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Linking the cell cycle to cell fate decisions

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Abstract

Pluripotent stem cells retain the ability to differentiate into a wide-range of cell types while undergoing self-renewal. They also exhibit an unusual mode of cell cycle regulation, reflected by a cell cycle structure where G1- and G2-phases are truncated. When individual pluripotent stem cells are exposed to specification cues, they activate developmental programs and remodel the cell cycle so that the length of G1 and overall cell division times increase. The response of individual stem cells to pro-differentiation signals is strikingly heterogeneous, resulting in asynchronous differentiation. Recent evidence indicates that this phenomenon is due to cell cycle-dependent mechanisms that restrict the initial activation of developmental genes to the G1-phase. This suggests a broad biological mechanism where multipotent cells are ‘primed’ to initiate cell fate decisions during their transition through G1. Mechanisms underpinning commitment towards the differentiated state and its relationship to the cell cycle are discussed.

Keywords

Cell cycle; pluripotency; cell fate; stem cell

Individual stem cells respond to differentiation cues with asynchronous kinetics

When pluripotent stem cells (PSCs) are exposed to differentiation-inducing signals, individual cells activate developmental pathways with asynchronous kinetics (Figure 1a). This phenomenon applies to all multipotent cells but an understanding of this phenomenon at the molecular level has been elusive. One anecdotal explanation for this observation has been that local differences in cell density creates variations in factor concentrations that in turn, support differentiation and self-renewal to varying degrees. Recent work now indicates however, that asynchronous differentiation and initiation of cell fate commitment is linked to the cell cycle. The central observation driving this concept is that G1 cells respond to

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specification signals more rapidly than cells at other cell cycle positions. This confers the ability of G1 cells to activate differentiation programs almost immediately following stimulation (1-3) and manifests in S-, G2- and M-phase cells activating differentiation programs with delayed kinetics. This delay is directly related to the time taken to transition into G1-phase, when developmental programs are activated. The model predicting this has been confirmed using the Fluorescence Ubiquitin Cell Cycle Indicator (Fucci) system (2), using the kinetics of developmental gene activation as a read-out (Figure 1b). The molecular mechanism controlling phase-specific cell fate commitment is not completely resolved and will be a major focus of this review (see Outstanding Questions Box).

The idea that cells initiate fate decisions in G1-phase is not a new concept. For example, cells make the decision to cycle or withdraw from the cell cycle during every round of cell division by a mechanism known as 'restriction point' (R-point) control (4). The R-point serves as a molecular switch that controls cellular 'decisions' relating to continued division or entry into the quiescent state (Go). This pathway involves the integration of extracellular mitogenic signals with the cell cycle machinery, converging on cyclin-dependent kinase (CDK) activity, the retinoblastoma protein (RB) family and E2F target genes (5). Other examples where cell fate decisions are coupled to G1 transition include mating type switching in budding yeast (6), the replication origin decision point (7) and size control mechanisms (8). In most of these cases, the general theme is that extracellular signals activate signaling pathways within the cell, resulting in the coupling of cell cycle-dependent transcriptional responses to cell fate decisions.

Why should cells preferentially make cell fate decisions from G1-phase and not other cell cycle phases (see Outstanding Questions Box)? Although speculative, it is feasible that transcriptional programs linked to cell identity can be rapidly reset following exit from M-phase. The transition from M-phase to G1 is associated with dramatic changes in nuclear architecture (9) including reformation of the nuclear envelope, chromosome decondensation and extensive chromosome repositioning in three dimensional space (10, 11). In the presence of pro-differentiation signals, G1-phase would potentially establish a favorable epigenetic and nuclear architectural environment that allows developmental programs to be activated (Figure 2). This general idea is supported by numerous observations. For example, the potential for a gene to be activated following M-phase is dependent on its relocation to the nuclear periphery in G1 (12). In the context of cell fate decisions, lineage-specific genes would be reorganized and recruited to the nuclear lamina by a mechanism dependent on the temporal signaling environment. This is likely to be associated with the dynamic nature of chromatin organization in early G1 cells and its continued refinement during the transition to S-phase (13). This is consistent with observations that topologically-associating domains (TADs) and promoter-enhancer loops are established in G1 (14). In this scenario, cell fate specification signals and the cell cycle machinery would act on permissive chromatin in G1 to elicit cell fate decisions.

