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12 Cell cycle checkpoints and DNA damage response in early mouse embryo*

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Abstract

Early mouse development can be defined as the period starting from fertilization and ending at gastrulation. This period is characterized by important variations in cell cycle regulation, which correlate with key developmental transitions. In this review, we summarize the data concerning cell cycle parameters, cell cycle checkpoints and DNA damage response during early embryogenesis. In particular,

*This article is dedicated to the memory of Charles Babinet

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we illustrate how mutant mice have provided an outstanding contribution to our knowledge of the differential requirement of checkpoints during the successive steps of early development. We also describe how experimental challenging of these checkpoints by various stress conditions have revealed several specialized embryonic strategies of genome maintenance that works during that period. Finally, we present the data obtained in embryonic stem cell lines as they represent a valuable experimental system to study the molecular mechanisms underlying the unique features of cell cycle regulation in early embryonic cells.

Introduction

During cell cycle progression, the transitions between the different phases rely on highly elaborated processes that checkpoints the correct finalization of each phase-specific task before proceeding to the next one. In most cells, these checkpoints are regulated by the extracellular context and are tightly linked to damage signalling pathways. Accordingly, their dysfunctions are associated with accumulation of genomic alterations leading to cell death or cellular transformation. Early mouse development is characterized by important variations in numerous cell cycle parameters, which correlate with known developmental transitions. It begins by a period, called preimplantation development, which is mainly devoted to the production of extraembryonic tissues. By the time of implantation, the mouse embryo is composed of cells having adopted very different strategies in terms of regulation of cell cycle progression. Both gene targeting experiments and experimental challenging of cell cycle checkpoints have been instrumental in determining the specificities of cell cycle regulation as well as the unique properties of various checkpoints in early mouse embryos. In this review, we first present the specificities of each step of mouse early development in term of cell cycle parameters. Then, in the following sections, we summarize the results concerning cell cycle checkpoints and DNA damage response in early embryos. Finally, in a last section, we summarize the studies performed on cell cycle regulation in embryonic stem (ES) cells as such analyses provide key information about the mechanisms at works during early embryonic development.

Cell cycle characteristics during early mouse development

From fertilization to the first mitosis

Ovulated oocytes are arrested in metaphase of second meiotic division. Fertilization triggers the resumption of meiosis and the extrusion of the

second polar body. Maternal and paternal chromosomes are set apart in pronuclei that migrate towards the center of the zygote. Before that, the packed condensed paternal chromatin is remodelled into a decondensed and transcriptionnally competent state. Indeed, in contrast to the nucleosomal organization of the maternal DNA, the sperm DNA presents a toroidal structure where histones are replaced by protamines, leading to a chromatin structure being sixfold more compact than metaphase chromosomes (for review, see [1]). Within the first hour post fertilization, the protamines are removed from the decondensing sperm nucleus and replaced with maternally provided histones. During the next six hours, different remodelling complexes modify the epigenetic marks present on the paternal DNA leading to a striking asymmetric pattern between male and female pronuclei (for review, see [2]). First, the paternal DNA is specifically and actively demethylated [3]. Second, histone H4 is preferentially hyperacetylated in the male pronucleus [4]. Lastly, the preferential association of the histone chaperone HIRA with the paternal DNA leads to its enrichment in the histone variant H3.3 [5]. These differences in the DNA organization level are thought to be responsible for the asymmetric transcription and replication kinetics of both pronuclei (for review, see [6]).

Replication patterns are tightly linked to chromatin structure. Hence, actively transcribed regions of euchromatic DNA are early replicated during S-phase with numerous replication foci scattered in the nucleus whereas heterochromatic regions are replicated in mid and late S-phase in a more discrete pattern [7]. In the zygote, DNA replication takes place during pronuclei migration and starts about seven hours after fertilization. It is first detected in the male pronucleus and lasts 4 to 8 hours [8-10]. The replication forks present temporally restricted patterns and both pronuclei replicate asynchronously with an S-phase that appears to be longer in the female pronucleus [11]. This suggests that the different epigenetic marks might influence the different usage or timing of replication origins activation during S-phase. Accordingly, Aoki and Schultz showed a stimulatory effect of histone deacetylases inhibitor trapoxin on zygotic peripheral DNA replication that is independent of transcription [12]. Another potential consequence of epigenetic mark asymmetry in the zygote is the onset of the first wave of transcription that starts first in the male pronucleus during the late S-phase [13]. Additionally, a higher rate of transcription is supported by the male compared to the female pronucleus and could result from a more permissive organization of paternal chromatin organization [4].

