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Research Article

Sea urchin embryo as a model for analysis of the signaling pathways linking DNA damage checkpoint, DNA repair and apoptosis

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Abstract. DNA integrity checkpoint control was studied in the sea urchin early embryo. Treatment of the embryos with genotoxic agents such as methyl methanesulfonate (MMS) or bleomycin induced the activation of a cell cycle checkpoint as evidenced by the occurrence of a delay or an arrest in the division of the embryos and an inhibition of CDK1/cyclin B activating dephosphorylation. The genotoxic treatment was shown to induce DNA damage that depended on the genotoxic concentration and was correlated with the observed cell cycle delay. At low genotoxic concentrations, embryos were able to repair the DNA damage and recover from checkpoint arrest, whereas at high doses they underwent morphological and biochemical changes characteristic of apoptosis. Finally, extracts prepared from embryos were found to be capable of supporting DNA repair *in vitro* upon incubation with oligonucleotides mimicking damage. Taken together, our results demonstrate that sea urchin early embryos contain fully functional and activatable DNA damage checkpoints. Sea urchin embryos are discussed as a promising model to study the signaling pathways of cell cycle checkpoint, DNA repair and apoptosis, which upon deregulation play a significant role in the origin of cancer.

Keywords. DNA damage, cell cycle checkpoint, sea urchin embryo, DNA repair, apoptosis, cancer biology.

Introduction

There is evidence that tumors and metastases originate from a minority of undifferentiated cells, which were called cancer stem cells, and resemble normal stem cells in their multipotency and their capacity to replicate almost indefinitely [1]. The hypothesis then arises that cancers can be viewed as a stem cell disease [2 ,3]. Obviously, the stem cells possess a life span long enough to accumulate the six to eight mutations required for a cell to become cancerous [4], supporting the concept that cancer stem cells could originate from the stem cell compartment [3]. The unlimited replication capacity of adult stem cells is normally tightly constrained by highly orchestrated processes controlling growth, elimination of abnormal cells or anarchic migration of the cells [5]. Accordingly, normal cell growth is controlled by surveillance mechanisms known as cell cycle checkpoints [6],

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which ensure the faithful transmission of undamaged genetic information. These genome-surveillance mechanisms monitor the integrity of the chromosomes and coordinate repair and cell cycle progression [7]. Usually, the parallel activation of programmed cell death (apoptosis) ensures that the abnormal cells are eliminated if no repair occurs (for review see [8]). Among the actors known to be implicated in the checkpoint response, the PI3-kinase-related protein kinases of the ATM/ATR family play a central role as a transducer in the DNA damage signaling response. ATM orchestrates the cell response by arresting/delaying the cell cycle through a phosphorylation cascade involving Chk1/2 and cdc25, by activating the DNA repair through a pathway that may implicate RPA, BRCA1 and H2AX phosphorylation, and by inducing the p53-dependent activation of the caspase apoptotic pathway [9]. An alteration of one actor implicated in these surveillance pathways would produce cells that escape the normal control of cell proliferation and cell death and acquire the genomic instability underlying cancer cells [10–12].

Deciphering the pathways that regulate proliferation, self-renewal, survival and differentiation of normal stem cells is a pre-requisite to the comprehension of mechanisms which are deficient or circumvented in cancer stem cells, resulting in delineating new therapeutic strategies to selectively kill the cancer stem cells, the ultimate condition to irreversibly cure cancers. The description of the molecular machines required to execute cell cycle control steps resulted primarily from genetic studies with yeast [13], and benefited from the discovery that a number of genes classified as oncogenes or tumor suppressor genes were actors in the checkpoint pathways [10]. Subsequent biochemical analyses of the pathways rely on the use of synchronized culture cells. Thus, timely progression through cell division was shown to require phosphorylation events orchestrated by cyclin-dependent protein kinases (CDKs), the universal cell cycle regulators. Their activity is controlled at multiple levels by synthesis of the regulatory cyclin subunits, phosphorylation of both the catalytic and cyclin subunits, binding to CDK inhibitors (CKIs), subcellular localization, and by specific ubiquitination targeting of cyclins and CKIs to proteasome-mediated proteolysis (for review see [14]). Amphibian or marine invertebrate oocytes or eggs have been used for a long time as relevant models for studies on cell cycle control biochemical mechanisms. The reason is that female gametes are physiologically cell-cycle arrested cells, which are synchronously induced to resume the cycle under a natural stimulus, *i.e.*, fertilization. Upon fertilization, the cells are naturally and synchronously triggered to enter cell cycle and go through mitotic divisions in a saline medium without addition of external drugs or growth factor. The sea urchin female gamete is an especially advantageous model in cell cycle studies since it has completed the entire maturation process and is arrested in the G1 phase of the cell cycle (reviewed in [15]). Therefore, fertilization in sea urchin allows the study of the molecular pathways involved in cell cycle entry without interference with a prior step of meiosis resumption, as it is the case in *Xenopus* embryos (reviewed in [16]). Furthermore, the whole genome of the sea urchin, *Strongylocentrotus purpuratus*, has recently become available (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/) [17] and was shown to contain the orthologs for the major genes known to be implicated in the cell cycle and its checkpoints [18, 19]. The archetype for a normal stem cell is the zygote, the initial totipotent embryonic cell from which all the cells of an organism originate [20], further favoring the use of embryos to study the transformation of a normal stem cell into a cancer stem cell. However, it was generally assumed that rapidly developing embryos lack checkpoints and induction of stress responses until after mid blastula transition [21]. This assumption relied mainly on the observation that cells in embryos proceed through rapid and continuous cycles of DNA replication (S-phase) and nuclear division (M-phase), without gap (G) periods, which allow for the repair of DNA damage and replication errors. Nevertheless, activation of the G2/M checkpoint has been demonstrated to occur during the first cell cycle of sea urchin embryos in response to toxic exposure [22, 23]. Furthermore, eukaryotic initiation factor-4 binding protein (4E-BP), the universal inhibitor of translation initiation known to be implicated in cell survival and/or apoptosis was overexpressed in sea urchin embryos under hypoxic stress or prolonged bleomycin exposure [24]. We show here that genotoxic drugs induce DNA breaks in sea urchin embryos leading to cell cycle delay or arrest. The embryo response depends on the importance of the DNA damage induced by the genotoxics. At low dose or short exposure time, the DNA damage provokes a delay in development and the induction of DNA repair. At high doses, the cell cycle is arrested and the embryo manifests cytological and biochemical hallmarks of apoptosis. Furthermore, we report the production of embryo extracts that are appropriate for studying DNA repair activities *in vitro*. The results demonstrate the presence of a fully functional and activatable DNA damage checkpoint in sea urchin embryos favoring the use of embryos as a powerful model to study the pathways of cell cycle checkpoints and the consequences of their deregulation on the origin of cancer.

Materials and methods

Handling of gametes and embryos. Sea urchins (*Sphaerechinus granularis*) were collected in the Brest area and maintained in running seawater. Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Sperm was collected 'dry' and kept undiluted at 4°C. Eggs, collected in 0.22-µm Millipore-filtered seawater, were dejellied by swirling for 30 s in filtered seawater (pH 5), rinsed twice in fresh seawater and suspended in filtered seawater at 5% (v/v) dilution. Diluted ($1/1000^{e}$) sperm was added to the eggs and withdrawn after 5 min. Experiments were only performed on batches exhibiting greater than 90% fertilization. Each experiment used gametes and eggs from a single female.

Embryo treatments. At the indicated time after fertilization, genotoxic agents bleomycin (Aventis) or methyl methanesulfonate (MMS) (Sigma) were added to the incubation medium at the indicated concentration. Genotoxic mother solutions were made in seawater and adjusted to pH 7.5 before addition to the embryo incubations. When needed, genotoxic agents were removed from the incubation medium by rinsing embryos twice in fresh seawater.

