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THINK AGAIN

Insights & Perspectives

The Anti-G0 Manifesto: Should a problematic construct (G0) with no biological reality be removed from the cell cycle? Yes!

Stephen Cooper[1,2]

[1] Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA

[2] The Center for Cell Cycle Analysis (C3A), Longboat Key, Florida 34228, USA

Correspondence
Stephen Cooper, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 481095620, USA.
Email: cooper@umich.edu

Abstract

It is widely accepted that there exists a "resting" or "quiescent" state where a growing cell leaves the cell cycle to enter what is often called the "G0-phase." I propose that there is no biological reality to the "G0-phase." The experimental basis for proposing a G0-phase is re-examined and re-analyzed here showing that the G0-phase is an anthropomorphic construct with no biological reality.

KEYWORDS
cell cycle, G0, G0/G1, G1/G0, G1pm, G1ps, growth arrest, postmitotic phase, presynthetic phase, quiescence

INTRODUCTION

Current status of the G0 proposal

The writing of this paper has been stimulated by the increasing use (Figure 1) of the idea that there exists a "G0"-phase where cells leave the normal cell cycle to enter a resting or quiescent phase. Because I am skeptical of the existence of this proposed "G0" phase, it is important for me to present the arguments re-examining the G0-phase very clearly, strongly, and unequivocally, so that it is possible to see the alternative view regarding the G0-phase proposal. Because critiques of the G0-phase are rare to non-existent, any such critiques have been obscured by the large number of papers citing the G0 idea (Figure 1). I hope that this paper may lead to reconsideration of the G0-phase proposal.

RESULTS

The defining of the G0 phase

The G0-phase of the cell cycle is usually defined as a "resting" phase that a cell enters from the G1-phase of the cell cycle. Entry into the "G0" phase is postulated to occur when cells are subjected to sub-optimal growth conditions that cause the cell to leave the cell cycle and enter an out-of-cycle "resting, G0-phase." Upon completion of the "resting, G0-phase," these cells are proposed to re-enter normal growth by returning to the G1-phase of the cell cycle. Since the G0 or G0/G1-phase has a G1-phase amount of DNA, it is proposed that there is a simple return to the G1-phase when cells are released from growth arrest or slow growth conditions.

Another definition or property of this postulated resting phase proposes that the G0-phase or resting phase is a period in the cell cycle in which cells exist in a "quiescent" state. Furthermore, the G0-phase has been viewed as either an extended G1-phase (hence the awkward but often used titles of G0/G1 or G1/G0) where the cell is neither dividing nor preparing to divide, or a distinct quiescent stage that occurs outside of the cell cycle. (This confusion between naming something G0 or G1 or G0/G1 or G1/G0 is an indication of problems with the recognition of a G0-phase.)

No matter how you define, name, or title the G0-phase (either G0, or G1/G0 or G0/G1), a search of the scientific literature indicates that in recent years there has been a dramatic increase in the use of this term to discuss a resting phase in the cell cycle (Figure 1). This rapid rise in the use of the G0-phase concept is the impetus for this presentation of an alternative view of the G0-phase.

It is the purpose of this paper to show that the G0-phase is an artifact of incorrect analysis of the data.

