Cells and polyamines do it cyclically

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Abstract

Cell-cycle progression is a one-way journey where the cell grows in size to be able to divide into two equally sized daughter cells. The cell cycle is divided into distinct consecutive phases defined as G₁ (first gap), S (synthesis), G₂ (second gap) and M (mitosis). A non-proliferating cell, which has retained the ability to enter the cell cycle when it receives appropriate signals, is in G₀ phase, and cycling cells that do not receive proper signals leave the cell cycle from G₁ into G₀. One of the major events of the cell cycle is the duplication of DNA during S-phase. A group of molecules that are important for proper cell-cycle progression is the polyamines. Polyamine biosynthesis occurs cyclically during the cell cycle with peaks in activity in conjunction with the G₁/S transition and at the end of S-phase and during G₂-phase. The negative regulator of polyamine biosynthesis, antizyme, shows an inverse activity compared with the polyamine biosynthetic activity. The levels of the polyamines, putrescine, spermidine and spermine, double during the cell cycle and show a certain degree of cyclic variation in accordance with the biosynthetic activity. When cells in G₀/G₁-phase are seeded in the presence of compounds that prevent the cell-cycle-related increases in the polyamine pools, the S-phase of the first cell cycle is prolonged, whereas the other phases are initially unaffected. The results point to an important role for polyamines with regard to the ability of the cell to attain optimal rates of DNA replication.

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Introduction

The fundamental unit of a multicellular organism, whether plant or animal, is the cell. The size of the organism depends on the number and size of cells. As all organisms in principle arise from one cell, the development of an organism depends on mechanisms whereby one cell can multiply into the number of cells that are required to build a specific organism. The basic cell growth and division cycle is called the cell cycle. The cell is an extremely complex structure composed of thousands of molecules of different sizes and with different roles in the life cycle of the cell. This chapter concerns the role for the polyamines in the cell cycle of mammalian cells.

Polyamines are present as essential components in all types of cells in multicellular organisms, and they are involved in several different cellular processes and structures. Polyamines are absolutely necessary for the growth of cells and tissues. The three main natural polyamines are putrescine, spermidine and spermine. They are aliphatic organic amines with various carbon chain lengths. At the pH in the cell, they are positively charged with the charge distributed along the entire length of the carbon chain. This distribution of the positive charge enables polyamines to interact in a specific way with polyanionic molecules in the cell, thus affecting their function. Extremely complicated processes involving biosynthesis, catabolism and transport over the cell membrane have evolved that maintain the cellular polyamine homoeostasis. With respect to polyamine homoeostasis during the cell cycle, biosynthesis has so far been mainly investigated. One means of trying to elucidate the role for polyamines during the cell cycle has been by using compounds that interfere with polyamine homoeostasis, resulting in decreased polyamine pools. Studies have also been performed with cell lines lacking biosynthetic enzymes. It has been determined that normal cell-cycle progression does indeed require certain levels of polyamines. However, not only polyamines are needed, but also an intricate web of proteins and enzymes, as well as other molecules. In this chapter, the cell cycle will first be described, together with some important cell-cycle regulatory mechanisms. The polyamines will then be introduced into this context and changes in polyamine homoeostasis during the cell cycle will be presented. Finally, cell-cycle effects caused by polyamine-pool depletion will be discussed as well as future research directions. This overview focuses on effects detected within one cell cycle of treatment with compounds that cause changes in the polyamine pools, something that we define as early cell-cycle effects. Early effects of polyamine-pool depletion may provide better evidence for the direct role for polyamines in cell-cycle regulation.

The cell cycle

The process by which one cell divides into two is fundamental to all growth, whether it is in connection with the developmental growth of an embryo into a young functional organism, further development into an adult, replacement
of injured cells in any life phase of the organism or uncontrolled growth of a cancer cell. The quiescent non-proliferating cell is said to be in a G₀ phase of its life cycle (Figure 1). Unlike terminally differentiated cells, G₀ cells retain the ability to enter the proliferative cell cycle as a response to extracellular factors. The purpose of the active cell cycle is to double the structural elements and functional capacities of the cell so that it can divide into two equal daughter cells at the end of the process. Thus the cell cycle includes two major processes: cell growth and cell division. The cell growth process is divided into three consecutive, biologically defined phases: G₁ (first gap), S (DNA synthesis) and G₂ (second gap), which are followed by the division phase called M (mitosis) (Figure 1). Completion of a specific sequence of metabolic events in each phase enables the cell to proceed to the next phase. The key event of the cell-growth process is the duplication of DNA taking place in S-phase.