Twenty hours post fertilization, both sets of chromosomes start to condense and align at a common metaphase plate. The first embryonic mitosis lasts 120 min, that is almost twice as long as the second one, which

takes approximately 70 min [14]. This difference in the duration of mitoses between the zygote and the 2-cell stage is reflected by the plateau of CDK1-Cyclin B activity and thus by the persistent phosphorylation of histone H1 that is observed during the first mitosis. Durations of prometaphase and metaphase plate formation are similar during the first and second cleavages indicating that the prolongation of the first mitosis relies on a prolonged metaphase. Interestingly, the lengthening of the metaphase is independent of the Spindle Assembly Checkpoint (SAC) activity and could be the result of the perdurance of proteins implied in the metaphase II arrest which is probably also independent from SAC activity [15].

Embryonic cleavages from 2-cell stage to blastocyst formation

The second division lasts approximately 20 hours and is characterized by a prolonged G2 phase. G1 phase is extremely short (1-2 h) [16], S-phase lasts approximately 6 hours, and G2 phase is very long (12 to 16 h) [17-19]. Strikingly, during this unusually long G2 phase occurs the major activation of the zygotic genome (ZGA) [20]. This promotes a dramatic reprogramming of gene expression pattern, coupled with the generation of novel transcripts that were not expressed in oocytes [21]. Activation of embryonic transcription is mediated by maternal proteins but, so far, only a small number of maternal-effect genes have been identified in mammals (*Dnmt1o* [22], *Dnmt3l* [23], *Fmn2* [24], *Hsf1* [25], *Mater* [26], *Npm2* [27], *Rgs14* [28], *Stella/Pgc7* [29], and *Zar1* [30]). Some of these genes are possibly involved in ZGA. Hence, embryos from *Hsf1* null females undergo the minor phase of zygotic genome activation that occurs at the end of the 1-cell stage but fail to progress to the 2-cell stage [25]. Similarly, embryos from *Mater* [26] or *Zar1* null females [30] arrest around 2- to 4-cell stages and do not undergo ZGA. How these genes regulate the complete activation of the zygotic genome remains to be elucidated. Chromatin organization in the zygote has also been shown to depend on a maternal effect gene, *Nucleoplasmin* 2 (*Npm2*). Npm2 was originally characterized in Xenopus as a nuclear oocyte specific protein that, *in vitro*, facilitates sperm protamines removal, nucleosome assembly and replication of the paternal genome [31]. In mouse, oocytes derived from *Npm2* deficient females do not condense chromatin around the nucleolus and present only uncoalesced nucleolar-like bodies. This absence of chromatin condensation does not seem to alter the global transcriptional silencing in fully-grown oocytes [32]. However, *Npm2* deficient females are subfertile and the majority of the embryos produced by these females fail to progress beyond the 2-cell stage [27]. The cause of the developmental failure of these embryos remain unclear and further analysis will be required to characterize

the function of NPM2 in the nuclear organisation of pronuclei chromatin as well as in other post-fertilization events.

The following four divisions are more homogeneous in terms of duration (Total=10-14 h; G1=1-2 h, S=7 h, G2/M=1-5 h) (for review, see [33 , 34]). At the 8-cell stage occurs the process of compaction characterized by a dynamic change in adhesive and polarization properties of the blastomeres. At this stage, the embryo proceeds to a second wave of transcription, the Mid-preimplantation Gene Activation (MGA). Many genes actively transcribed during MGA are involved in commitment and early differentiation events of the first embryonic lineages [21, 35].

Embryonic implantation and gastrulation

At the blastocyst stage, external cells will give rise to the trophectodermal (TE) cells whereas internal cells will constitute the inner cell mass (ICM) which will further segregate into the epiblast and the primitive endoderm. ICM and TE cells differ not only by their molecular signatures but also by their cell cycle parameters. Indeed, ICM cells are characterized by a high proliferative capacity contrary to a sub-population of TE cells, the trophectodermal giant cells (TGC), which undergo endoreplication cycles and can acquire more than 500 haploid genomes [36, 37].