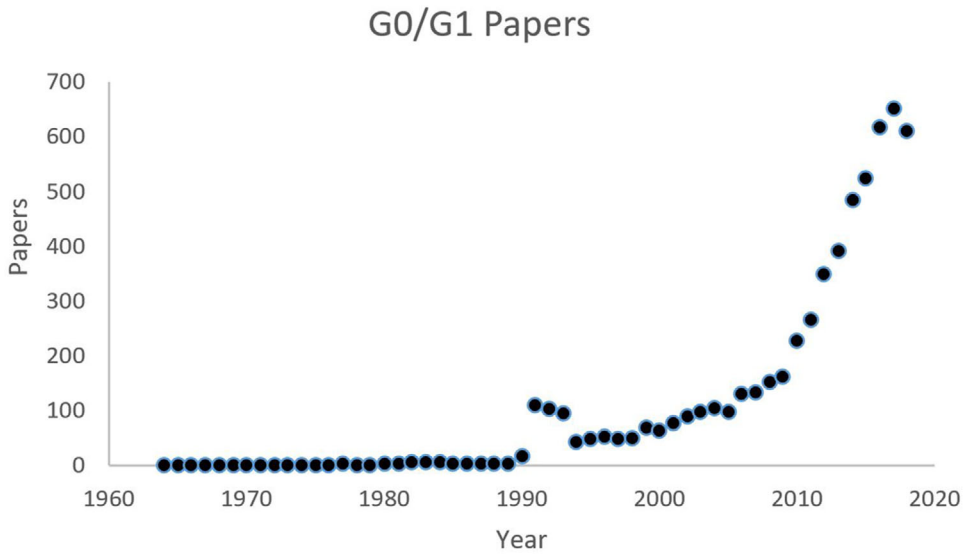

Figure 1 The annual appearance of papers using the G0/G1 terminology. A search of the scientific literature by year was carried out using the G0/G1 or G1/G0 terms as the simple G0 term has many non-biological and non-cell-cycle contexts. The "Web of Science" was the source of the papers.

Why a "manifesto"?

One definition of manifesto is "A public declaration of principles, policies, intentions or views.…" Because I am not writing a "balanced" analysis, I hope that it will be clear that the G0-phase is a problematic concept that should be re-examined in future work on cell growth and the cell cycle.

This paper is not a review, or a synthesis of different ideas, or even a presentation of two sides of an issue. This paper does not present arguments for the existence of a G0-phase, rather I leave this to others who use the concept of the G0-phase in their research. The analysis presented here is a critique of the widely held proposal that there exists a G0-phase or a "quiescent" phase that a cell enters when passing through the cell cycle.

A previous "manifesto" has dealt with the widely used whole-culture methods that are proposed to synchronize cells.[1] This Synchrony-Manifesto[1] points out that it is impossible to synchronize cells by whole culture methods.

Critique of the G0-phase concept

The easiest way to present a critique of the G0-phase is to analyze, using a simple *Gedanken* (thought) experiment, how an acceptance of the G0-phase has arisen and why this wide-spread acceptance is very likely in error.

In bacteria, as in the classic bacterium *E. coli*, it is well known and accepted that the C-period (the time in the bacterial cell cycle when DNA is synthesized and which is equivalent to the S phase of the eukaryotic cell cycle) and the D-period (the time after termination of DNA replication before cell division, and which is equivalent to the G2-phase of the eukaryotic cell cycle) are both relatively invariant in time. When cells are studied over many different growth rates the lengths of the C and D periods are relatively invariant.[2–5] Although the term "relatively" invariant is used here, in practice the C and D periods are quite constant over a wide range of growth rates.[3]

Although the data from eukaryotes are not as comprehensive as those from bacteria (prokaryotes), there is evidence that the S and G2 phases are also invariant in eukaryotes. In one classic and superb example, Liskay and Prescott[6] showed that when a eukaryotic cell growing at two different growth rates were compared, the increase in the interdivision time in the slower growing condition was precisely equal to the increase in the length of the G1-phase of the slower growing cell compared to the more rapidly growing cell. This means that the time required for the sum of the S and G2 phases in these cells was unchanged. Because of the invariance of the time for S and G2 it was clear that the increased interdivision time was related solely to the increase in the fraction of cells with a G1-phase amount of DNA. As the interdivision time of these cells increased, there was no change in the time for S and G2 phases and there was an increase in the fraction of cells with a G1-phase DNA content.

Additional evidence has accumulated to show that the invariance of S and G2 phases is a common phenomenon.[7] As growth rates are slowed, there is a concomitant increase in the G1-phase fraction of eukaryotic cells.

A complete analysis of this comparison of invariant C and D periods and S and G2 periods has been presented as a unified view of the passage of both eukaryotic and prokaryotic cells through the cell cycle.[5]

To pursue this *Gedanken* experiment in more detail, consider a cell that for the most part follows the rule that the S-phase and G2-phase (or the C and D phases in bacteria) are largely or relatively invariant as the growth rate (or the interdivision time) is allowed to vary over a wide range.