Cytological analysis. Embryos were cultured at 16°C under constant stirring and observed at time intervals by phase-contrast microscopy for developmental progression. Thousands of embryos were incubated for each experimental determination from which around 100 were scored for the developmental stage [23]. At various times after fertilization, 0.2-ml aliquots of the embryo suspension were fixed overnight in 1 ml methanol/glycerol (3:1, v/v) in the presence of the DNA dye Hoechst (bisbenzimide, 0.1 µg/ml), mounted in 50% glycerol and observed under fluorescence microscopy [25].

For transmission electron microscopy, embryos were fixed for 2 h in a mixture of 2.5% glutaraldehyde, 1% paraformaldehyde buffered to pH 7.2 with 0.2 M sodium cacodylate and 20% sea water. They were then rinsed three times in 0.2 M sodium cacodylate, pH 7.2 containing 450 mM sucrose and 20% sea water. After the dehydration series in ethanol, samples were embedded in epoxy resin (SPURR). The resin was cut and ultra-thin sections were mounted on a grid and labeled with uranyl acetate and lead citrate (Reynolds). Observations were made under transmission electron microscope (JEOL 1200EX).

CDK1/cyclin B activation *in vivo*. The activation state of CDK1/cyclin B at various times after fertilization was determined from activity measurements of the protein kinase affinity purified from the embryos as already described [23]. Briefly, low-speed (16 000 *g*, 10 min) supernatants were prepared from 1 ml pelleted embryos homogenized in 400 µl ice-cold buffer [60 mM β-glycerophosphate pH 7.2, 15 mM *p*-nitrophenyl phosphate, 25 mM 4-morpholinepropanesulfonic acid (MOPS), 15 mM ethylene glycol tetraacetic acid (EGTA), 15 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM disodium phenylphosphate, 10 µg/ml soybean trypsin inhibitor (SBTI), 100 µM benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.1% Triton]. The CDK1/cyclin B complex was isolated from the extract supernatants by affinity chromatography on $p13^{suc1}$ Sepharose beads prepared as described in [23] and the activity of the bound kinase was determined using histone H1 substrate under standard conditions [26].

Western blot analyses. Electrophoretic resolution of the $p13^{suc1}$ - affinity purified proteins was performed under SDS denaturing conditions on a 15% polyacrylamide gel according to Laemmli [27]. Proteins were electro-transferred from the gel onto nitrocellulose membranes (Schleicher and Schuell). The membranes were saturated in Tris buffer saline containing 0.1% Tween 20 and 5% BSA (Serva) during 1 hour. The membranes were then incubated overnight at 4°C in monoclonal anti-phosphotyrosine horseradish peroxidase-conjugated antibody (ref PY99, Santa Cruz) at 1:500. The antigen-antibody complexes were revealed by the chemiluminescence system (ECL) according to the manufacturer's instructions (Amersham). The total amount of CDK1, taken as a loading control, was verified after immunorevelation using a mouse monoclonal antibody directed against human PSTAIRE (ref 7962, Sigma).

Analysis of DNA damage and repair *in vivo*. The DNA profile was analyzed by pulse-field gel electrophoresis (PFGE) [28]. At 20 min after fertilization, the embryo suspension was treated for 30 min in the presence of different genotoxic agents at the indicated concentrations. After treatment, aliquots (50 µl) of pelleted cells were embedded in agarose plugs [1% low melting point agarose (Appligene), 0.5× Tris-borate-EDTA buffer (TBE, Sigma)] and the plugs were incubated for 48 h in 0.5 M EDTA, 1% *N*-laurylsarcosyl and proteinase K (1 mg/ml; Amresco) at 20°C in the case of bleomycin treatment, and at 20°C or 50°C in the case of MMS treatment. The 50°C treatment was reported to convert the heat-labile alkylation sites induced by MMS to double-strand breaks observable after PFGE [29]. Agarose plugs were washed twice with washing buffer (10 mM Tris, pH 7.5, and 0.1 M EDTA) and incubated with RNase [10 mM Tris, pH 7.5, 0.1 M EDTA and 0.1 mg/ml RNase A (Sigma)] at 37°C for 1 h. Electrophoresis was carried out with a CHEF-DR II system (Bio-Rad) in 1% agarose gels. Gels were run at 14°C with increasing pulse times from 60 to 240 s over 24 h at 100 V. DNA was revealed by ethidium bromide staining and quantified by densitometry. Regression analysis of DNA fragmentation was realized and expressed as the fraction of activity released (FAR), which is the ratio of fragmented DNA to total DNA [28].

DNA repair activity *in vitro*. Extracts were prepared from embryos at 60 min after fertilization by the previously described protocol [30]. Briefly, embryos were washed twice in 10 volumes of lysis buffer (10 mM HEPES, 250 mM NaCl, 25 mM EGTA, 5 mM $MgCl_2$, 110 mM glycine, 250 mM glycerol, 1 mM DTT and 1 mM phenylmethansulfonyl fluoride), then resuspended in lysis buffer at a ratio of 1:1 and homogenized with a syringe fitted with a 23-gauge needle. The lysate was centrifuged twice at 16 000 *g* for 10 min at 4°C and the supernatant was stored at –80°C. An ATP regenerating system (final concentration: 2 mM ATP, 20 mM phosphocreatine and 50 µg/ml creatine kinase) was added to the extracts before the DNA repair assay. A non-homologous end-joining (NHEJ) assay was performed using the duplex oligonucleotides (P1/P3 and P2/labeled-P4; see below) and the method described in detail by Fan and Wu [31]. Briefly, repair activity is reflected by the appearance of a 62-base labeled oligonucleotide after electrophoresis, resulting from ligation of the precursors P3 and P4. The assay was conducted using 2 pmol of each oligonucleotide incubated in the presence of embryo extract (100 µg protein) for 180 min at 37°C. Base excision repair (BER) was analyzed using the protocol described by Cabelof *et al.* [32]. In brief, restoration of a restriction site was used to test the repair of a G:U mismatch-containing oligonucleotide (labeled-P5/P7; see below). The assay was conducted using 2 pmol of duplex oligonucleotide P5/P7 mimicking BER substrate, or control substrate (labeled P6/P7 containing the original restriction site), incubated in the presence of the embryo extract (100 µg protein) for 10 min at 37°C. After purification, oligonucleotides were digested with 50 U *Hpa*II (New England Biolabs) for 30 min. For both assays, oligonucleotides were resolved on denaturing gels (50% urea, 20%, acrylamide, 30% formamide) and analyzed by PhosphorImager scanning. The oligonucleotides were purchased from Eurogentec. Their sequences are indicated below:

P1 (5-ACAAAGTTTGGATTGCTACTGACCGCTCTC GTGCTCGTCGCTGCGTT-3, 47-mer)
P2 (5-GCCTCGCGACGCATG-3, 15-mer)
P3 (5-GCGACGAGCACGAGAGCGGTCAGTAGCAATCCA AACTTTGT-3, 41-mer)
P4 (5-CATGCGTCGCGAGGCAACGC-3, 20-mer)
P5 (5-ATATACCGCGGUCGGCCGATCAAGCTTATT-3, 30-mer)
P6 (5-ATATACCGCGGCCGGCCGATCAAGCTTATT-3, 30-mer)
P7 (5-AATAAGCTTGATCGGCCGGCCGCGGTATAT-3, 30-mer).