Endoreplication cycle represents a high degree of cell cycle specialisation. It is characterized by a succession of S-phase and a gap-like phase, without intervening mitosis. It requires a specific regulation of replication origin firing and the down regulation of pathways promoting G2 and M phases. In this regards, analysis of the different key regulators of cell cycle progression in TGC revealed a continuous degradation of Geminin and Cyclin A2, probably due to a constitutive activation of the E3 ligase complex so called Anaphase Promoting Complex or Cyclosome (APC/C), thereby allowing a continuous loading of the replication specific helicase complex MCM2-7 and preventing mitotic entry respectively [38]. Genetic analysis unravelled the essential role of cyclins E in the regulation of S-phase in TGC. Indeed, loss of function of both *Cyclin E1* and *Cyclin E2* induces developmental defects of the placenta due to TGC endoreplication defects [39, 40]. One possible explanation is that cyclins E can be compensated by cyclins A in the embryo proper and not in TGC. The reason why this substitution cannot occur in trophoblast is unknown but at least two hypotheses can be proposed. The first one is that cyclins A are not expressed in TGC. Alternatively, if expressed, they might be able to substitute to cyclins E with respect to their E2F activating function, i.e. the phosphorylation of Rb, but not for their more direct S-phase promoting role

in endoreplication such as loading of MCM complexes (for review, see [41]). Considering their key role in TGC, regulation of cyclins E levels is expected to be critical for endoreplication in these cells. Accordingly, elevated cyclins E levels have been reported in TE mutant cells displaying defects of endoreplication. For examples, mutations affecting components of ubiquitin and ubiquitin-like modifications pathways such as *Cul1* [42] or *Cul3* [43], two members of the SCF complexes, *Csn2* [44], a subunit of the COP9 signalosome and *Uba3* [45], the catalytic subunit of the NEDD8-activating enzyme, induce high levels of cyclins E and early embryonic lethality. Apart from cyclins E, other pathways appear to be essential for the regulation of endoreplicative cycles. Indeed, disruption of *Mat1*, which encodes for a subunit of the CDK7-CyclinH-Mat1 complex implicated in the regulation of RNA polymerase II phosphorylation, leads to an embryonic lethality around the time of implantation associated with an arrest of TGC endocycles [46].

Whereas the endoreplication cycles produce highly polyploid TGCs that will contribute to the formation of extraembryonic tissues, ICM cells of the blastocyst remain diploid and proliferate to form the epiblast that will give rise to the embryo proper. By the time of implantation, cell division pace of epiblast cells increase dramatically during the egg-cylinder stage at 6.5 days *post-coitum* (*dpc*). During gastrulation, analysis of the cell cycle parameters is particularly limited due to the permanent movement of the cells and the acquisition of new cellular identities. Nevertheless, careful analysis performed on mice and rat gastrulating embryos revealed the existence of a proliferative zone in close proximity to the primitive streak region characterized by remarkably fast cell cycles (2-3 h) [47, 48]. To date, the characterization of this cell population, noticeably concerning its cell cycle parameters and regulatory mechanisms, has not been described so far.

Checkpoints specificities in early embryos

Cell cycle checkpoints are essential to ensure the correct transmission of the genetic material to the daughter cells. Briefly, they aim to 1) sense that the external environment conditions are not detrimental for the propagation of life, 2) protect DNA from *de novo* mutations, 3) ensure that parental DNA is correctly copied and 4) control that the two daughter cells inherit a complete set of chromosomes. Thus, cell cycle checkpoint possess several activities as to sense and detect abnormalities, delay the entry into cell cycle phase until the preceding phase is not properly achieved and stimulate the activity of various repair response pathways. As illustrated in the previous section, early mouse development is characterized by important modifications

of cell cycle parameters that are likely to reflect differences in checkpoint activities. In this section, we review how phenotypical analyses of mutant mice for components of the core cell cycle machinery as well as the various checkpoints have contributed to our understanding of cell cycle regulation during early embryogenesis. In the following section, we will then focus on the specificities of the DNA damage response during early embryonic development.

The spindle assembly checkpoint

The SAC is essential to ensure the fidelity of chromosome segregation during mitosis by inhibiting the onset of anaphase until all chromosomes are attached via their kinetochores to the microtubules in a bi-oriented manner (for review, see [49]). Several evidences suggest that SAC is functional during early mouse development. This is based, on the one hand, on the localization of checkpoint proteins (as MAD2 or BUBR1) to unattached kinetochore (evoked in [15, 50, 51]) and, on the other hand, on the observation that an efficient arrest in metaphase can be induced upon exposure of embryos to drugs that interfere with microtubule dynamics [52-55]. Moreover, genetic mouse models strongly support the idea that SAC is functional in early embryos since disruption of genes encoding members of these pathways generally gives rise to an embryonic lethality phenotype at either a pre- or peri-implantation stage. For example, invalidation of genes encoding for the kinetochore structure proteins (*CenpA* [56], *CenpC* [57], *CenpE* [58]) or for microtubule-kinetochore attachment stabilizer proteins (*Incenp* [59], *Survivin* [60]) leads to abnormal mitotic figures and to a peri-implantation lethality. A similar phenotype was observed following inactivation of genes encoding for SAC core components (*Bub1* [61], *BubR1* [35], *Mad2* [62] and *Rae1* [63]); for all these mutants, failure to arrest in metaphase upon drug-induced spindle microtubule disruption could be demonstrated.