Consider a numerical example regarding a eukaryotic cell growing with a 20-hour doubling time with a 10-hour G1-phase, a 5-hour S-phase, and a 5-hour G2/M-phase. To a rough approximation (leaving out statistical variation and the mathematics of the cell-age distribution) we would expect that approximately 50 percent of the cells would show a G1-phase DNA content.

Now slow down growth. To be precise, slow down growth but do not arrest cell growth. Consider that in the new growth condition the cells have a 1000-hour interdivision or doubling time. If the S and G2 phases are time-invariant and remained at 5 hours and 5 hours respectively, the G1-phase would be 990 hours. This is because the total interdivision time of a cell is the sum of the time in the G1-phase, the time in the S-phase, and the time in the G2-phase. If the DNA distributions were measured in these cells it would be observed that approximately 99% of the cells would have a G1-phase amount of DNA. Because the interdivision time for cells with a 1000-hour doubling time would be approximately 40 days, it would be extremely difficult to show that all cells are growing and dividing. To show all cells are growing and eventually dividing one would have to observe the cells continuously (perhaps by using videography of the growing cells) for up to 40 days to see whether the cells all grew and divided during that period of time.

As proposed above, the cells under consideration in this *Gedanken* experiment are still growing but just growing extremely slowly. That is, the cells are not arrested or in any non-growing or "quiescent state." Because it would be seen that 99% percent of the cells would have a G1-phase DNA content the conclusion that would be made, and which is normally made from the G0-phase proposal, is that the cells were "arrested" with a G1-phase amount of DNA or are in a "G0-phase" of non-growth or quiescence.

This is the common explanation for such experiments such as putting cells in low serum which would presumably lead to slowing the growth rate of cells. But as can be seen in this simple analysis, there is no arrest and the cells are merely growing slowly. The dominance of the G1-phase cells leads to the proposal that the cells are now arrested in a G0-phase, or as often put, in a G0/G1 (or G1/G0)-phase.

In support of the ideas presented here, it is important to recall the classic experiments of Rubin and Steiner[8] who studied the growth of cells that were either overgrown or starved of serum. They concluded:

> ''Up to 96% of the cells in post confluent cultures growing in conventional medium become labeled upon continuous, prolonged exposure to 3H-thymidine. Seventy-eight percent of the cells in serum-deprived cultures growing at a very low rate become labeled. These and other considerations suggest that the inhibition of cell multiplication by high population density or serum deprivation is caused by a lengthening of the time cells remain in the pre-replicative G1-period rather than by shifting cells into a qualitatively distinct G0-period.''

This is a clear and simple example of how a large number of papers have proposed or accepted the existence of a G0-phase. Simply explained, slowing growth leads to an increase in the fraction of cells with a G1-phase amount of DNA but these cells are not arrested and the increase in the G1-phase DNA content cells are merely the result of relatively invariant S and G2 phases.

Some have proposed that a more multi-sided rather than a one-sided view of the G0 problem would be better for scientific discussion, suggesting that I should write more about the "utility" and the "applicability" of the G0 concept.

My thought about this suggestion is that I cannot think of a single utilitarian need for the G0 concept, or any applicability of the G0 concept. That slower growth leads to a longer G1 phase is simply the result of the expansion of the interdivision time increasing the G1 phase more than increasing the S and G2 phases.

Thus, a cell with a 25-hour interdivision time with G1, S, and G2 phases of 15, 5, and 5 hours respectively, when the growth is slowed to perhaps 100-hour interdivision time, the resulting phase distribution would possibly be 80, 10 and 10 hours making it thus appear that there is a longer G1 phase which is then termed a G0 phase.

This example shows why it is not necessary to term the longer G1 phase a G0-phase.

Critique of the Zetterberg-Larsson model

Zetterberg and Larsson[9,10] have summarized a number of experiments proposing that the G1-phase of the mammalian cell division cycle could be subdivided into two phases. Cells from the early phase could enter the G0 phase, and those in the later phase could not. The point of division between these two phases was associated with the restriction point.[11] I will present a reinterpretation of those experiments and provide an alternative explanation for the concept of the G0-phase and division of the G1-phase into different parts at the restriction point.