Caspase activity assay. Acetyl-DEVD-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA), a fluorogenic substrate for caspase, and acetyl-DEVD-aldehyde (acetyl-DEVD-CHO), a caspase inhibitor, (BD Bioscience) were used for the estimation of caspase activity in embryo extracts. At different times after fertilization, 1 ml from a

5 % suspension of control or treated embryos was pelleted. Pellets were suspended in 500 µl ice-cold buffer A (100 mM HEPES, pH 7.5, 10 mM DTT) and homogenized by short sonication (5 s, amp. 40). Embryo extracts were clarified by a 14 000 *g* centrifugation for 15 min at 4°C. Caspase assays were carried out at 20°C for 3 h in 1 ml buffer A containing 10 µM substrate, in the presence of embryo extract (100 µg protein). At the end of the incubation, the fluorescence of 7-amino-4-methylcoumarin that had been cleaved from Ac-DEVD-MCA by caspase was detected by excitation at 380 nm and emission at 460 nm with a luminescence spectrophotometer (LS 50 Perkin Elmer).

Results

Embryonic sea urchin cells contain an activatable DNA damage checkpoint. In mammalian cells, genotoxics like MMS, which induces DNA base alkylation [29], or bleomycin, a radiomimetic agent that causes double-strand DNA breaks [33], are commonly used to trigger DNA damage checkpoints. In this study, the genotoxic agents were applied to sea urchin embryos to investigate the DNA damage checkpoint. MMS or bleomycin when added to embryos 20 min after fertilization provoked a delay in the occurrence of the first cleavage as observed under phase-contrast microscope (Fig. 1). The delay in the first cytokinesis was dependent on the concentration of the genotoxic agents, and ranged from several minutes to complete arrest. Depending on the experiments using different animals, an average 30 min delay was observed with concentration ranges 1–4 mM MMS or 0.4–4 mM bleomycin (Fig. 1a). Complete cell cycle arrest, as judged by the absence of cell division was reproducibly obtained for doses of 10 mM MMS or 10 mM bleomycin (Fig. 1a). The chromatin morphology, as illustrated in Figure 1b, was comparable during all phases of the cell cycle in the genotoxic-treated and the control embryos at the interphasic state (uncondensed chromatin) and at the three main mitotic steps (chromosome condensation, metaphasic alignment and chromatin segregation). Thus, genotoxic treatments specifically affected cell cycle kinetics without major metabolic injury. Figure 1b further shows that

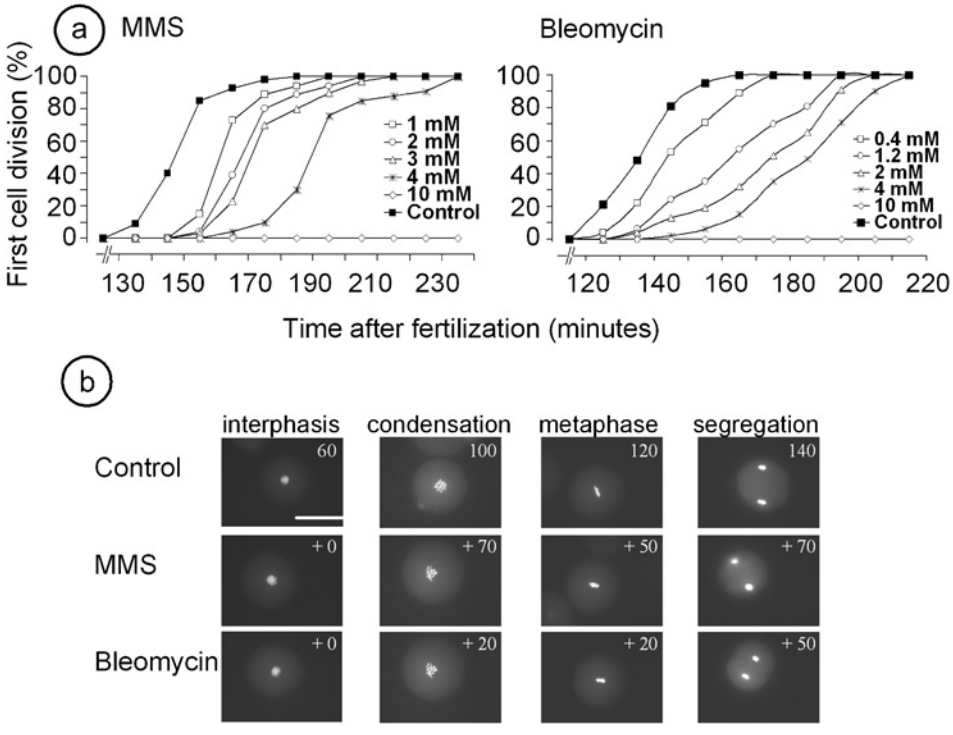

Figure 1. Genotoxic treatment affects the kinetic of the first cell division. Sea urchin eggs were fertilized and the embryos were transferred 20 min later to fresh seawater (5 % suspension) without (Control) or in the presence of MMS (MMS) or bleomycin (Bleomycin). (*a*) For each treatment, 100 embryos were scored for the first cleavage by phase-contrast microscopy as a function of time after fertilization. Control embryos are represented by closed symbols and genotoxic-treated embryos at the indicated concentrations by open symbols. Each kinetic was obtained for the eggs isolated from a single female and is representative of at least three independent experiments. (*b*) The chromatin morphology was observed by fluorescence microscopy after Hoechst staining of the control embryos, 4 mM MMS-treated embryos or 1.2 mM bleomycin-treated embryos. The four main cell cycle stages (interphasis, chromatin condensation, metaphase alignment, chromosome segregation) are illustrated. Each photograph shows an embryo representative of at least 80 % of the embryo population at the related time. The time in minutes after fertilization is indicated for each stage in the control lane; for the treated lanes, the number (mean of three experiments) in each panel symbolizes the time delay (in minutes) as compared to the control to reach the identical stage. Bar: 100 µm.

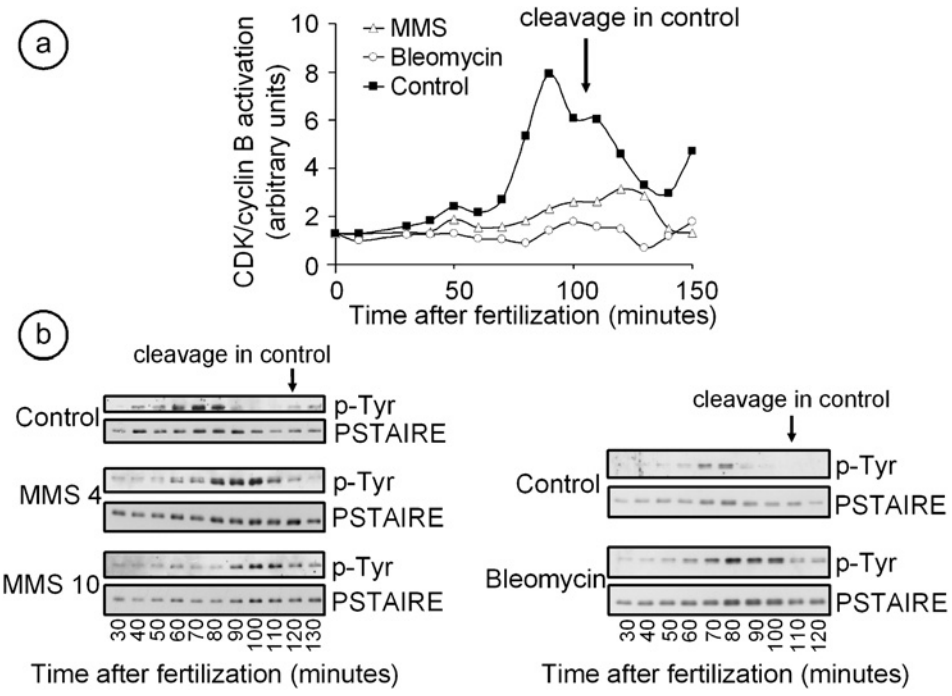

Figure 2. Genotoxic treatment affects CDK1/cyclin B activation *in vivo*. Sea urchin eggs were fertilized and embryos were transferred 20 min later to fresh seawater or in the presence of MMS or bleomycin. At the indicated times after fertilization, batches of embryos were processed for CDK1/cyclin B affinity purification on p13-Sepharose beads as indicated in Materials and methods. (*a*) The CDK1/cyclin B activation state estimated by the kinase activity of the purified complex was measured in control embryos (Control), in 1 mM MMS-treated embryos (MMS) or 0.4 mM bleomycin-treated embryos (Bleomycin). In this experiment, the first cleavage indicated by an arrow occurred around 110 min after fertilization for control embryos. (*b*) The phosphorylation state of CDK1 was analyzed by Western blot on purified complex using phosphotyrosine (p-Tyr) antibodies. The experiment was done on control (Control), MMS-treated embryos at 4 mM (MMS4) or at 10 mM (MMS10) and bleomycin-treated embryos at 10 mM (Bleomycin). The total amount of purified CDK1, revealed by PSTAIRE antibodies, is taken as the loading control. Arrows indicate the time for first cleavage in control embryos.