Starting from a quiescent state in G₀, the progression of cells into G₁ depends absolutely on the availability of specific growth factors and a suitable extracellular environment (Figure 2). Quiescent cells initially require growth factors to initiate early regulatory processes referred to as competence [1]. There are a number of different growth factors, such as PDGF (platelet-derived growth factor) and EGF (epidermal growth factor) [2–4]. Different cells are stimulated by different growth factors depending on which receptors they display on their surface. Binding of a growth factor to its receptor initiates immediate effects on the activation of signal transduction pathways [e.g. the MAPK (mitogen-activated protein kinase) pathway], resulting in activation of immediate early genes (also known as primary response genes) (Figure 2). Transcription of FOS and JUN has been shown in numerous model systems to be induced within minutes of growth-factor stimulation of cells in G₀ as a
consequence of growth-factor action. Another immediate early gene is MYC, which is induced slightly after JUN and FOS (Figure 2). The rapid protein synthesis-independent induction of immediate early genes is followed by the subsequent protein synthesis-dependent induction of secondary response genes. The induction of secondary response genes is distinct from that of primary response genes in requiring de novo protein synthesis, i.e. cycloheximide treatment inhibits the accumulation of their respective mRNA. Thus the generally accepted model of growth-factor-induced gene expression has two major components: the initial induction of immediate early genes, followed by a compulsory delay allowing translation of their mRNAs to produce the transcription factors that then induce the secondary response genes. However, there are also newer results indicating that gene induction can be divided into three steps: the immediate early, the delayed primary and the secondary response,
where the first two steps do not require protein synthesis. The D-type cyclins belong to the secondary response genes and have a major role during G₁ progression (Figure 2).

D-type cyclins regulate the kinase function of CDK (cyclin-dependent kinase) 4 and 6 (Figure 2). Cyclin D/CDK4,6 complexes phosphorylate different proteins, thereby driving the cell through the G₁-phase, during which very rapid protein synthesis takes place. The cell eventually reaches the R-point (restriction point) (Figure 2) [5]. Once the cell passes the R-point it is committed and has to complete a full cell cycle [1]. At the R-point, the E2F transcription factors are released from the pRB (retinoblastoma protein) and related proteins p107 and p130. E2F are transcription factors required for the transcription of genes involved directly or indirectly in DNA replication [6]. Cells in G₀/G₁ express non-phosphorylated forms of pRB. Cyclin D/CDK4,6 complexes initiate the phosphorylation of pRB [7], whereas cyclin E, together with CDK2, orchestrate continued phosphorylation of pRB, resulting in its inactivation which leads to release of E2F. The release of E2F facilitates the activation of genes critical for S-phase progression.

In the presence of IGF-1 (insulin-like growth factor-1) or insulin, the cell will enter the S-phase and commence DNA synthesis without any additional requirements for growth factors (Figure 2) [8]. In the absence of IGF-1 or insulin, the cell will retain the ability to enter into S-phase for a short while; however, if the hormone peptides are not provided, the cell will exit the S-phase and enter the G₀-phase [9]. Binding of IGF-1 to the IGF-1 receptor activates, e.g. the PI3K (phosphoinositide 3-kinase)/Akt pathway, which is an important survival stimulation pathway necessary for the completion of the cell cycle (Figure 2).

As the cell enters and proceeds through S-phase, cyclin E/CDK2 activity rapidly diminishes and instead cyclin A exerts the activating function of CDK2 (Figure 2). In the middle of S-phase, cyclin A switches partner to CDK1, which in turn switches partner to cyclin B during the G₂-phase. The different cyclin/CDK complexes phosphorylate different proteins consecutively, and in that way irreversibly drive the cell cycle towards cell division.

Working against the cell-cycle stimulatory effects of CDKs are the CDK inhibitory proteins p15INK4B, p16INK4A, p19INK4D, p21WAF1/CIP1, p27KIP1 and p57KIP2 [7,10]. A number of phosphatases are also involved in modulating cell-cycle progression [11]. There are also checkpoint controls, besides the R-point, where the cell can be halted in the cell cycle if different kinds of damage are sensed. Damage induced in DNA after exposure of cells to ionizing radiation activates checkpoint pathways that inhibit progression of cells through the G₁- and G₂-phases, and induce a transient delay in the progression through S-phase [12].