The SAC pathway is based on the regulatory inhibition of the effector complex, APC/C, which carries an E3 ubiquitin ligase activity indispensable to initiate anaphase through the degradation of several proteins. Thus, it is not surprising to observe a metaphase arrest in blastocyst embryos deficient for the APC/C complex subunit APC10 [64 , 65]. Deficiency for APC2, another subunit of APC/C also leads to an early embryonic lethality ; however the phenotypic characterization of the corresponding embryos has not been performed [66]. APC/C activity during mitosis is mainly regulated by its co-activator CDC20, which is sequestred by the unsatisfied SAC. Interestingly, a gene-trap insertion into *Cdc20* locus induces, at the homozygous state, a

metaphase arrest at the 2-cell stage [67]. Finally, disruption of *Emi1*, another inhibitor of APC-CDC20 activity leads to an early preimplantation arrest around the morula to blastocyst transition [68]. As soon as all kinetochores are properly attached in a bi-orientation manner to the spindle, the APC/C complex is activated to target to proteosomal degradation the two main inhibitors of anaphase onset: Securin and Cyclin B1. Until APC/C activation, Securin is associated to Separase preventing its cleavage activity on cohesins. Furthermore, Separase activity is also inhibited by the phosphorylation mediated by CyclinB1-CDK1 complex. Upon ubiquitin-dependent degradation of Cyclin B1 and Securin, a complete activation of Separase activity leads to cohesins degradation and thus to sister chromatid disjunction. Contrary to *Securin*-null mice which are normal and fertile [69, 70], mutant mice for *Separase* die around preimplantation period [71, 72].

Altogether, these studies indicate that the SAC is operational early during development. However, it should be noticed that, except in the case of *Cdc20* mutants, the onset of phenotypic defects occurs relatively late, by the blastocyst stage at the earliest. The presence of maternally inherited products that can transiently compensate for the absence of zygotic product might account for such observation. Alternatively, since the 6th first divisions occur without the presence of a centrosome (for review, see [73]), it may also reflect a transition in cell division that occurs at the end of preimplantation development.

Plasticity of CDKs pathway

During cell cycle progression, different cyclin-dependent kinases (CDK) complexes are sequentially activated to ensure the correct transition between G1, S, G2 and M phases. CDK2, CDK3, CDK4 and CDK6 are involved during interphase progression whereas CDK1 drives the cell through mitosis. For the two last decades, genetic mouse models have demonstrated that these key cell cycle regulators as well as their co-activator subunits, the cyclins, are highly dispensable for mouse embryonic development (for review, see [74]). This is in part due to functional redundancy as suggested by the observation that combined genes inactivation lead to a phenotype more severe than single knock-out. For example, *Cdk4* and *Cdk6* double mutants fail to develop beyond 14.5 to 16.5 dpc whereas simple knockouts are viable [75, 76]. Moreover, embryos lacking all interphase Cdks (*Cdk2*, *Cdk3*, *Cdk4* and *Cdk6*) are able to develop until mid-gestation due to compensatory mechanisms involving CDK1[77]. Indeed, CDK1 is able to bind all cyclins and to form biochemically active complexes, therefore highlighting the high degree of plasticity of CDKs [78]. Finally, CDK1 appears to be the only

CDK essential for early embryonic development since its deletion induces a developmental arrest before the 2- to 4-cell stage [77]. Thus CDK1 can compensate for the absence of one or several CDKs but its absence cannot be compensated by others CDKs.

Similar observations have been done concerning the cyclins and their role during development. For example, whereas the single mutations of *Cyclin E1* or *E2* are viable, the double mutants die at mid-gestation because of a loss of placenta tissues [39]. Analogous results were observed for D-type cyclins family [76]. So far, only inactivation of *Cyclin A2* [79 , 80] or *Cyclin B1* [81] leads to an early developmental arrest. Mouse embryos lacking *Cyclin A2* complete preimplantation development but die soon after implantation [79]. Of note, these mutant embryos develop normally from the 4-cell stage to early postimplantation in the absence of detectable *Cyclin A2* gene product, indicating that Cyclin A2 is critically required only by the time of implantation [80].