the genotoxic agent induced the cell cycle delay as early as the chromosome condensation stage (*i.e.*, entry into M-phase), a feature reminiscent of the DNA damage checkpoint activation. The checkpoint triggering was further supported by the analysis of CDK1/cyclin B activation *in vivo* (Fig. 2). As already reported [34], control embryos presented a characteristic peak of CDK1/cyclin B activation at 90 min after fertilization, around 20 min before the cleavage occurred. Figure 2a illustrates that during the same period, no CDK1/cyclin B activity peak was detectable in the genotoxic-treated embryos, indicating that the complex activation had been impaired. The fact that, at the doses tested, the embryos finally underwent cell division, although with a delay kinetic (see Fig. 1a) implies that activation of CDK1/cyclin B complex has been delayed in the treated embryos as compared with the control. The phosphorylation state on tyrosine of CDK1 was analyzed in control and genotoxic-treated embryos (Fig. 2b). In control embryos, CDK1 tyrosine phosphorylation increased progressively up to 70 min after fertilization and abruptly disappeared around 90 min, indicative of the CDK1/cyclin B complex activation, as already well documented [23]. When the embryos were incubated in the presence of MMS or bleomycin, the tyrosine dephosphorylation of CDK1 was either delayed (4 mM MMS) or barely detectable (10 mM MMS or 10 mM Bleomycin) in correlation with the dose-dependent delay or arrest in the cell division occurrence (see Fig. 1a). Therefore, genotoxics dramatically affect the activating dephosphorylation of CDK1 on tyrosine, a critical feature in the DNA damage checkpoint pathway [10].

Altogether, the experiments demonstrated that the cell cycle DNA damage checkpoint is operative and can be triggered in sea urchin embryos.

Induction of DNA repair in the genotoxic-treated embryos. To test whether genotoxics affect the sea urchin embryo DNA as they do in mammalian cells [35], the embryo DNA profile was examined by PFGE under different experimental conditions (Fig. 3). Intact chromosomal DNA isolated from control embryos was reproducibly found to resolve as large bands at the top of the gel (Fig. 3a, both panels, lanes 0, brackets). DNA isolated from embryos treated with 10 or 20 mM MMS displayed a smear

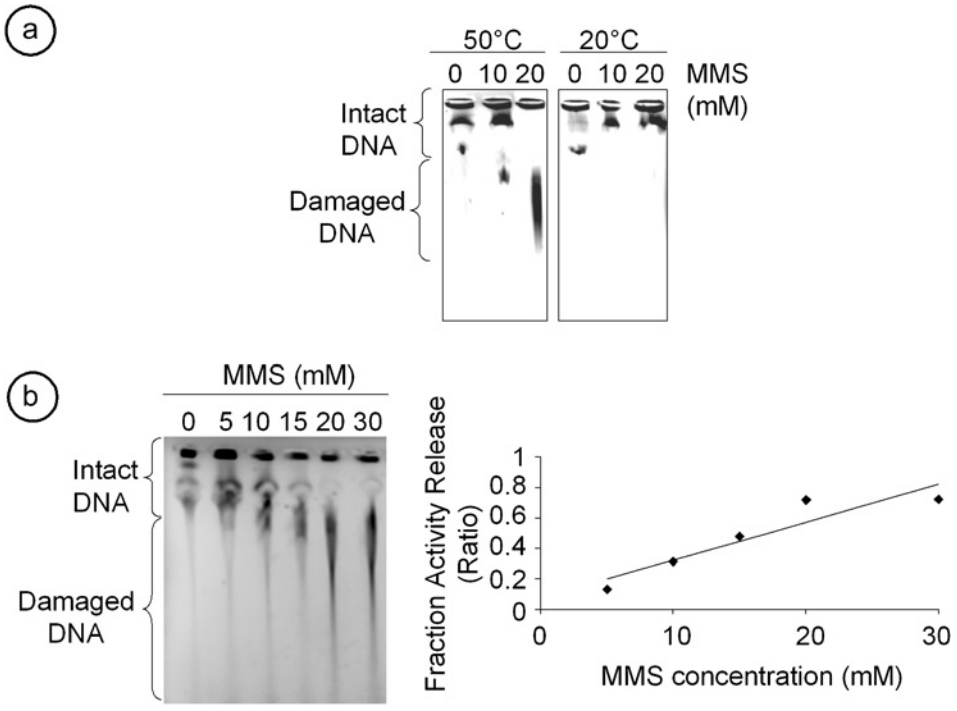

Figure 3. Genotoxic treatment induces embryo DNA damage. Sea urchin eggs were fertilized in fresh sea water (5% suspension) and, 20 min later, the embryos were treated for 30 min in the presence of MMS at the indicated concentrations. Pulsed-field gel electrophoresis (PFGE) was realized on 50-µl aliquots of pelleted embryos embedded in agarose plugs as described in Materials and methods. DNA was visualized on gel after BET staining. (*a*) MMS produces heat-labile DNA damage. The agarose plugs from control embryos (0) and MMS-treated embryos at 10 mM (10) or at 20 mM (20) were incubated at 20°C or 50°C before electrophoresis. (*b*) DNA damage increases with MMS concentration. The DNA profile on PFGE was analyzed after embryo treatment at increasing MMS concentration. Left panel: Gel after BET staining. Right panel: Quantification of the intensity of DNA damage after densitometric analysis of the gel, and is expressed as fraction activity release as described in the Material and methods.

profile created by high molecular weight DNA fragments entering the gel (Fig. 3a, 50°C left panel, lanes 10 and 20), indicating that the sea urchin embryo DNA was damaged by MMS. Likewise, PFGE analysis demonstrated that bleomycin induced double-strand breaks in sea urchin embryo DNA as judged by the occurrence of smear DNA profile (illustrated below). It is noteworthy that when the preparation of DNA from MMS-treated embryos was not pre-heated at 50°C before electrophoresis, a treatment which reveals the heat-labile site induced by MMS-induced DNA alkylation (see Materials and methods), no smear bands could be observed (Fig. 3a, compare 50°C left and 20°C right panels), indicating that no huge unspecific DNA damage had occurred. Further analyses of the DNA damage induced by MMS in embryos showed that the size and mobility of the smear increased with the concentration of the genotoxic (5–30 mM) applied to the embryos (Fig. 3b, left panel). Quantification of the smear after densitometry of the gel (Fig. 3b, right panel) illustrated the dose-dependent intensity of the DNA damage, which is then correlated with the dose-dependent activation of the checkpoint shown in Figure 1a. Altogether, this demonstrates that exposure to MMS or bleomycin induces DNA damage in sea urchin embryos as well as in mammalian cells.