These observations point towards a set of general principles that make G1-phase special with regards to cell fate choice (Figure 2). First, they indicate that G1 represents a permissive phase for initiating cell fate decisions through control of 'decision' genes at the transcriptional level. Second, they indicate that cells are unresponsive to inductive cues

outside of the G1-phase. MEK/ERK, PI3K/AKT, TGF β /BMP and WNT activation do not appear to be cell cycle regulated in pluripotent cells (15) and so it is unlikely that periodicity in signaling pathways can account for G1-specific effects. Why target genes are less responsive to developmental signals outside of G1 remains unclear. The overarching question that remains then is why are cell fate decisions restricted to G1-phase? This question will be tackled later in this review.

Cell cycle regulation in pluripotent stem cells

To understand how pluripotent stem cells initiate cell fate decisions in G1, it is first important to understand how the cell cycle differs between PSCs and differentiated, somatic cell types. Pluripotent stem cells of peri-implantation stage mammalian embryos undergo rapid cell divisions that drive the expansion of embryonic volume and cell number during this stage of development. By counting cell numbers during pre-implantation and early post-implantation stages of development it has been estimated that generation times of PSCs in the murine embryonic epiblast can be as brief as 4.4 hours (16). Then, as gastrulation proceeds and as early cell fate decisions are made, cell cycle length increases to 16 hours or more (16, 17). This general trend has been reported throughout the animal kingdom and has been particularly well-documented in flies, fish and frogs where rapid cell/nuclear division is a general feature of uncommitted cell populations during early embryogenesis (18-22). Recent work has provided some mechanistic insight into how the cell cycle lengthens during the mid-blastula transition (MBT) in *Xenopus* embryos where changes in the nuclear to cytoplasmic ratio impact concentrations of factors required for DNA replication (23). The resulting dilution effect delays the onset of S-phase by extending G1-phase. Changes in the nuclear to cytoplasmic volume ratio have long been suspected to be a determinant of cell cycle length in early development so this represents an important observation. Whether this mechanism holds for mammalian embryos still needs to be determined. Another key issue is whether rapid division is just a convenient mechanism to quickly increase embryonic cell number prior to gastrulation or, if it is something inherent to the mechanisms associated with the pluripotent state. Several laboratories have tackled this question using cultured PSCs as a model. Here, the general conclusion is that rapid cell proliferation rates are not an absolute pre-requisite for maintenance pluripotency (24, 25).

Flow cytometry analysis of cells from murine epiblasts has identified an unusual cell cycle structure that provides some hints as to why PSCs exhibit rapid cell cycles (22). Approximately 60% of pluripotent cells in the embryonic epiblast are actively replicating DNA (S-phase) while only a small proportion are in G1 and G2. The mechanism associated with rapid cell division in the early mammalian embryo therefore seems to be linked to rapid transition through the G1- and G2-phases. The high proportion of cells actively replicating DNA may mislead the reader into thinking that S-phase is unusually long but in fact, it is comparable to that of a somatic cell. Instead, the gap phases are significantly truncated relative to differentiated cells. In most somatic cell types, gap phases comprise a major fraction of the cell cycle length and importantly, is the period when the decision to arrest or proliferate occurs. In contrast, pluripotent cells of the embryo lack a fully formed G1-phase and advance rapidly into S-phase following completion of M-phase. Quite dramatically, cell cycle duration changes at around the time of gastrulation when pluripotent cells become

committed to one of the three germ layers (mesoderm, ectoderm or endoderm). During this stage of development the gap phases become extended, accounting for increased cell division times. This coincidence between rapid proliferation rates and broad differentiation potentiality again suggests a mechanistic link between the cell cycle gap phases and mechanisms of cell fate commitment.

Peri-implantation mammalian embryos are difficult to work with for many purposes so unraveling the mechanisms associated with cell cycle restructuring during early development has been difficult. This problem has been alleviated however, by using cultured PSCs that have a similar mode of cell cycle regulation to their in vivo counterparts. Models that have been used to understand the link between cell cycle control and pluripotency include embryonal carcinoma cells (ECCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and epiblast-like stem cells (EpiSCs). Similar to their in vivo counterparts, cultured PSCs exhibit a high proportion of cells in S-phase and a low proportion in G1 (22)- the latter being indicative of a short gap phase. Similarities in cell cycle properties between pluripotent cells in vivo and in vitro argue that ECCs, ESCs, iPSCs and EpiSCs are suitable models for studying cell cycle-related embryonic events. Over recent years this concept has been validated to the point where we now have a good understanding of events underpinning the cell cycle and how this may relate to cell fate outcomes in the early embryo.