The Rb pathway

As previously mentioned, the first cleavages are characterized by a very short G1 phase and the absence of G1/S checkpoint. Consistently, preimplantation development is relatively independent of exogenous growth factors (for reviews, see [82 , 83]). In somatic cells, activation of several signal transduction pathways, such as Ras and Myc, by environmental stimuli regulates the activity of CDK4 and CDK6, which in turns, control G1/S transition. Both kinases, in association with cyclins D, regulate Rb phosphorylation and dissociation from the transcription factor E2F that, when released, can transactivate genes essential for S-phase initiation such as cyclins E. *Rb*, as well as the other pocket proteins encoded genes *p107* and *p130*, has been shown to be dispensable for early embryonic development. Indeed, *p130* and Rb deficiencies cause embryonic lethality during the second half of gestation and *p107* deficient mice are viable and fertile, although growth retarded [84-86]. Interestingly, the defects observed in *p107* and *p130* deficient mice or embryos are abolished following a single cross with C57BL/6 mice, indicating that requirements for these two pockets proteins is highly dependent on the genetic background [87, 88]. During preimplantation development, the status of Rb in the control of G1/S transition has been monitored by two different groups. Iwamori and collaborators described barely detectable levels of Rb mRNA by RT-PCR in the zygote and 2-cell stage embryos and conclude that low levels of Rb are necessary for shortened G1 phases. Accordingly they observed that forced expression of Rb by plasmid injection into zygotes induced a developmental

arrest before the morula stage [89]. In another study, Xie and collaborators observed Rb phosphorylation at Ser795 at the 2-cell stage and throughout preimplantation development [90]. Therefore, both low level of RB protein together with its constitutive phosphorylation may account for the inability of the RB pathway to inhibit cell cycle progression during preimplantation development. It should be noted that no functional analysis of p107 and p130 during preimplantation development has been reported to date, and therefore, whether these pocket proteins are regulated by similar mechanisms in early embryo remains to be determined.

The DNA damage response during early development

Although studied for many years, the regulation of DNA damage response during early development is poorly characterized. It is known for a long time that mouse preimplantation embryo is highly sensitive to ionizing radiation and that radiation-induced lethality is higher during that period than during any other period of development [91]. Recently, different studies aimed at determining onset of the different DNA damage responses in early embryos has been performed (see below). These studies lead to the idea that some of the DNA damage response pathways are defective during early embryogenesis, probably because of the incompatibility between the high proliferation rate of embryonic cells and the necessity to delay cell cycle progression to repair the damages. According to these reports, the nature of the response largely relies on the quantity of damages and on the stage of development at which they are induced. In this regard, it appears that a switch in the activation of the repair pathways occurs at the morula stage since the DNA detection pathways seem to be defective before this stage.

Crosstalk between male and female pronuclei

Analysis of zygotes produced by *in vitro* fertilization with irradiated sperm revealed that cell cycle progression can proceed without apparent G1/S and G2/M checkpoints. Nonetheless, the first round of replication is delayed by few hours and this lengthening is dependent of p53 [92]. As in the mammalian zygote genomes of the incoming sperm and residing oocyte form two separate pronuclei, damage can be delivered through irradiated sperm while the responses can be analyzed in the damage-free female pronucleus. Cell cycle analysis of sperm-irradiated zygote revealed that a crosstalk exists between male and female pronuclei since an S-phase delay was observed in both pronuclei. Furthermore, this observation demonstrates that suppression of DNA synthesis in mouse zygote is due to a true checkpoint activity rather than a mechanical block of progression of the replication machinery at the

damage site. Because S-phase delay observed in the sperm-irradiated zygote is dependent on p53, the status of the p21 cyclin-dependent kinase inhibitor was analyzed. Indeed, p21 is a potent negative regulator of G1/S and G2/M transitions that can be activated by p53. Interestingly, the cell cycle delay observed is independent of p21 as sperm-irradiated *p21*$^{-/-}$ zygotes present also an S-phase delay. Therefore, p53-dependent suppression of DNA replication in sperm-irradiated zygote must rely on other mechanisms. A likely hypothesis would be that p53 acts through Replication Protein A (RPA). Indeed, RPA is, together with DNA polymerase and primase, a core component of the replication machinery and p53 has been shown to bind RPA and suppress its ability to associate with single stranded DNA [93]. Whether such pathway is at works in sperm-irradiated zygote and blocks replication fork movement remains to be demonstrated.