In mammalian cells, activation of the checkpoint is accompanied by the mobilization of the repair machinery allowing the resumption of cell cycle [7]. We then tested the reversibility of the genotoxics-induced damages in sea urchin embryos. In correlation with the DNA damage induced by a 30-min treatment in the presence of MMS, short (30 or 60 min) incubation in high dose (10 mM) MMS led to the checkpoint activation, as judged by the significant delay in the occurrence of the first cell division (Fig. 4, compare the 3-h column control lane with the 30-min and 60-min MMS lanes). Similarly, checkpoint triggering was induced by short incubations in a high dose (10 mM) of bleomycin, with the delay in cell division increasing with the incubation time (from 30 to 120 min, Fig. 4). Moreover, when the embryos were washed free of the genotoxics and further incubated in fresh seawater, the cell cycles resumed and the embryos developed at least to the blastula stage (about 400 or 800 cells) (Fig. 4, 20-h column). Cell cycle resump-

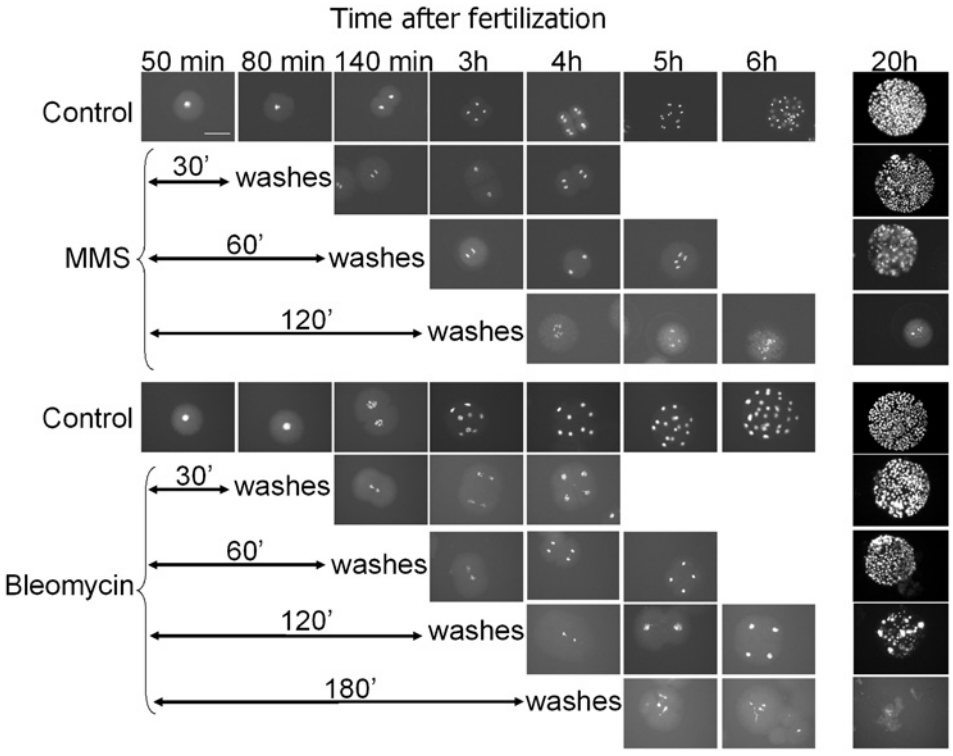

Figure 4. The reversibility of cell cycle arrest depends on the time of treatment in the presence of the genotoxic. At 20 min after fertilization, batches of embryos were incubated in fresh seawater in the absence (control), in the presence of 10 mM MMS (MMS) for 30, 60, or 120 min, or in the presence of 10 mM bleomycin (Bleomycin) for 30, 60, 120 or 180 min. After the treatment, the genotoxics were removed by two successive washes in fresh seawater. The chromatin morphology was observed by fluorescence microscopy after Hoechst staining on batches of embryos taken at different times from 50 min to 20 h after fertilization. Each photograph shows an embryo representative of at least 80% of the thousand present in the population at the related time. Bar: 100 μm.

tion suggested either that the checkpoint has been by-passed or that the lesions have been repaired.

Sea urchin embryos were therefore tested for their ability to repair DNA *in vitro*. Two main DNA repair processes were analyzed: BER and NHEJ. The BER system repairs modified bases generated by alkylating agents such as MMS and endogenous hydrolytic and oxidative processes, whereas NHEJ repairs DNA double-strand breaks, the more toxic DNA damage for the cell, which is induced by ionizing radiation or radiomimetic drugs such as bleomycin (for review see [36]). When oligonucleotides mimicking double-strand breaks were introduced into the extracts, NHEJ repair occurred, as judged by the occurrence after denaturing gel analysis of a high (62 bases) nucleotide (nt) band (arrow in Fig. 5, upper panel), resulting from the ligation of two short linear duplex precursors. Quantification of the joining activity was estimated by densitometric analysis of the autoradiograph. The ligated product at 62-nt represented around 8.7% of the total labeled signal, comparable to the activity reported for 5 nM ligase complex tested under the same experimental conditions [31]. Figure 5 (lower panel) shows that the embryo extracts were also able to support a base-mismatch repair. Whereas a control oligonucleotide, 30 bases long, containing a restriction site, was efficiently cleaved into a 12-base fragment (lane 1), the same oligonucleotide containing a base mismatch (C→U) could not be cleaved (lane 2). After incubation in the presence of embryo extracts, the mismatch-containing oligonucleotide could now be cleaved as well as the control one (Fig. 5, lower panel, lanes 3 and 4), indicating that the restriction site had been restored by incubation in the extract, by means of BER. Therefore, two of the main DNA repair activities are functional in embryos and can be easily measured in extracts *in vitro*.

It was then essential to demonstrate that the embryos' DNA repair activities were mobilized after genotoxic-induced damage and allowed the resumption of cell cycle through repair of the lesions. The DNA state was then examined by PFGE experiments after a short (30 min) high-dose (10 mM) treatment of the embryos in the presence of the MMS or bleomycin, and further incubation in fresh seawater (Fig. 6). DNA was found to be repaired as early as 60 min after washing, as judged from the disappearance of the smeary profile and migration of the DNA as a single band at the top