As discussed already, changes in proliferation rates that occur during early stages of cell fate commitment can be explained by increased time spent transitioning through G1. This raises numerous questions about why a short G1 is an inherent feature of pluripotency, especially because PSCs are known to initiate cell fate decisions from here (1, 26-28). If PSCs are susceptible to differentiation signals in G1, it follows that truncation of this gap phases would reduce the propensity for differentiation. Numerous studies have established the molecular changes underpinning cell cycle remodeling during differentiation. Initial reports focusing on murine ESCs (mESCs) showed that the retinoblastoma tumor suppressor (RB) is inactive and then upon differentiation, this critical proliferation-regulator is activated in a cell cycle-dependent manner (22, 29). These cells are somewhat similar to PSCs from the inner cell mass (ICM) of pre-implantation stage embryos but exhibit subtle differences. Unlike somatic cells, cyclin-dependent kinases (CDKs) in mESCs exhibit no phase-specific activity (22). Instead, they are constitutively active throughout the cell cycle. The only CDK that shows any periodicity in mESCs is the mitotic oscillator CDK1/cyclin B; all other CDK-cyclin complexes show unusually high, constitutive activity (22). This observation explains inactivity of the RB pathway, the constitutive activity of downstream E2F-regulated target genes and why the cell division of mESCs is mitogen independent. Instead of the RB gene being mutated, as is frequently the case in tumor cells, RB activity is biochemically inactivated by sustained CDK-dependent phosphorylation in mESCs. CDK inhibitory molecules (CDKIs) are absent in mESCs, also contributing to rapid cell division and unrestrained CDK activity (30, 31). Overall, these findings indicate that mESCs multiply through unrestrained cell division and why they are refractory to external growth-modulatory signals such as contact inhibition and growth factor depletion. This is highly reminiscent of tumor cells where cell cycle controls are often uncoupled from extrinsic mitogenic signals.

As indicated above, the initial studies of cell cycle regulation in PSCs focused on mESCs maintained in fetal calf serum (FCS) and leukemia inhibitory factor (LIF). What these studies did not take into account was that multiple forms of PSCs exist during peri-implantation development and that cell cycle control could potentially differ between alternate pluripotent states. In recognition of this, interest has more recently shifted to the characterization of different stages of pluripotency such as those representing primitive ectoderm, the pluripotent cell from early post-implantation stage embryos. In the murine system these cells are referred to as epiblast-like stem cells (EpiSCs [32, 33]) or more broadly, 'primed' PSCs. Human ESCs (hESCs) belong to the 'primed' PSC category and like EpiSCs (34) are developmentally equivalent to primitive ectoderm. A third sub-type of pluripotent cell, commonly referred to as 'naive' or 'ground state' PSCs, has been isolated from human and mouse sources and closely resemble PSCs from the ICM (34). With regards to the cell cycle structure of PSCs, 'primed' cells have short gap phases and spend the majority of time in S-phase. Upon differentiation, the cell cycle is restructured so that gap phases are extended and division rates slow down. Although all PSCs studied share these cell cycle features independent of source, the molecular mechanisms driving the cell cycle machinery seems to vary (35). In contrast to FCS/LIF maintained mESCs, hESCs display phase-dependent CDK activity, CDKIs are expressed at higher levels and RB protein is phosphorylated in a cell cycle dependent manner (35), suggesting that 'primed' cells have an intact R-point pathway. Cell cycle analysis of 'naive/ground-state' PSCs is surprisingly limited but it is anticipated that they have a cell cycle structure similar to that of other PSCs. In summary, different classes of PSCs have a similar cell cycle structure comprising short gap phases but details of their molecular regulation differ somewhat. The available information suggests that activation of cell cycle-dependent CDK activity and normal growth control mechanisms are initiated when the 'primed' pluripotent state is established. The link between cell cycle and cell fate commitment has been primarily based on studies in 'primed' cells but it will be interesting to establish if transition from the 'naive' to 'primed' state is also coupled to G1 transition.

Signaling pathways connect the cell cycle to developmental genes in G1-phase

Several reports have described the process by which PSCs initiate the differentiation program from G1-phase (1, 27, 28) but two recent reports (2, 15) go on to address the molecular mechanism underpinning this. Both of these reports utilize the Fucci system so as to avoid problems associated with the use of synchronizing drugs and centrifugal elutriation. One of these reports (2) describes how SMAD2,3 promotes differentiation by its recruitment to developmental genes during G1-phase (Figures 2,3). As cells progress through G1, SMAD2,3 is removed from target genes and translocates into the cytoplasm by a cyclin D-dependent mechanism. This places cell cycle regulated biochemical activities at the center of developmental gene activation in PSCs. Furthermore, G1 is partitioned so that mesoderm and endoderm commitment occurs in early G1, when SMAD2,3 is nuclear, while ectoderm commitment occurs in late G1; coinciding with nuclear exclusion of SMAD2,3. This model shows that mesoderm and endoderm lineage commitment is temporally separated from ectoderm differentiation. The partitioning of G1 into periods of preferential commitment to

different germ layers was completely unanticipated and provides an elegant way of initiating cell fate decisions based on cell cycle position.

