitopes is most pronounced in S phase when cells exhibit elevated levels of cyclins E and A. Our data indicate that subnuclear organization of the HINFP/p22ONPAT pathway is rapidly established as ES cells emerge from mitosis and that p22ONPAT is subsequently phosphorylated in situ. Our findings establish that the HINFP/p22ONPAT gene regulatory pathway operates in a cell cycle dependent microenvironment that supports expression of DNA replication-linked histone genes and chromatin assembly to accommodate human stem cell self-renewal.

Introduction

Self-renewal of human embryonic stem (ES) cells is expedited by an abbreviated cell cycle that is characterized by a very brief G1 phase (Becker et al., 2006) Defining the molecular basis of pluripotency and the capability of human ES cells for self renewal is critical for the rational design of biomedical applications of human ES cells as a cellular source for tissue replacement. Cell cycle progression in both human ES and somatic cells is controlled during G1 and S phase by growth factor dependent signaling pathways that promote proliferation and surveillance mechanisms that monitor the integrity of the genome and chromatin packaging. Seminal studies by Pardee and colleagues have defined the restriction (R) point in the G1 phase of somatic cells that represents the stage when commitment towards initiation of DNA synthesis at the G1/S phase transition becomes growth factor independent. Studies in our laboratory have examined the functional coupling between the R point and signaling pathways that control the activation of histone gene expression ('S point') which is essential for the packaging of newly replicated DNA. One essential difference between human ES and somatic cells is the length of G1 phase during which cells commit to S phase entry. Therefore, we have focused on defining molecular parameters of the regulatory pathways that control the onset of S phase in ES cells. We have shown that the G1 phase of human ES cells is about 2.5 to 3 h as compared to a typical G1 phase in human somatic cells of 9 to 12 h. Similar to somatic cells, ES cells express histone gene regulatory factors (e.g., HINFP and p22ONPAT) and exhibit S phase specific expression of distinct members of the DNA replication dependent histone gene family. In somatic cells, critical events for the onset of S phase include the cell cycle dependent phosphorylation of p22ONPAT by cyclin E/CDK2 at nuclear foci related to Cajal bodies and the subsequent recruitment of p22ONPAT by transcription factor HINFP to histone H4 gene promoters. In this study, we examined the subnuclear organization and phosphorylation of p22ONPAT during cell cycle progression in ES cells. Cell cycle stages were identified by western blot analyses and immunofluorescence microscopy.

Results

Cell cycle progression in human embryonic stem cells

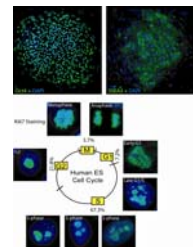


Figure 1: Expression of stem cell specific markers validates the pluripotent nature of H9-hES cells (upper panels). H9 cells were grown on coverslips for 4 days and then fixed and stained using primary antibodies for stem cell specific markers. The upper panels show merged images of representative hES colonies (10X objective) stained with Oct-4 (left top panel) or stem cell specific embryonic antigen-3 (SSEA-3) (right top panel) that were counterstained with DAPI. The cell cycle dependent distribution of the nuclear specific Ki-67 reflects cell cycle distribution in H9 ES cells (lower panels). H9 ES cells were grown on coverslips and nuclear staining patterns of Ki-67 were quantified (Solovoi et al., 2005) using immunofluorescence microscopy. A total of 400 cells were analyzed for each experiment and at least two independent counts were taken. The lower panel shows cell cycle phase-specific staining patterns (100X) that were arranged in relation to a diagram depicting the abbreviated cell cycle of human ES cells (Becker et al., 2006). The average percentages depicted reflect the fraction of cells that exhibit the indicated staining pattern.

Differential expression of cyclins A, B and E in asynchronous H9 ES cells

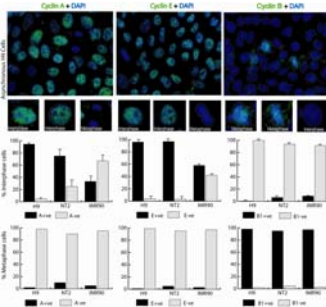


Figure 2: Immunofluorescence microscopy was used to detect cyclins A, B and E in asynchronous H9 ES cells (first row). The micrographs show images at different magnifications (63X and 100X) with representative staining patterns observed in representative colonies (63X objective), as well as single cells in interphase or metaphase (100X objective) as indicated. All images show merged staining patterns for the indicated cyclins and DAPI. The graphs show quantification of cyclin expression during interphase or metaphase in H9, NT2 or IMR90 cells. The bar graphs show the fraction of cells that express the indicated cyclin in either interphase (second row) or metaphase (third row). The black bars represent the percentage of cells positive for either cyclin, while gray bars represent cells that are negative for cyclin staining. For each determination we counted at least 200-300 interphase cells. The mitotic distribution of cyclins was determined in 100 metaphases for each cell line. Values for three independent experiments were averaged and the error bars reflect the range of the values. The cyclin E antibody detects signal in IMR-90 cells that do not stain with Ki-67; these Ki-67 negative cells were interpreted as quiescent cells and were excluded from our analyses.

In situ localization and phosphorylation of p22ONPAT.