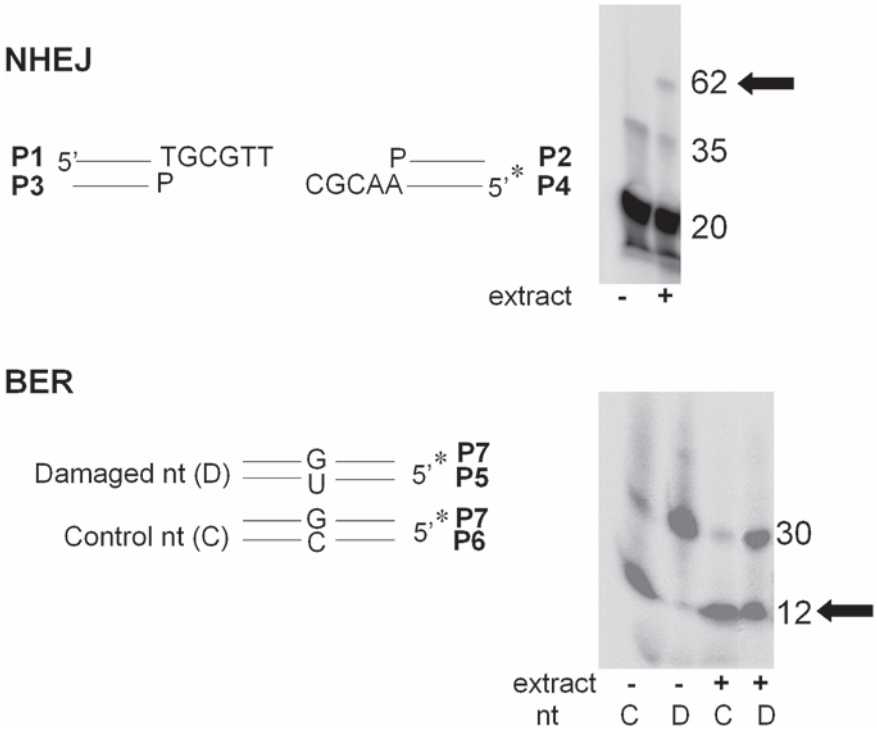

Figure 5. Embryo extracts repair DNA *in vitro*. Extracts were prepared from 60-min fertilized embryos as described in the Materials and methods. (Upper panel) Non-homologous end-joining (NHEJ) activity was measured by incubation of the duplex oligonucleotides (nt) mimicking DNA breaks (illustrated at the top left) in the absence (–) or presence (+) of the embryo extracts (100 µg protein). (Lower panel) Base excision repair (BER) activity was measured using the duplex oligonucleotides (nt) illustrated at the bottom left. Control (C) or damaged (D) oligonucleotides were subjected to digestion by the restriction enzyme *Hpa*II either directly (–) or after pre-incubation in the presence of the embryo extract (100 µg protein). The resulting oligonucleotides were analyzed by denaturing gel electrophoresis and visualized after autoradiography of the gel. Arrows indicate the position of the expected relevant product of reaction. Note that labeling at the level of 35-nt (in NHEJ) corresponds to incomplete denaturation of the P2/P4 precursor duplex.

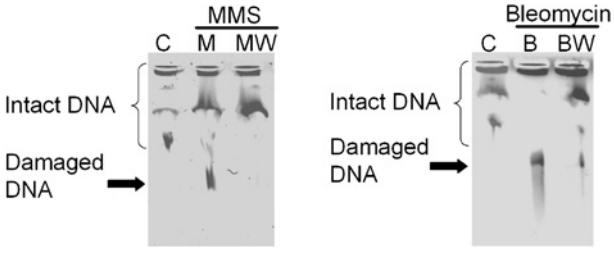

Figure 6. DNA repair occurs in embryos after genotoxic treatments. Sea urchin eggs were fertilized and, 20 min later, the embryos were incubated for 30 min in the absence (C) or in the presence of 10 mM MMS (MMS) or 10 mM bleomycin (Bleomycin). At the end of the incubation, one batch of the treated embryos (M or B) was immediately prepared for PFGE and another was transferred to fresh seawater and further incubated for 60 min before preparation for electrophoresis (MW or BW). PFGE was realized on 50-µl aliquots of pelleted embryos as described in the Materials and methods. The DNA was visualized on gel after BET staining.

of the gel, which was comparable to the profile in control embryos (compare lanes M and W in Fig. 6, left panel and lanes B and W in Fig. 6, right panel). Therefore, in addition to the activation of the cell cycle checkpoint to arrest cell cycle, the sea urchin embryos respond to DNA damage by an activation of the DNA repair machinery.

Induction of apoptosis in the genotoxic-treated embryos. Incubation of the embryos for 120 min or more in 10 mM MMS, or 180 min or more in 10 mM bleomycin induced complete arrest in cell cycle. The embryos remained at the one cell stage for at least a 20-h period (see Fig. 4), indicative of permanent checkpoint activation probably related to permanent DNA damage. Although cytologically intact, the cells started to look unhealthy at the chromatin morphology level (Fig. 4, lanes 6 h or 20 h for treated embryos), and the main proteins underwent extensive degradation as judged from the electrophoretic profile after Ponceau red staining (data not shown). To further address this point, embryos were treated for 5 h in 10 mM MMS or bleomycin and thereafter maintained in fresh seawater for 4 days. Whereas control embryos had developed normally until the prism stage (Fig. 7, control column), both the MMS- and bleomycin-treated embryos, remained undivided until 90 h after treatment and progressively presented typical apoptotic figures. The DNA of

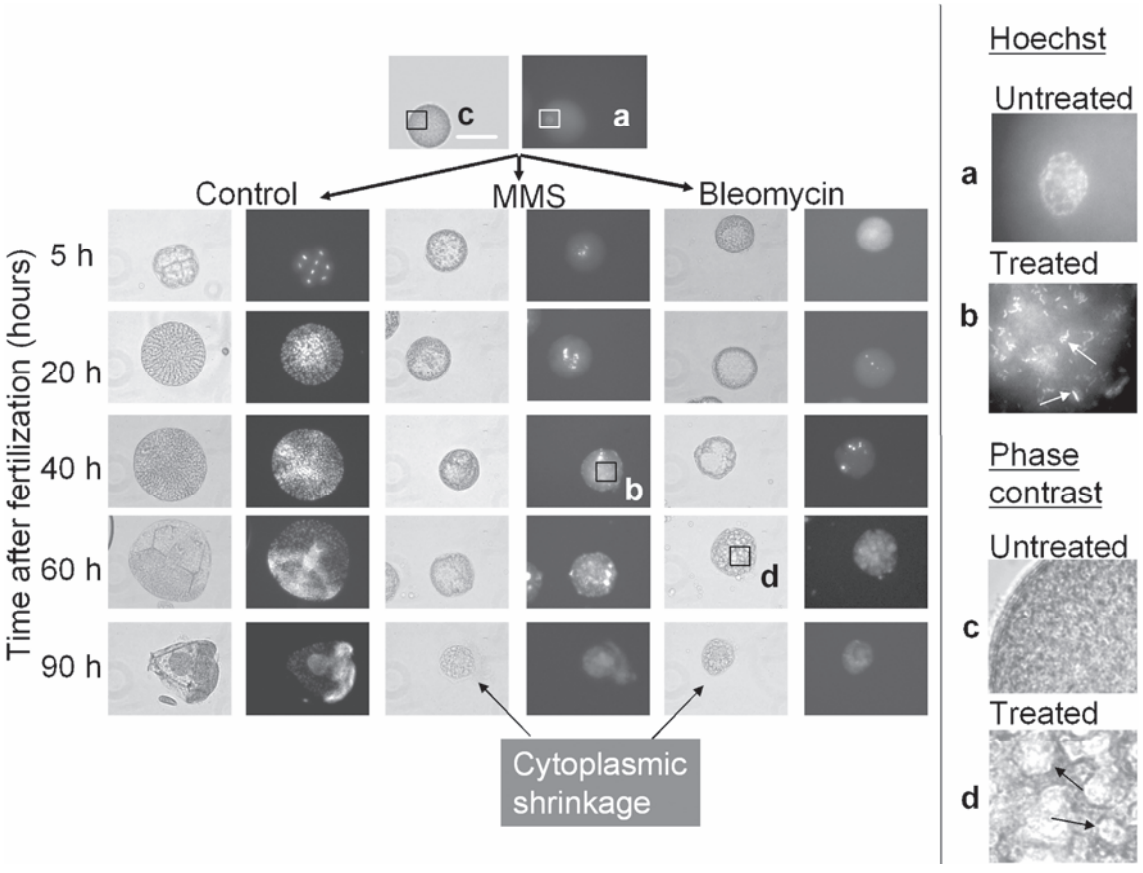

Figure 7. Genotoxics induce cell death in sea urchin embryos. Sea urchin eggs were fertilized and 20 min later the embryos were transferred to fresh seawater in the absence (Control) or presence of 10 mM MMS (MMS) or 10 mM bleomycin (Bleomycin) for 5 h. Control or treated embryos were then thoroughly washed and further incubated in fresh seawater. Left panel: The cytoplasmic and DNA morphologies of the embryos were followed for 4 days under, respectively, phase-contrast microscopy (left column for each treatment) and fluorescence microscopy after Hoechst staining (right column for each treatment). Bar: 100 μm. Right panel: Comparison at high magnification (×9) of the DNA (Hoeschst) and cytoplasmic (phase-contrast) morphologies in untreated (untreated) or genotoxic-treated (treated) cells. The photographs (a–d) correspond to the zones highlighted by squares on the left panel. Arrows indicate the characteristic apoptotic features (DNA fragmentation and cytoplasm budding) as described in text.

these one cell-stage-arrested embryos appeared condensed and fragmented (Fig. 7, compare insert a and insert b), whereas the cytoplasm displayed figures of budding (Fig. 7, compare insert c and insert d). At 90 h, the treated embryos underwent cytoplasmic shrinkage (Fig. 7, lane 90 h). To further confirm the occurrence of apoptosis in genotoxic-treated embryos, electron microscopy analysis was conducted (Fig. 8). At 20 h after fertilization, the MMS-treated and bleomycin-treated embryos contained small round bodies surrounded by membranes and containing intact organelles, a characteristic of apoptotic bodies [37, 38].