A second report took a different approach by evaluating transcript levels for developmental genes in the cell cycle of PSCs. Most developmental genes are 'bivalent' meaning that they are marked by overlapping domains of H3K4 and H3K27 tri-methylation near their respective transcription start sites (36). Surprisingly, the second report showed that a high proportion of developmental genes are weakly transcribed during G1 in hPSCs (15). This observation is counter-intuitive because developmental genes are silenced in PSCs by a polycomb-dependent mechanism (36), but this study indicates that transcriptional 'leakiness' occurs during G1. This implies that developmental genes are transcriptionally primed in G1 and that this phase represents a 'window of opportunity' for differentiation (Figures 2,3). When signaling networks switch from supporting self-renewal to differentiation, periodicity of transcription for the developmental genes is amplified while retaining some cell cycle-dependent periodicity. In addition, 5-hydroxymethylation of cytosine (5hmC) increases in parallel to increased transcription during G1, suggesting a link between cytosine methylation status and the activation of developmental genes in the cell cycle. These reports provide important information about the mechanisms governing cell cycle dependent fate decisions in PSCs. The coupling of external signals to the cell cycle machinery and cell fate decisions are reminiscent of other cell fate decisions made in G1; the R-point being a good example. G1-specific binding of SMAD2,3 at developmental genes in G1 (2), their transient transcriptional activation (15) and coincident epigenetic changes (15) may be due to the reorganization of the genome that occurs following M-phase and/or due to activation of specific CDK activities associated with G1 progression (Figure 2). Even though chromatin may continue to be permissive for gene activation in S- and G2-phases, the clearance of transcription factors from developmental genes in S-phase precludes their activity outside of G1 and hence, accounts for their cell cycle regulation. With this information an understanding of G1-specific cell fate decisions is now within sight.

G1 length and cell fate decisions in other stem cell populations

The general idea that G1 length is connected to the self-renewing state is reiterated by studies in neural stem cells. During neurogenesis of the ventricular zone, for example, the length of G1 increases from 3 hours to 13 hours and this has a corresponding impact on total cell cycle length (37). Shortening the length of G1 by ectopic elevation of CDK activity delays neural commitment while the converse is true when CDK activity is reduced (38). In neurogenesis, the length of G1 therefore controls the balance between self-renewal and differentiation (39, 40). Taking the parallel between neural stem cells and PSCs one step further, early neurogenic genes such as NGN2 are specifically expressed in late G1 (41, 42). This is reminiscent of results in PSCs where developmental genes and associated transcription factors are preferentially activated in the G1-phase (2, 15). Also in common with PSCs, CDK4/cyclin D complexes have also been implicated in G1-specific fate commitment of neural stem cells (2, 39). This suggests a common mechanism that couples G1 to cell fate decisions in different stem cell populations. There is ongoing work characterizing the cell cycles of other stem/progenitor cells, such as hematopoietic and

pancreatic stem/progenitor cells (43-45), but it is unclear whether G1 length is connected to mechanisms of differentiation (see Outstanding Questions Box).

Concluding remarks

Several examples have been used to explain how signaling pathways converge on the cell cycle machinery to regulate developmental genes and execute cell fate decisions. The lower part of Figure 3 (Key Figure) illustrates how different cell cycle-dependent mechanisms could serve to activate and restrict the activation of developmental genes to G1. The 'commitment window' (Figures 2,3) indicates the time when cells are responsive to extracellular signals that impact cell fate determination. The activity of transcription factors and chromatin remodeling enzymes that target developmental genes would overlap with this period. Both of these seem to be important in PSCs and neural stem cells. A permissive chromatin state can be envisaged to persist from early G1 through to mitosis, the former of which is associated with chromosome de-condensation and remodeling and, increased transcriptional activity (46). G1 CDK2/4 activity starts in early-mid G1-phase and persists through G1 into early-S-phase. Although the details have yet to be determined this implies that an inhibitory step could close the window of potential for developmental gene activation when cells enter S-phase. This loss of potential also involves the inactivation of CDK activity. Clearly, all of these mechanisms must be tightly coordinated to ensure a robust mechanism for control of cell fate. This returns us to the question of why developmental decisions are mechanistically tied to a specific phase of the cell cycle. Once an understanding of all facets of regulation depicted in Figure 3 is obtained, we may be in a position to fully answer this question.

Acknowledgments

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Outstanding questions

- How are developmental genes activated during G1 phase?