At the end of mitosis, the cell divides into two daughter cells that continue directly into the G₁-phase if the necessary growth factors are provided. There seem to be certain differences in the molecular events at the G₀/G₁ transition.
compared with the M/G₁ transition, mainly resulting in prolonged transition before the commencement of S-phase in the former transition.

**Polyamines and the cell cycle**

After having described the cell cycle and some of the mechanisms that govern cell-cycle progression, changes in polyamine metabolism and polyamine levels that have been observed during the cell cycle will be added into this process. Possible functions of the polyamines during the cell cycle will be discussed in the next section in relation to the cell-cycle changes that follow polyamine-pool manipulation.

A cell in G₀, which is not proliferating, contains lower polyamine levels than when it is progressing through the cell cycle. The sizes of the polyamine pools are determined by biosynthesis, catabolism and uptake over the cell membrane. The increase in polyamine pools that take place when a cell is stimulated to proliferate is mainly due to activation of biosynthesis. Most work on polyamine biosynthesis during the cell cycle has been performed on ODC (ornithine decarboxylase) and AdoMetDC (S-adenosylmethionine decarboxylase).

ODC and AdoMetDC activities are very low in quiescent G₀ cells [13]. The ODC activity increases rapidly when quiescent G₀ cells are stimulated with serum (Figure 2) [14,15]. The ODC and AdoMetDC activities display two peaks during the G₁-to-M progression, one in conjunction with the G₁/S transition and the second in conjunction with the S/G₂ transition and G₂-phase (Figure 2) [16,17]. Thus it seems that when quiescent cells are stimulated to proliferate, three enzyme activity peaks are seen during the first cell cycle, whereas only two are found in actively proliferating cells. This is, however, not totally clear and needs further investigation.

ODC and AdoMetDC are supposed to belong to the group of secondary response genes, as cycloheximide treatment inhibits the massive accumulation of their mRNA after serum stimulation [15]. Actinomycin D treatment inhibits this mRNA accumulation as well. There is, however, evidence that there is a very low level of stable ODC mRNA in quiescent cells, which provides the basis for the peak in activity found early after growth stimulation of G₀ cells [18]. Hogan [18] showed that this early peak in ODC activity did not depend on mRNA synthesis, but only on protein synthesis. Another study has shown that the peak in ODC activity, found when quiescent G₀ cells were stimulated to proliferate by serum, appeared to be regulated differently than the first ODC peak in actively cycling cells [14]. The importance of this early peak after growth stimulation may give rise to speculation. It is well-known that c-myc is a transcription factor for ODC, and that MYC belongs to the immediate early genes [19]. However, polyamines are also known to participate in a positive-feedback loop in the regulation of the MYC gene [20]. Thus this early peak in ODC activity may be necessary for an optimal activation of MYC to ensure an optimal G₀/G₁ transition and optimal c-myc-induced gene transcription (Figure 3).
A general phenomenon of proliferating cells appears to be the peak in ODC and AdoMetDC activities found in conjunction with the G₁/S transition [17,21–23]. The temporal correlation between these activities and the onset of DNA replication suggests that polyamines are important for processes during S-phase, something that will be discussed below. The activity peak found during late S-phase and G₂-phase also seems to be a general phenomenon consistent with a role for the polyamines at the end of the cell cycle [17,22].

Several different mechanisms, such as the level of mRNA synthesis, the translational efficiency of mRNA and the stability of the enzyme, are part of the complex regulation of ODC activity [24,25]. One important regulating mechanism for enzyme stability involves antizyme, which induces ubiquitin-independent proteasomal degradation of ODC [26]. Antizyme activity was shown to fluctuate in an inverse correlation with ODC activity during the cell cycle, thus implicating a role for antizyme in the biphasic activity of ODC during the cell cycle (Figure 2) [27]. It has been suggested that the second peak in ODC activity depends on a cap-independent internal ribosome entry site in ODC mRNA that functions exclusively in the G₂/M-phase [28], a suggestion that has been contradicted by others [29].

Although polyamine biosynthetic activities show distinct biphasic changes during the cell cycle, such clear biphasic changes in polyamine levels have not always been found [17, 22, 30]. It is clear that polyamine pools increase during
the cell cycle and in principle double in size from G\textsubscript{1} to the end of the G\textsubscript{2}-phase (Figure 2); however, different studies show different kinetics in the changes of the pools [17, 22, 30].