The molecular hallmark of apoptosis is the activation of cysteine proteases named caspases, which are central regulators of apoptosis [9]. Caspase activity was therefore assayed in extracts from control and genotoxic-treated embryos (Fig. 9). Whereas no significant caspase activity could be detected in control embryos from 5 to 50 h after fertilization, the MMS-treated embryos displayed a peak of caspase activity at around 20 h after treatment. The specificity of the activity was ascertained by its inhibition in the presence of caspase inhibitor in the incubation medium (Fig. 9).

Thus, over the limit of possible reversion of DNA damage by DNA repair, sea urchin embryos engage a cell death program when they are treated with genotoxics.

Discussion

The results reported here demonstrate for the first time that the complete DNA damage checkpoint pathway is fully functional and activatable during the early divisions of sea urchin embryos. In mammalian cells, alkylating agents (such as MMS), double-strand

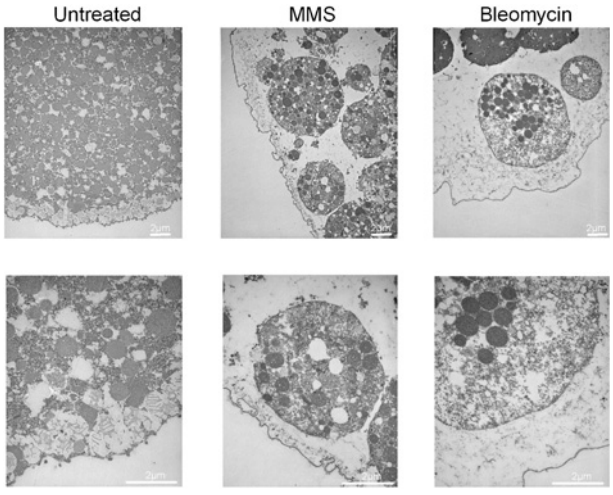

Figure 8. Apoptotic bodies arise in genotoxic-arrested sea urchin embryos. Sea urchin eggs were fertilized and 20 min later the embryos were transferred to fresh seawater in the absence or presence of 10 mM MMS or 10 mM bleomycin for 5 h. Control or treated embryos were then thoroughly washed and further incubated in fresh seawater. At 20 h after fertilization, batches of control eggs (Untreated), 10 mM MMS-treated (MMS) or 10 mM bleomycin-treated (Bleomycin) embryos were processed for transmission electron microscopy observation as described in the Material and methods. Each photograph shows an embryo representative of the population at the related time and treatment. Photographs in the lower lane are 3.2 magnification of the upper lane.

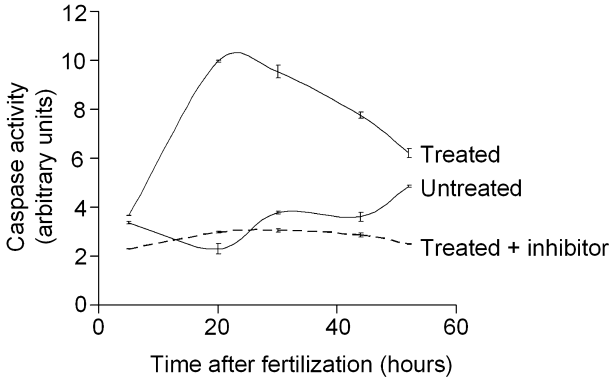

Figure 9. Genotoxics activate caspase in sea urchin embryos. Sea urchin eggs were fertilized and 20 min later the embryos were transferred to fresh seawater in the absence or presence of 10 mM MMS for 5 h. Control or treated embryos were then thoroughly washed and further incubated in fresh seawater. At different times after fertilization, 50-µl aliquots of pelleted control (Untreated) or 10 mM MMS-treated (Treated) embryos were processed for caspase activity assay as described in the Material and methods. The caspase activity assay was performed in triplicate for each extract in the absence or presence of the caspase inhibitor (Treated + inhibitor). Caspase activity (expressed in arbitrary unit) was measured by the intensity of the fluorescence detected by the photometer.

break inducing agents (such as bleomycin) were shown to activate a complex response known as the DNA damage response, implicating a signal transduction network leading to cell cycle arrest, DNA repair or apoptosis [7]. Accordingly, we have shown that MMS or bleomycin treatments of sea urchin embryos induce cell cycle delay or arrest as evidenced by the delay or absence of cytokinesis correlated with delay or absence of the peak of CDK1/cyclin B activation and CDK1 tyrosine dephosphorylation. The DNA from treated embryos presents the characteristic lesions described generally for MMS or bleomycin [28, 29]. The embryos are also able to activate the repair of damage, as demonstrated by pulsed-field analysis of the DNA state. In addition, two main DNA repair processes are measurable in embryo extracts: BER, and NHEJ. Finally, in the case of excessive or sustained DNA damage induced by high dose or long exposure time to genotoxics, the embryos switch on the program of cell death, as shown by the appearance of characteristic apoptotic morphological and biochemical features (apoptotic bodies, caspase activity). Therefore, the sea urchin embryo, already used as a relevant model for studies of cell cycle control, is a pertinent and advantageous model for analysis of checkpoints and their regulation. Major challenges in cancer biology are to understand how stem cells transform into cancer stem cells, and how the cancer stem cells generate tumors and cancer (see Introduction). In these processes, main clues may be found by understanding the involvement of cell cycle checkpoints, DNA damage and apoptosis control in the origin of cancerization [10]. Since human stem cell, the most relevant model for such studies, are not readily available and are not synchronized in their division, the sea urchin early development model, which involves authentic stem cells [20], constitutes a powerful alternative and complementary model. Embryonic cells offer the advantage of being naturally dividing stem cells, easily and quantitatively available from sea urchins. They are not affected in their cell cycle regulators in contrast with most available cell lines, which are transformed cells and therefore already affected in the cell cycle checkpoints. Quantitative amounts of cells or cell extracts can be obtained at specific stages of cell division, as also reported for *Xenopus* (reviewed in [39]) and have already been used for biochemical analysis, for instance, extracts reproducing *in vivo* cell cycle regulation [40–43]. Further valuable advantages of the sea urchin embryo model are (1) the experimental facility for phase-specific determination due to the transparency of the embryos, (2) the possibility for functional analysis *in vivo* using fluorescent probes, and (3) the tools provided by the recent availability of the sea urchin complete genome [17] in which the genes were found that are implicated in the cell cycle and the cell cycle checkpoints (CDKs, p21/27, ATM/ATR, Chk1/2, p53, …) as well as DNA

metabolism [18] and the basic apoptotic machinery (caspases, Apaf-1, IAP and the bcl2 family proteins) [19].

Thus, we propose the sea urchin embryo as a pertinent alternative and complementary model for studies on cell cycle checkpoints and their de-regulation, a feature at the origin of the transformation of a normal cell into a cancerous one.