Pluripotent stem cells activate developmental genes in G1 but the mechanism linking cell cycle progression to gene activation, cell signaling pathways and differentiation are still unclear.

- Why is G1 phase special with regards to cell fate commitment capacity?

This review highlights work pointing towards a role for cell cycle specific changes in chromosome architecture. It assimilates data in the literature pointing to why developmental programs and major changes in gene expression can be initiated from G1.

- Is the link between G1 transition and cell fate commitment a conserved feature of multipotent cells besides pluripotent stem cells and neural stem cells?

It seems likely that mechanisms discovered in pluripotent cells and neural stem cells will apply to other multipotent stem cells. More work is required to address this question.

Trends Box

- Pluripotent and neural stem cells have a short G1 cell cycle phase. Committed cells extend their G1 phase and cell cycle length.
- Stem cells initiate fate decisions by activating developmental genes in G1 phase. In pluripotent stem cells, developmental genes are cell cycle regulated and respond to extracellular differentiation cues in G1.
- Developmental signaling pathways connect to target genes in G1 phase, allowing for activation of transcriptional programs that direct cell fate.
- Cyclin-dependent protein kinases (CDKs) control the activation of developmental genes in G1. CDKs target transcription factors such as SMAD2,3.
- The epigenetic landscape changes at developmental genes in G1. This is likely to be important for initiation of developmental programs.

Figure 1. Initiation of the differentiation program in pluripotent stem cells is coupled to cell cycle progression

(a) Stem cells exposed to cell fate specification cues differentiate as an asynchronous wave.

(b) The asynchronous differentiation program can be accounted for by the activation of developmental genes in G1-phase of the cell cycle. Cells in G1 respond rapidly to differentiation cues whereas cells in S-, G2- and M-phase experience a delay, indicated by the kinetics of transcriptional activation.

Figure 2. Chromatin resets in G1-phase to open a window of opportunity for cell fate commitment

In the S-, G2- and M-phases, stem cells are refractory to differentiation signals. Upon entry into G1, cells become permissive for cell fate specification, respond to extracellular signals and activate developmental genes required for progression towards one of the three embryonic germ layers. This 'window', or 'primed state', occurs when topologically-associating domains (TADs) reform after M-phase, when potentially active genes become proximal to the nuclear periphery and conceivably coincides with formation of promoter-enhancer loops structures (partly adapted from [14]). Transition through G1 also coincides with activation of G1 cyclin-dependent kinase activities (CDK2/4) and the recruitment of transcription factors (TF) to developmental genes. The 'window of opportunity' for activation of developmental genes in G1 is shown ('commitment window'). From S-phase to the end of M-phase, TFs disengage from target genes and close the developmental window ('unprimed state').

Figure 3. “Key Figure” Multiple cell cycle-regulated mechanisms contribute to the cell cycle-dependent activation of developmental genes

Top three rows: Potential mechanisms for G1-dependent ‘priming’. **(i)** Cell cycle-regulated binding of transcription factors to developmental genes. This is suggested from work in pluripotent (2) and neural stem cells (41, 42) and indicates the engagement of transcription factors with developmental genes in G1-phase. The green square and orange oval shapes represents G1-specific binding factors at developmental genes (for example, SMAD2,3 or NGN2). Other factors (red circles) may bind throughout the cell cycle and serve to recruit cell cycle regulated DNA binding factors. **(ii)** Cell cycle-dependent changes in the epigenetic landscape around developmental genes. This could include histone modifications or DNA methylation changes. One example is the conversion of 5-methyl cytosine (5mC) to 5-hydroxymethyl cytosine (5hmC) at developmental genes in G1 (15). **(iii)** Changes in chromosome architecture around developmental genes. One scenario could be the recruitment of enhancers to proximal promoter regions by DNA looping. Some or all of these changes are likely to be orchestrated by the activity of cyclin-dependent protein kinases and their transcription factor targets. **(iv)** Cell cycle phases corresponding to features above (i-iii) and below (v-ix). Bottom five rows: temporal activity of factors that potentially impact G1- specific cell fate commitment. **(v)** Window of time when conditions are suitable for activation of developmental genes (‘commitment window’). **(vi)** Time in the cell cycle when transcription factors bind and activate developmental genes. **(vii)** Period of the cell cycle when chromatin is broadly permissive for developmental decisions. **(viii)**. Periodicity of CDK activity responsible for the recruitment of transcription factors to cell fate genes in G1. **(ix)** It is hypothesized that an inhibitory signal or inactivation of CDK2/4 is required for decommissioning of developmental genes as cells transition through early S-phase.