The discussion above pertains to mammalian cells; however, similar biphasic changes in polyamine biosynthesis and polyamine levels have been found in synchronized tobacco BY-2 cells [31].

**Polyamine-pool depletion and cell-cycle progression**

Polyamine-pool depletion is achieved in cell lines and *in vivo* by treatment with compounds that inhibit polyamine biosynthesis and/or stimulate polyamine catabolism [32,33]. Since polyamine-pool depletion always results in inhibition of cell proliferation, many of these compounds are being exploited in the treatment of proliferative diseases such as cancer [32–34]. However, the stop in cell-cycle progression in cells treated with compounds that deplete the polyamine pool is not immediate, but instead the cell-cycle progression is gradually more and more affected. In general it takes several cell cycles before cell proliferation is totally halted. The early effects seen in the first cell cycle after the beginning of the treatment are described below in relation to the specific cell-cycle phases.

**G\textsubscript{0}/G\textsubscript{1} transition and G\textsubscript{1} progression**

It has been shown that non-proliferating mouse Balb/c-3T3 fibroblasts in G\textsubscript{0} contained basal levels of polyamines that allowed an apparently normal G\textsubscript{0}/G\textsubscript{1} transition, although polyamine biosynthesis was inhibited [35]. Even polyamine-depleted cells that were stimulated to proliferate by the addition of serum were able to enter the G\textsubscript{1}-phase [35]. The actual G\textsubscript{0}/G\textsubscript{1} transition was not investigated, but the time point at which cells entered S-phase was the same in control and inhibitor-treated cells. These studies were performed using the ODC inhibitor DFMO (2-difluoromethylornithine) [36]. Similar results were found when CHO (Chinese hamster ovary) cells were treated with DFMO [37]. DFMO treatment does not, however, lower the spermine pool [36]. Using the AdoMetDC inhibitor CGP48664 (4-amidinoindan-1-one 2′-amidinohydrazone) [38], it was shown that G\textsubscript{1}-phase progression was not inhibited in CHO cells [39]. Treatment with CGP48664 decreased the spermidine and spermine pools, whereas the putrescine pool increased. When plateau-phase CHO cells were seeded in the presence of the polyamine analogue DENSPM (diethylnorspermine) [40], the progression to S-phase appeared to occur normally, although the levels of all three polyamines decreased [41].

These results and others [42] indicate that cells have a certain basal level of polyamines, which enable them to progress from G\textsubscript{0} through the G\textsubscript{0}/G\textsubscript{1} transition and the G\textsubscript{1}-phase of the first cell cycle, after seeding in the presence of compounds that inhibit polyamine biosynthesis (Figure 4).
A large number of experiments with polyamine biosynthesis inhibitors have revealed an effect of polyamine depletion on the key event of the cell cycle, i.e. the replication of DNA [42]. Schaefer and Seidenfeld [35] showed that when non-proliferating mouse Balb/c-3T3 fibroblasts in $G_0$ were seeded in the presence of DFMO, S-phase progression was impaired after an apparently normal $G_0$ and $G_1$ progression [35]. Laitinen et al. [43] showed impaired S-phase progression during the first cell cycle after seeding CHO cells lacking ODC activity in medium without putrescine. They also showed that polyamine depletion did not significantly interfere with the nucleosomal organization of chromatin during S-phase. Using a bromodeoxyuridine DNA flow cytometric method, it was shown that the $G_1$/S transition was not affected

**Figure 4. Cell-cycle kinetic effects during the first cell cycle after seeding cells in the presence of compounds that prevent the normal cyclic changes in polyamine biosynthesis**

The blue line denotes the R-point.

**$G_1$/S transition and S-phase progression**

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after seeding cells in the presence of compounds that lower the polyamine pools, but that S-phase progression was impaired [37,39,41].

Thus it seems clear that the peak in polyamine biosynthesis occurring in conjunction with the G\textsubscript{1}/S transition is important for a proper S-phase progression (Figures 2 and 4); however, the mechanism is not clear. Polyamines themselves may be important as immediate stabilizing molecules for newly synthesized DNA, as polyamines have shown important DNA-stabilizing functions [44]. A destabilized DNA may present a physical hindrance for effective DNA replication [45]. Using the single-cell gel electrophoresis assay, it was shown that DNA strand breaks were induced within one cell cycle after seeding cells in the presence of compounds that reduced the polyamine pools [46]. This would seem to confirm the DNA-stabilizing function of the polyamines.