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1 Reya, T., Morrison, S. J., Clarke, M. F. and Weissman, I. L. (2001) Stem cells, cancer, and cancer stem cells. Nature 414, 105 – 111.
2 Beachy, P. A., Karhadkar, S. S. and Berman, D. M. (2004) Tissue repair and stem cell renewal in carcinogenesis. Nature 432, 324 – 331.
3 Ratajczak, M. Z., Kucia, M., Dobrowolska, H., Wanzeck, J., Reca, R. and Ratajczak, J. (2006) Emerging concept of cancer as a stem cell disorder. Cent. Eur. J. Biol. 1, 73 – 87.
4 Nowak, M. A., Komarova, N. L., Sengupta, A., Jallepalli, P. V., Shih, Ie., M., Vogelstein, B. and Lengauer, C. (2002) The role of chromosomal instability in tumor initiation. Proc. Natl. Acad. Sci. USA 99, 16226 – 16231.
5 Clarke, M. F. and Fuller, M. (2006) Stem cells and cancer: two faces of eve. Cell 124, 1111 – 1115.
6 Hartwell, L. H. and Weinert, T. A. (1989) Checkpoints: controls that ensure the order of cell cycle events. Science 246, 629 – 634.
7 Zhou, B. B. and Elledge, S. J. (2000) The DNA damage response: putting checkpoints in perspective. Nature 408, 433 – 439.
8 Norbury, C. J. and Zhivotovsky, B. (2004) DNA damage-induced apoptosis. Oncogene 23, 2797 – 2808.
9 Nyberg, K. A., Michelson, R. J., Putnam, C. W. and Weinert, T. A. (2002) Toward maintaining the genome: DNA damage and replication checkpoints. Annu. Rev. Genet. 36, 617 – 656.
10 Kastan, M. B. and Bartek, J. (2004) Cell-cycle checkpoints and cancer. Nature 432, 316 – 323.
11 Massague, J. (2004) G1 cell-cycle control and cancer. Nature 432, 298 – 306.
12 Sawyers, C. (2004) Targeted cancer therapy. Nature 432, 294 – 297.
13 Hartwell, L. H. and Kastan, M. B. (1994) Cell cycle control and cancer. Science 266, 1821 – 1828.
14 Murray, A. W. (2004) Recycling the cell cycle: cyclins revisited. Cell 116, 221 – 234.
15 Gilbert, S. F. (2000) Developmental Biology, Part 2, Chapter 7, Sinauer Associates, Sunderland.
16 Masui, Y. (2001) From oocyte maturation to the in vitro cell cycle: the history of discoveries of maturation-promoting factor (MPF) and cytostatic factor (CSF). Differentiation 69, 1 – 17.
17 Sodergren, E., Weinstock, G. M., Davidson, E. H., Cameron, R. A. and the Sea Urchin Genome Sequencing Consortium (2006) The genome of the sea urchin *Strongylocentrotus purpuratus.* Science 314, 941 – 952.
18 Fernandez-Guerra, A., Aze, A., Morales, J., Mulner-Lorillon, O., Cosson, B., Cormier, P., Bradham, C., Adams, N., Robertson, A. J., Marzluff, W. F., Coffman, J. A. and Geneviere, A. M. (2006) The genomic repertoire for cell cycle control and DNA metabolism in *S. purpuratus.* Dev. Biol. 300, 238 – 251.
19 Robertson, A. J., Croce, J., Carbonneau, S., Voronina, E., Miranda, E., McClay, D. R. and Coffman, J. A. (2006) The genomic underpinnings of apoptosis in *Strongylocentrotus purpuratus.* Dev. Biol. 300, 321 – 334.
20 Sell, S. (2004) Stem cell origin of cancer and differentiation therapy. Crit. Rev. Oncol. Hematol. 51, 1 – 28.
21 Epel, D. (2003) Protection of DNA during early development: adaptations and evolutionary consequences. Evol. Dev. 5, 83 – 88.
22 Hansen, E., Even, Y. and Geneviere, A. M. (2004) The alpha, beta, gamma, delta-unsaturated aldehyde 2-trans-4-trans-decadienal disturbs DNA replication and mitotic events in early sea urchin embryos. Toxicol. Sci. 81, 190 – 197.
23 Marc, J., Belle, R., Morales, J., Cormier, P. and Mulner-Lorillon, O. (2004) Formulated glyphosate activates the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. Toxicol. Sci. 82, 436 – 442.
24 Le Bouffant, R., Cormier, P., Mulner-Lorillon, O. and Belle, R. (2006) Hypoxia and DNA-damaging agent bleomycin both increase the cellular level of the protein 4E-BP. J. Cell Biochem. 99, 126 – 132.
25 Boulben, S., Monnier, A., Le Breton, M., Morales, J., Cormier, P., Belle, R. and Mulner-Lorillon, O. (2003) Sea urchin elongation factor 1delta (EF1delta) and evidence for cell cycle-directed localization changes of a sub-fraction of the protein at M phase. Cell. Mol. Life Sci. 60, 2178 – 2188.
26 Marc, J., Mulner-Lorillon, O., Boulben, S., Hureau, D., Durand, G. and Belle, R. (2002) Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. Chem. Res. Toxicol. 15, 326 – 331.
27 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680 – 685.
28 Joshi, N. and Grant, S. G. (2005) DNA double-strand break damage and repair assessed by pulsed-field gel electrophoresis. Methods Mol. Biol. 291, 121 – 129.
29 Lundin, C., North, M., Erixon, K., Walters, K., Jenssen, D., Goldman, A. S. and Helleday, T. (2005) Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable *in vivo* DNA double-strand breaks. Nucleic Acids Res. 33, 3799 – 3811.
30 Collas, P. and Poccia, D. (1998) Methods for studying *in vitro* assembly of male pronuclei using oocyte extracts from marine invertebrates: sea urchins and surf clams. Methods Cell Biol. 53, 417 – 452.
31 Fan, W. and Wu, X. (2004) DNA polymerase lambda can elongate on DNA substrates mimicking non-homologous end joining and interact with XRCC4-ligase IV complex. Biochem. Biophys. Res. Commun. 323, 1328 – 1333.
32 Cabelof, D. C., Raffoul, J. J., Yanamadala, S., Guo, Z. and Heydari, A. R. (2002) Induction of DNA polymerase beta-dependent base excision repair in response to oxidative stress *in vivo.* Carcinogenesis 23, 1419 – 1425.
33 Povirk, L. F. (1996) DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: bleomycin, neocarzinostatin and other enediynes. Mutat. Res. 355, 71 – 89.
34 Monnier, A., Morales, J., Cormier, P., Boulben, S., Belle, R. and Mulner-Lorillon, O. (2001) Protein translation during early cell divisions of sea urchin embryos regulated at the level of polypeptide chain elongation and highly sensitive to natural polyamines. Zygote 9, 229 – 236.
35 Hoeijmakers, J. H. (2001) Genome maintenance mechanisms for preventing cancer. Nature 411, 366 – 374.
36 Friedberg, E. C. (2003) DNA damage and repair. Nature 421, 436 – 440.
37 Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239 – 257.

38 Perl, M., Chung, C. S. and Ayala, A. (2005) Apoptosis. Crit. Care Med. 33, S526 – 529.
39 Philpott, A. and Yew, P. R. (2005) The *Xenopus* cell cycle: an overview. Methods Mol. Biol. 296, 95 – 112.
40 Collas, P. (1998) Cytoplasmic control of nuclear assembly. Reprod. Fertil. Dev. 10, 581 – 592.
41 Collas, P. (2000) Formation of the sea urchin male pronucleus in cell-free extracts. Mol. Reprod. Dev. 56, 265 – 270.
42 Gliksman, N. R., Parsons, S. F. and Salmon, E. D. (1993) Cytoplasmic extracts from the eggs of sea urchins and clams for the study of microtubule-associated motility and bundling. Methods Cell Biol. 39, 237 – 251.
43 Zhang, H. and Ruderman, J. V. (1993) Differential replication capacities of G1 and S-phase extracts from sea urchin eggs. J. Cell Sci. 104, 565 – 572.