Zetterberg and Larsson[9,10] used time-lapse video recording to study the growth of eukaryotic cells. They watched cells growing normally over many generations (by playing back the video tape) and thus they knew how long in time each cell was from birth by division and thus each cell could be assigned a particular cell-cycle age. By noting when a particular cell arose by division they could assign to each cell, at a particular time, the extant cell-cycle age of that cell in the culture.

Zetterberg and Larsson[9] then removed serum from the growing cells for one hour (1 h). After one hour without serum, the normal serum concentration was restored. Time-lapse observation of the treated cells continued after serum restoration. The time until each of the serum-starved cells divided was measured. Changes from the normal time of division (i.e., if there were no serum removal) were observed and were correlated with "cell-cycle-age" at the moment of serum removal.

They observed that the youngest cells in the culture when serum was removed, those within 3.5 h of birth (said to be in the G1pm or "postmitotic" phase), had an 8-hour delay in cell division. Cells past this 3.5 h mark (G1 cells in this phase are called G1ps or presynthetic, that is, closer to the start of DNA synthesis) had a normal, undelayed division. Cells in the S and G2 phases also had a normal, undelayed division.

The division delay observed in the G1pm cells was approximately 8 h, significantly longer than the 1-h serum removal time. Zetterberg and Larsson proposed that cells early in the G1 phase (G1pm) are different from cells in the latter part of the G1 phase (G1ps). They proposed that the G1pm cells can make a decision to leave the cell cycle and enter the G0 phase. G1ps cells cannot enter the G0 phase. To explain the division delay in the G1pm cells they proposed that 8 hours are required for the cells to return from the G0-phase to the normal cell cycle. The Zetterberg-Larsson results led to the proposal that there is a particular point in the G1 phase at which cells change from G1pm to G1ps, and more importantly, that only cells in the G1pm part of the cell cycle can enter a "G0-phase."

While the Zetterberg-Larsson experiment appears to define a "G0-phase," a subsequent analysis of growth led to an alternative explanation which has been published in extreme detail.[12]

Briefly, the Zetterberg-Larsson results postulate that there would be a re-ordering of the order of cell divisions. Thus, considering only the first division delay, those cells would divide out of order. This change in the order of cell ages violates what I have proposed as an immutable law of cell-cycle growth, the "Law of Conservation of Cell Age Order."[13] (An alternative name for this law is the "Law of Cell Age Order Invariance.")

On the law of conservation of cell age order

To give a simple explanation of the "Law of Conservation of Cell Age Order," consider that at any time in a culture there are cells of different ages. Label these different age groups 1, 2, 3, 4, and 5 in order of ascending age. The cells of group 1 are the youngest cells, with ages from 0.0 to 0.2, the cells of group 2 have ages from 0.2 to 0.4, and so forth. Although the groups are divided into equal fractions by cell age, there are not equal numbers of cells in each group. There are more cells in the youngest group than in the oldest group because of the exponential age distribution. The "oldest" cells in the culture at time zero will divide to give the cell number increment during the first 20 percent of a division cycle, and the "youngest" cells will divide during the last 20% of the division cycle. If we eliminate, for this *Gedanken* experiment, statistical variability of cell interdivision times, the predicted order of cell division during unperturbed exponential growth (i.e., without the intervention of a short period of serum starvation) would be that cells would divide in normal order, with the oldest cells first, and then the mid-cycle cells, and finally the youngest cells dividing. The cell number increases by divisions occurring first in group 5 (the oldest cells at time zero), then groups 4, 3, 2, and 1, in the next cycles and this order repeats in the daughter and granddaughter cells.

What would one expect from the setback in cell division by cells in the G1pm group of cells? Consider the experiment where they gave a short incubation (one hour) in serum-free medium so that only cells in in a specific age group are now "set back" into G0, from which it is postulated that it takes 8 h to leave the G0-phase. It would be predicted that the "normal" cell age order would be altered. Certain cells that would have divided between other cell divisions at a particular cell age would not divide at that time and the normal cell age order would be lost.