Late onset of damage detection

DNA damages response in early embryos is probably partial since the S-phase delay observed in sperm-irradiated zygote is clearly not sufficient for all DNA breaks to be efficiently repaired. Hence, gamma-irradiated 1- and 2-cell stages embryos continue to divide but fail to reach the blastocyst stage. This ability to proceed to two or three divisions whereas DNA breaks are still present may rely on a defective detection of DNA damages [92]. In somatic cells, detection of DNA breaks usually involves the phosphorylation of the PI3 kinases ATM, ATR and DNA-PK. In turn, these kinases phosphorylate multiple substrates including the histone variant H2aX (referred hereafter as γ-H2aX) within minutes after irradiation. γ-H2aX localized to damaged sites and is involved in the recruitment of the DNA repair machinery (for review, see [94]). Examination of mouse embryos after gamma irradiation revealed that H2aX phosphorylation is not observed in the 1- and 2-cell stages embryos, while it can be easily induced in oocytes. Following irradiation of 1- or 2-cell stages embryos, γH2aX-dependent detection of DNA breaks can only be visualized from the 4-cell stage onwards [95]. Strikingly, immunofluorescence analysis revealed that the absence of γH2aX in zygotes and 2-cell stage embryos is not due to the absence of ATM or DNA-PK that are present in early embryos and activated after irradiation. This suggests that other factors, necessary for H2aX phosphorylation by these kinases might be absent before the 4-cell stage or, alternatively, that an inhibitor of these pathways is present [95 , 96].

The insufficient function of checkpoints in 1- and 2-cell stage embryos may be caused by an absence of proteins involved in the cell cycle arrest upon ZGA. According to this hypothesis, microarray analysis of

preimplantation stage mouse embryos failed to detect *p21* mRNA until embryos reach the 8-cell stage [97]. *p21* induction at this time of development might explain why sperm irradiated mouse embryos stop to divide at the morula to blastocyst transition. Indeed, *p21*$^{-/-}$ sperm-irradiated embryos lack this early developmental arrest. Instead, they exhibit extensive apoptosis in the ICM at the late blastocyst stage together with a high level of developmental failure after implantation. In contrast, *p53*$^{-/-}$ sperm-irradiated embryos fail to implant suggesting that the protective function of p21 operates at a later stage than p53. According to these data, Adiga and collaborators proposed that early embryogenesis is associated to a progressive hierarchy in the DNA damage responses starting from a p53-dependent p21-independent S-checkpoint in zygote, to a p53-dependent p21-dependent G2/M checkpoint at E2.5 and finally to an apoptotic response in the ICM of blastocyst embryos [97].

Most of DNA repair pathways are dispensable before implantation

Analysis of genetic mouse models concerning DNA damage detection and repair pathways have led to the conclusion that these pathways are not essential to preimplantation development (for reviews, see [34 , 98]). Indeed, requirement for genes of theses pathways is manifested at the earliest at the end of the preimplantation period in ICM cells. In the corresponding mutant embryos, normal cell cycle progression until the end of preimplantation period might be due to the presence of maternal stores. Alternatively, requirement for these pathways may change drastically by the time of implantation. Even after implantation, some pathways appear to be dispensable since mice deficient for either *Atm* [99] or *Chk2* [100] are viable. In contrast, embryos lacking *Atr* [101] or *Chk1* [102] die soon after implantation and exhibits high degrees of DNA fragmentation. Finally, the two main pathways for double strands break repair seem to be differentially required during early development. Hence, while non-homologous end-joining is extremely active at the 1-cell stage [103], inactivation of several genes implied in this process like *DNA ligase IV* [104], *DNA-Pkcs* [105] or *Xrcc4* [106] does not lead to an early embryonic lethality. In contrast, early embryonic lethality was observed following inactivation of several genes involved in DNA double strand break repair such as *Nbs1* [107] and *Rad50* [108].

Remove rather than repair the damaged cells

Later on during development, the onset of gastrulation is associated with a dramatic increase in the rate of proliferation and growth. As already mentioned, the cell cycle is extremely shortened during this period. In somatic cells, DNA damage response involves a cell cycle arrest necessary for damages to be repaired before entering to the following phase. If the cell cannot deal with a too large amount of damages, apoptotic pathways are activated and the cell is eliminated. Just before gastrulation, embryos are highly sensitive to DNA damage. Hence, embryos irradiated with low dose undergo a massive p53- and ATM-dependent apoptotic response in the embryo proper without any apparent alteration of cell cycle progression. The high proliferation rate observed at this stage of development is certainly not compatible with cell cycle arrest or delay and therefore, the main surveillance mechanism consists in removing damaged cells in order to prevent their contribution to critical lineages in the embryo [109].