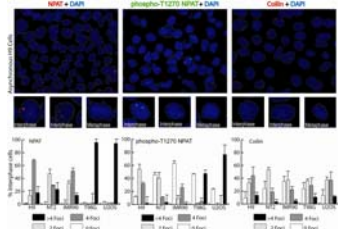


Figure 3: In situ immunofluorescence microscopy was used to examine the organization and phosphorylation of p22ONPAT in relation to the Cajal body component colin/p80 (upper panels) in asynchronous ES cells. Phosphorylation of p22ONPAT was determined using an antibody that recognizes the phospho-T1270 epitope. The micrographs show representative staining patterns observed in representative ES colonies (63X objective; top row), and single interphase or metaphase cells (100X objective; second row) as indicated. All images show antibody staining merged with the DAPI counterstain. We quantified the distribution of p22ONPAT, phosphorylation of p22ONPAT at the CDK2 related epitope T1270, or colin/p80 in asynchronously growing H9, NT2 teratocarcinoma cells, ES cells and a panel of somatic cell types, including IMR90, T98G and U2OS cells. The number of foci observed for each antibody was quantified in each cell type. Cells were categorized as having 0, 2, 4 or >4 foci per cell, because these foci are known to occur in pairs and even numbers prevailed in our analyses. The bar graphs (lower panels) show the percentage of interphase cells with the indicated number of foci for each cell line. No immuno-reactivity was observed during metaphase in any of the cell lines. For each determination we counted at least 200-300 cells per experiment. The values depicted in the graphs represent counts for three independent experiments, and the error bars represent the range of the values.

Cell cycle regulation of cyclins A, B and E in synchronized human ES cells.

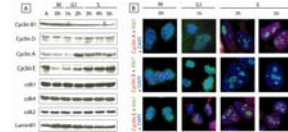


Figure 4: Mitotically synchronized human ES cells were examined by western blot analysis (Panel A) or immunofluorescence microscopy (Panel B) for the levels and presence of cyclins. H9 ES cells were synchronized by 16 hr nocodazole treatment and total cellular protein was harvested at the indicated times after release from the mitotic block (0 h to 5 h). Protein (25-30 µg) was separated by 10% SDS-PAGE and immunoblotted with the indicated antibodies (Panel A). In-situ immunofluorescence microscopy for detection of cyclins A, B and E (Panel B) was performed as described in Figure 2. The micrographs show merged images of cells stained for the DNA marker DAPI (blue), the cell cycle marker Ki-67 (green) and cyclin antibodies (red). The three rows differ in staining for cyclin A (first row), cyclin B (second row), or cyclin E (third row).

Cell cycle dependent phosphorylation of p22ONPAT occurs after a cellular increase in the number of foci containing p22ONPAT.

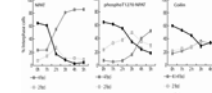


Figure 5: Mitotically synchronized H9 ES cells were examined for the number of foci containing p22ONPAT, phospho-p22ONPAT or colin as described in Figure 5. The percentage of cells with 0, 2, 4 or >4 foci were assessed at each cell cycle stage. The line graphs show the percentage of interphase cells with the indicated number of foci as a function of time following release from a nocodazole block. For each determination we counted at least 200-300 cells per experiment, and each point of the graph depicts the average of two independent experiments.

Focal organization and phosphorylation of p22ONPAT during G1 and S phase progression of human embryonic stem cells.

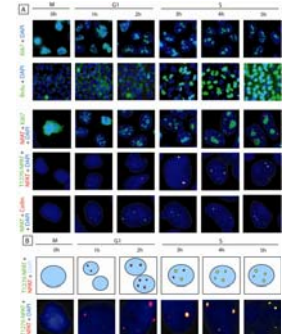


Figure 6: Panel A shows mitotically synchronized H9 ES cells (as in Figure 4) that were examined for foci containing p22ONPAT (rows 3, 4 and 5), phospho-p22ONPAT (row 4) or colin (row 5) as described in Figure 3. Staining patterns were examined in comparison to cell cycle stage specific nuclear staining patterns of Ki-67 (rows 1 and 3) or the onset of S phase as determined by BrdU staining (row 2). Panel B shows interpretative diagrams that depict a doubling of p22ONPAT foci prior to the in situ appearance of phospho-p22ONPAT (row 1). The micrographs presented below the diagrams (row 2) show enlargements of the foci containing p22ONPAT and phospho-p22ONPAT that are presented in Panel A (row 4).

Conclusions

- >The principal finding of this study is that cell cycle regulation of histone gene expression in human ES cells is supported by a functional p22ONPAT pathway as reflected by cell cycle dependent phosphorylation of p22ONPAT at specific subnuclear foci. The spatial organization of p22ONPAT containing histone gene regulatory complexes into distinct foci is similar to what has been observed for somatic cells.
- >The current data strongly suggest that at least a subset of Cajal bodies contains an integrated supramolecular architectural complex in which histone gene transcription factors, the co-activator p22ONPAT, histone gene clusters and the U7 snRNP related 3' end processing machinery are all associated contemporaneously.
- >The upregulation of the levels of cyclins E and A is consistent with the cyclin E dependent phosphorylation of p22ONPAT as cells progress into S phase as part of a mechanism to ensure activation of histone gene expression in conjunction with DNA replication. It appears that the human ES cell cycle is driven not only by a cyclin B/CDK1 oscillator as has been suggested for murine ES cells (Stead et al., 2002), but also by the cell cycle dependent phosphorylation of p22ONPAT, which is mediated by cyclin E/CDK2 in somatic cells.

Acknowledgments

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