Thus, considering that over two generations the cell age order of division would be 5,4,3,2,1,5,4,3,2,1, the altered sequence would be 5,4,3,2,5,4,1,3,2, as the youngest cells during treatment would be dividing later than normally expected.

This can be defined as a break from the normal cell age order and a violation of the Law of Conservation of Cell Age Order. (For a detailed analysis, see.[12])

This apparent violation of the "Law of Conservation of Cell Age Order" is in fact only an apparent violation. That is because when all cells in a culture are treated identically the "Law of Conservation of Cell Age Order" proposes that it is impossible to produce an altered order of cell divisions. How is this "violation" of the Law of Conservation of Cell Age Order prevented? The return to the normal cell age order occurs when the cells that were not delayed in the first division have a delay in the next cell division. Thus, the sequence of cell divisions would be 5,4,3,2,…1,…5,4,3,2,1, because the cells not delayed in the first cell cycle would be delayed in the second cell cycle. That is, after a normally timed cell division, these cells that appeared "unaffected" by the serum starvation would then have a division delay in the next cell cycle. This was predicted to occur based on the ideas present in the Law of Conservation of Cell Age Order.[12]

By some wonderful concatenation of events, the editors of the Journal (Bioessays) where the initial analysis of the Zetterberg-Larsson Experiments and the proposal of the Law of Conservation of Cell Age Order was published, asked Dr. Peter Fantes to comment on, and critique this proposal of a general law, the Law of Conservation of Cell Age Order, and the ideas discussed regarding the Zetterberg-Larrson Experiment. Unexpectedly, the choice of Fantes to comment on the published article led to an exciting contribution to the discussion by Fantes and in fact Fantes gave a superb support of the ideas on the Law of Conservation of Cell Age Order and the critique of the G0 concept (addendum to[12])

The wonderful result of the invitation to comment led Fantes to point out an experiment in the extant literature that I was not contemporarily aware of, that in fact there was a delay in the second division in those cells that did not have an initial division delay.[10] That is, those cells in the G1ps phase of the cell cycle that did not have an initial delay in division were predicted to have a delay in the next division. To quote Fantes in detail:

> However, Larsson *et al*.[10] also show that cells too late in the starvation cycle to be mitotically delayed in the first cycle have an extended second cycle. This is, **paradoxically**, (*emphasis added)* in agreement with Cooper's contention that cells other than the delayed G1pm cells are affected by serum starvation. The kinetics show that cells later in the first cycle (and therefore nearer to the next S phase, and not showing a first-cycle delay) at the time of starvation are more delayed in the second cycle. It is therefore not clear whether Cooper's proposal is consistent with the combined data of

> Larsson et al.[9,10] The most extreme expression of Cooper's ideas, the 'Law of Cell Age Invariance', which states that no batch treatment of cells can reverse the order of division, is attractive as a general rule, but remains to be critically tested by experiment. A reanalysis of Larsson's observations and their presentation in a way suitable for Cooper's analysis might be highly informative.
>
> Larrson, Zetterberg and Engstrom[10] report in a subsequent paper, not discussed by Cooper, that cells delayed for one mitosis by starvation undergo a shortened second cycle. In other words, there is evidence for cell-cycle order conservation or time homeostasis. The extent of shortening is less than the original delay, as seen in other recovering systems, but it is possible that these cells will catch up over several cycles with untreated control cells. In this case the cell-number curve for starved cells may eventually coincide with the control curve, and not resemble that predicted by Cooper.

I point out this comment by Fantes because one of the hallmarks of a good theory is that the theory can predict results that were not expected. That the prediction of the Law of Conservation of Cell Age Order is "paradoxical" should be replaced by the suggestion that my prediction has been supported by experiment that thus there is an important support for the Law of Conservation of Cell Age Order.

This interesting and productive denouement supporting the proposal made here should be considered when thinking about the proposed existence of a G0-phase.