Embryonic stem cells: a model for embryonic checkpoints analysis

Several pluripotent stem cell lines have been established from early mammalian embryos. Twenty-seven years ago, mouse ES cell lines were first derived from blastocyst outgrowth [110 , 111]. Since, non-human primate and human ES cell lines were obtained using similar procedures [112 , 113]. Finally, in the last months, derivation of pluripotent epiblast stem cell (EpiSC) lines from the late epiblast layer of postimplantation mouse and rat embryos has been reported [114 , 115]. While these various cell lines exhibit differences (growth factors requirement, gene expression pattern, …) that are likely to reflect their diverse temporal origins, they can be considered as the *in vitro* counterparts of ICM/epiblast cells. Thus, they provide a valuable experimental system to study the molecular mechanisms underlying the unique features of cell cycle regulation of these cells.

Senescence and G1/S transition deficiencies in embryonic stem cells

In terms of response to DNA damage and cell cycle arrest, ES cells differ markedly from differentiated somatic cells, as do epiblast cells. Hence, ES cells proliferate at a fast rate (cell cycle length of ~10 h) and exhibit a short G1 phase. Contrary to most lineage-committed cell-types, ES cells proliferate without apparent limit, are not subject to contact inhibition and, to date, there is no means of inducing cell-cycle arrest and quiescence in these cells. When put in culture, most mammalian cells undergo a limited number of cell

divisions before entering a state of irreversible proliferative arrest termed replicative senescence [116]. The number of cell divisions in culture or Hayflick-limit varies with cell type and species and is controlled through the activity of three essential signaling pathways: the telomerase pathway, the p53 pathway, and the Rb pathway (for review, see [117]). The unique regulation of these pathways in ES cells may account for the fact that ES cells are one of the rare non-transformed cell types able to bypass senescence (for review, see [118]). Hence, ES cells present high levels of telomerase enzyme expression and activity [113 , 119]. This is in sharp contrast with differentiated somatic cells, in which the lack of telomerase activity leads to a progressive telomere shortening and subsequently cellular senescence. Interestingly, telomerase-deficient mouse ES cells cease to grow after 460-480 divisions strongly suggesting that telomerase activity contributes to the unlimited proliferation properties of ES cells [120]. In differentiated somatic cells, telomere shortening activates the p53-mediated response. In ES cells, this pathway seems inactive because of the cytoplasmic sequestration of p53 and a low efficiency of p53 translation to the nucleus in response to ribonucleotides depletion or DNA damage [121].

Rapid self-renewal of ES cells is supported by a shortened G1 cell cycle phase [122]. As a consequence, these cells are unable to undergo normal G1/S checkpoint activation after DNA damage. In differentiated somatic cells, one of the key events in the G1 phase is the phosphorylation of the pocket proteins (Rb, p107, p130) by the CDK/cyclin D and E complexes. Mouse ES cells are deficient in cyclin D-associated activity and rely on CDK2 activity to drive G1/S transition [123]. Components of the G1/S checkpoint, such as Rb are permanently inactivated and the hypophosphorylated form of Rb is barely detectable during the very short G1 phase (1.5 h) [124 , 125] (for review, see [126]). At the onset of differentiation, the proliferation rate decreases with an extension of the G1 phase, associated with the establishment of the Rb/E2F pathway regulating the G1/S checkpoint. Interestingly, combined inactivation of all three genes of the Rb family [$p107^{-/-}$, $p130^{-/-}$/, $Rb^{-/-}$ triple knockout (TKO) cells] do not seem to compromise the proliferation of ES cells [125, 127]. However TKO MEFs, like wild type ES cells, escape replicative senescence, are not subject to contact inhibition, are immortal and lack G1 checkpoint indicating that a defective Rb pathway is a central aspect of the peculiar cell cycle properties of ES cells.

A high mutation frequency in the pluripotent embryonic cells would be detrimental, not only to the individual but also to the species. To prevent such detrimental condition, different mechanisms for maintaining genomic