It remains to be clarified whether it is only the decrease in polyamine pools that results in the delayed S-phase progression during the first cell cycle after seeding cells in the presence of compounds that deplete polyamines, or whether there are also other molecular changes as a consequence of low polyamine levels that may impinge on the replication machinery. One signal transduction pathway important for successful DNA replication is the PI3K/Akt pathway [47]. It has been shown that DFMO treatment for 24 h reduced the phosphorylation of Akt in neuroblastoma LAN-1 cells [48]. In another study, the phosphorylation of Akt was not affected in human chondrocytes treated with DENSPM for 24 h [49]. These seemingly contradictory results may only be a question of differences in polyamine-pool reduction. Further studies of how the PI3K/Akt pathway and other signal transduction pathways are affected by polyamine depletion before cells enter S-phase is a future arduous research project that is necessary to achieve an understanding of the molecular mechanisms behind the delay.

**G\textsubscript{2} and M-phase progression**

A second peak in polyamine biosynthesis takes place at the end of S-phase and in G\textsubscript{2}-phase. However, the importance of this activity for G\textsubscript{2} and M-phase progression has been less well studied, presumably because no real overt effects have so far been found in the G\textsubscript{2} and M-phases during the first cell cycle after seeding cells in the presence of compounds that lower the polyamine pools. The study by Laitinen et al. [43] mentioned above, showed that polyamines may regulate S-phase progression without, however, affecting the nucleosomal organization of chromatin. They also found that the nucleosomal organization of chromatin was not affected in G\textsubscript{2} and that mitosis was not affected, but merely occurred later due to the prolonged S-phase. Similar results have been observed in breast cancer cell lines and a normal breast epithelial cell line treated with DENSPM [50,51], i.e. despite a prolongation of the S-phase, there was no effect on the length of the G\textsubscript{2}+M-phase. This indicates that when a cell, seeded in the presence of a compound that lowers its polyamine pools,
has traversed the S-phase, albeit at a reduced rate, there are no more negative changes caused by polyamine depletion during that first cell cycle (Figure 4). This notion clearly has to be investigated.

**What happens during the second cell cycle and further on?**

Cells seeded in the presence of compounds that deplete the polyamine pools will finally stop proliferating after one to several cell cycles, depending on the initial polyamine content and the concentration of the compound used. Many results indicate that the final stop point in the cell cycle depends on the status of the p53 gene [50–53]. In the presence of wild-type p53, cell-cycle progression is halted in the G1-phase, whereas cells containing mutated p53 continue cycling; however, at a progressively slower rate with all cell-cycle phases being affected. It has been shown that the induction of cell-cycle arrest in cells containing wild-type p53 involves the p53/p21WAF1/CIP1/pRb pathway [53], and an increase in p27KIP1 has also been found [54]. It also appears that the MAPK signal transduction pathway is involved in the induction of p53/p21WAF1/CIP1/pRb [55].

**Conclusions**

There is no doubt that the polyamines are important for the ability of the cell to keep up optimal rates of cell-cycle progression. Many results point to the S-phase being the most sensitive phase to polyamine depletion. The majority of the research of polyamines in cell-cycle regulation was carried out a number of years ago. With all the new knowledge of different cell-cycle regulatory systems, including signal transduction pathways, as well as a better knowledge of different mechanism of DNA replication, research into the mechanisms exerted by the polyamines in cell-cycle regulation should be intensified. Particularly, it would be a very important finding if any cell-cycle regulatory pathways could be elucidated as being responsible for the polyamine depletion-induced effects on the cell cycle.

**Summary**

- **Polyamine biosynthesis is activated when quiescent G0 cells are stimulated by mitogens.**
- **Polyamine biosynthesis varies bicyclically during the active cell cycle, with one peak in conjunction with the G1/S transition and a second at the end of S-phase and G2-phase.**
- **When cells in G0/G1 are seeded in the presence of compounds that prevent the increases in polyamine pools, the S-phase of the first cell cycle is prolonged, whereas the other phases are unaffected.**
- **Polyamine depletion finally results in cell-cycle arrest after one to several cell cycles, depending on the efficiency of polyamine depletion.**
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