On the cells used to study the cell cycle

An objection to the proposal made here is that I consider only cells grown in culture, and not cells growing within an organism. Perhaps, it is suggested (by a reader of this paper in an earlier version), that looking at the pattern of growth within an organism would yield results suggesting the presence of a G0 phase. I have written forcefully[14] that the best (and in my view, scientifically reproducible method) is to use cells grown in steady state in culture rather than cells observed in vivo where the complications of influence by adjacent cells would make these reults unreliable and un-reproducible.

A word about "quiescence"

Quiescence is the reversible state of a cell in which it does not divide but retains the ability to re-enter cell proliferation. Some adult stem cells are maintained in a quiescent state and can be rapidly activated when stimulated, for example, by injury to the tissue in which they reside.

It is widely observed that cells in a growing organism stop growing and remain in a non-growing state with a G1-phase amount of DNA. This observation has been proposed as a "natural" support of the G0-phase because cells are now arrested and do not grow and remain in this proposed "G0-state."

The critique of the G0-phase concept can be applied to this observation of "quiescence" by noting that this non-growing state of cells is not a "cell-cycle phenomenon" but merely a "mass growth cessation" with no cell cycle control or relationship.

I have proposed (along with others) that cells initiate DNA synthesis (S-phase) when a certain amount of cell growth has occurred. When cells cease making mass, the resulting cells end up all with a G1-phase amount of DNA. Thus, when cells are prevented from making mass those cells that are in the S phase (making DNA) or in the G2 phase (finished replication of the genome but not yet divided) will all proceed to divide. The cells in S phase will complete replication (once replication is started it is proposed that replication will be completed) and thus the cells in the G2-phase and the S-phase eventually divide and produce newborn cells with a G1-phase amount of DNA. These cells join with the pre-existing cells that were in G1 phase and which did not initiate DNA replication under conditions of cessation of mass growth.

It is of interest to look back at the very first proposal of the G0-phase which which resulted from the study of liver cells before and after partial hepatectomy. Thus, considering human beings, the liver remains about the same size throughout adult life. The liver does not grow in size. But when radioactive thymidine is added to normal liver it is observed that there are a few cells that are making DNA because there is incorporation of radioactive thymidine. How can this happen? I conjecture the following scenario. Out of the millions and billions of liver cells there are cells that happen to accidentally die and dissolve and disappear from the liver. This removal of cells from the liver now allows all other cells in the liver to grow and produce a very slight increase in mass. Those cells in liver that were very close to starting DNA replication will now reach some "initiation mass" and start replication.

When, however, part of the liver is removed (partial hepatectomy) the liver is now able to regenerate new liver mass. Thus, there is now a massive growth of new cells and there is now a much larger incorporation of radioactive thymidine into the liver cells. This "experiment" was used to suggest that the non-growing cells were in "G0-phase" and could be released from this G0-phase by stimulation of growth.

The interpretation of this liver experiment as suggesting the existence of a G0-phase is merely the result of non-growth of mass until the liver is stimulated to grow. There is no indication of as mechanism of G0-phase entry.

CONCLUSION AND SUMMARY

This article is written to ask workers in the field of cell-cycle studies to reconsider the proposal of a G0-phase. I suggest that these terms, G0, or G0/G1, or G1/G0 be discarded because the proposed "out-of-cycle" resting or "G0" phase does not actually exist, has no

applicability or utility, and thus hinders clear thinking about the cell cycle.

ACKNOWLEDGEMENTS

Over the 57 years that I have been working on, and thinking about, the cell cycle (starting with my working in the laboratory of Ole MaalØe in 1963), I have been influenced by many scientists and collaborators. I cannot convey my thanks directly to all who have given me ideas and support, so let me mention some of the key members of the field of cell-cycle studies that have been important in my coming to the ideas presented in this paper. Thus, I thank, in no particular order, Charles Helmstetter, Alan Leonard, Moselio Schaechter, Frederick Neidhardt, Patrick Dennis, Peter Fantes, Eric Boye, Jay Keasling, Joachim-Volker Höltje, and others too numerous to mention. And I thank two anonymous reviewers who have made suggestions that have vastly improved this paper.

CONFLICT OF INTEREST

The author states that he has no conflict(s) of interest.

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