integrity operate in these cells as well as in ES cells. For example, ES cells are hypersensitive to ionizing radiation (IR) and other DNA-damaging agents, and this is partly due to their lack of G1/S checkpoint. Indeed, since cells with damaged DNA can proceed into S-phase, damaged DNA is replicated leading to an unsustainable mutational burden that induces a strong apoptotic response. As already mentioned, p53 is inoperative in ES cells and therefore the p53-dependent G1 arrest after DNA damage is not fully functional. Recently, an alternative mechanism that contributes to the absence of a G1 arrest after IR exposure of ES cells has been identified [122]. In unperturbed cycling cells, transition from G1 to S phase is facilitated by the activity of the CDC25A phosphatase that removes inhibitory phosphates on the Cdk2/cyclin E complex. After DNA damage by IR in differentiated somatic cells, ATM phophorylates CHK2, which in turn, phosphorylates CDC25A causing its ubiquitin-mediated degradation. In ES cells, this pathway involving ATM, CHK2, CDC25A and CDK2 appears to be compromised because of the lack of available functional CHK2, which is sequestered at centrosome and unavailable to phosphorylate CDC25A phosphatase. Noticeably, ectopic expression of CHK2 is sufficient to allow the phosphorylation of CDC25A, the activation of downstream targets, and the restoration of a G1 arrest. Importantly, it also protects from gamma irradiation-induced apoptosis [122], suggesting that sequestration of CHK2 to the centrosome contributes to the facilitated apotosis of damaged ES cells.

An additional mechanism to prevent the ES cells with damaged DNA from self-renewal has been proposed. Lin and collaborators have shown that p53 binds to the promoter of *Nanog*, a gene required for ES self-renewal, and suppresses *Nanog* expression after DNA damage [128]. This, in turn, drive ES cells to differentiate, leading to the activation of the classical p53 mediated DNA-damage response, and the elimination of the compromised cell. p53 would therefore have a novel role in maintaining genomic stability of ES cells through the DNA damage-induced differentiation of ES cells.

Uncoupling the mitotic checkpoints and apoptosis in ES cells

Surprisingly, ES cells exhibit an unusual tolerance towards chromosomal aberrations such as loss of heterozygosity, uniparental disomy, and aneuploidy. In mouse and human ES cells, while the SAC is functional, it fails to prevent rereplication and polyploidy after drug-induced spindle microtubule disruption or DNA double strand breaks. After prolonged SAC activation, ES cells are resistant to apoptosis (caspase 3-dependent or -independent), exit mitosis and become polyploid. Upon initiation of differentiation, a rapid transition from tolerance to intolerance to polyploidy is observed as SAC activation or DNA damage in differentiated ES cells

results in robust apoptosis similar to that observed in mouse embryonic fibroblasts and other differentiated somatic cells [129].

Another explanation for the high rate of chromosomal aberrations in mouse ES cells may originate from the fact that the decatenation checkpoint is highly inefficient in these cells. This checkpoint, which is distinct from the G2/M DNA damage checkpoint, normally delays entry into mitosis if the chromosomes have not been sufficiently decatenated or disentangled by topoisomerase II, the enzyme required for chromosome decatenation and condensation. Its mediators are thought to include the ATR kinase, BRCA1, Polo-like kinase 1 and the Werner's syndrome helicase [130-132]. Interestingly, the decatenation checkpoint was also found inefficient in neural progenitor cells and hematopoietic stem cells while its efficiency increased when ES cells were induced to differentiation, suggesting that decatenation checkpoint deficiency is a feature of the undifferentiated state [133].

Concluding remarks

Altogether, these observations indicate that the unique cell cycles properties of ES cells are intimately linked to their highly proliferative and pluripotency status. In undifferentiated ES cells, checkpoints are either non-functional or, when functional, they are uncoupled with cellular responses, such as apoptotic elimination of damaged cells, that are normally observed in differentiated somatic cells. Commitment towards specific cell lineages is accompanied by deceleration of cellular expansion and acquisition of functional checkpoints. *In vivo*, the advantage for such strategy is unclear. After implantation, the period of extreme proliferation might not be compatible with cell cycle arrest and accurate repair of DNA damages, as this would certainly interfere with embryonic patterning. In addition, transient tolerance for aneuploid cells might also be important for early embryo survival when cell numbers are low. Interestingly enough, specialized cell cycle regulation is not limited to early embryos and ES cells, but might be shared, at least to certain degree, with other stem cells and progenitors. The emerging studies on conditional inactivation of cell cycle regulators will certainly bring important information on this question. Interestingly, acute deletion of ATR in adult leads to dramatic reductions in tissue-specific stem and progenitor cells and exhaustion of tissue renewal and homeostatic capacity [133]. In the future, it will be important to determine whether critical requirement for ATR function in epiblast cells and adult stem cells is due to common cell cycle regulation strategies. Similarly, if the tolerance to chromosomal anomalies observed in ES cells is shared by adult stem cells, it is possible that such mechanism contributes to chromosomal instability

observed in human tumors. Thus, beyond the comprehensive view of mouse development, understanding cell cycle regulation in early embryo may bring important clues for the comprehension of cancer progression as well as for the mastering of therapeutical use of embryonic and adult stem cells.

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