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A Developmental Role for Ataxia-Telangiectasia Mutated in Protecting the Embryo from Spontaneous and Phenytoin-Enhanced Embryopathies in Culture

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Ataxia-telangiectasia (A-T) is characterized by impaired recognition and repair of DNA damage and increased sensitivity to ionizing radiation (IR), cancer, and neurodegeneration. We previously showed pregnant knockout mice lacking the A-T gene product ataxia-telangiectasia mutated (Atm) are highly susceptible to the embryopathic effects of IR, which damages DNA, possibly via generation of reactive oxygen species (ROS). Here we show that Atm more broadly protects against both spontaneous and phenytoin-enhanced embryopathies. In the absence of drug exposure, cultured embryos from pregnant *Atm* knockout mice showed more embryopathies than wild-type littermates, with a gene dose-dependent decrease in susceptibility from −/− to +/− to +/+ embryos ($p < 0.05$). A similar but significantly enhanced gene dose-dependent pattern of embryopathic susceptibility was evident in *Atm* knockout embryos exposed to the ROS-initiating teratogen phenytoin ($p < 0.05$). These results provide the first evidence that Atm has a broad developmental importance beyond IR embryopathies, possibly by protecting the embryo from constitutive and xenobiotic-enhanced oxidative stress, with even heterozygotes showing increased risk. This developmental role of Atm further implicates DNA damage in ROS-mediated teratogenesis and DNA damage response and repair as risk factors for individual susceptibility.

Key Words: **Atm; ataxia-telangiectasia; oxidative stress; reactive oxygen species; phenytoin; development; embryopathy; developmental toxicology.**

Ataxia-telangiectasia (A-T) is an autosomal recessive and progressive disorder characterized by a wide variety of clinical manifestations (Meyn, 1999), including enhanced sensitivity to ionizing radiation (IR), progressive cerebellar ataxia, premature aging, immunodeficiency, gonadal dysgenesis, genetic instability, and enhanced tumorgenesis (Kamsler *et al.*, 2001; Rotman and Shiloh, 1999). This pleiotropic disorder occurs due to complete loss of the ataxia-telangiectasia–mutated (*Atm*) gene product, the ATM protein, which is a serine/threonine kinase. Recent studies suggest that ATM is recruited to and activated at sites of DNA double-strand breaks (Andegeko *et al.*, 2001), and the ATM kinase activity is enhanced immediately following exposure of cells to double-strand break-inducing agents such as IR, bleomycin, radiomimetic drugs like neocarzinostatin (Andegeko *et al.*, 2001), restriction endonucleases, and inhibitors of topoisomerase such as camptothecin (Thacker, 1994) and/or the topoisomearse II inhibitor, etoposide (Andegeko *et al.*, 2001). Furthermore, ATM is an important signal transducer that directs the repair of DNA double-strand break damage by phosphorylating numerous target proteins like p53 that halt the cell cycle and initiate DNA repair or apoptosis (Banin *et al.*, 1998; Canman *et al.*, 1998).

Cells from A-T patients have elevated amounts of residual chromosomal damage following irradiation (Pandita and Hittelman, 1992), are deficient in repairing DNA double-strand breaks (Rotman and Shiloh, 1999), and are markedly impaired in IR-induced activation of G_1-S, intra-S, and G_2-M cell-cycle checkpoints (Shiloh, 2001). Adult *Atm*-null knockout mice accumulate oxidative DNA damage (Kamsler *et al.*, 2001; Quick and Dugan, 2001), and with increasing age, *Atm*-null knockout mice show many features of the human A-T syndrome (Quick and Dugan, 2001). In pregnancy, *Atm*-null mice have not been reported to exhibit birth defects or other developmental abnormalities, suggesting that Atm may not be required for normal development.

However, the developing embryo has relatively little protection against reactive oxygen species (ROS) (Nicol *et al.*, 2000; Wells *et al.*, 2005), and embryonic oxidative DNA damage caused by both constitutive (Nicol *et al.*, 2000) and xenobiotic-enhanced (Wells *et al.*, 2005) ROS formation has been implicated in embryonic death, birth defects, and postnatal lethality in mouse models. Further evidence implicating ROS-dependent embryopathies includes proteratogen bioactivation by embryonic prostaglandin H synthases (Wells *et al.*, 2005; Winn and Wells, 1997) to free radical intermediates

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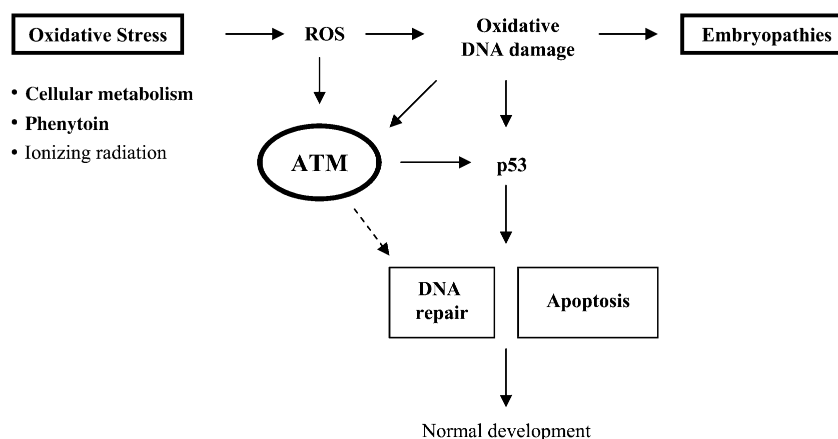

FIG. 1. Postulated developmental role for ATM in protecting the embryo from constitutive and phenytoin-enhanced oxidative stress. ROS are formed during normal cellular metabolism, and ROS formation is enhanced by numerous teratogenic agents including the anticonvulsant drug phenytoin and IR. ATM serves as a ROS sensor, either directly or by detecting damage to cellular macromolecules. Upon activation, ATM serves as a transducer for multiple signaling pathways, including p53 and complementary DNA damage response pathways, initiating apoptosis or repair of oxidative DNA damage that ultimately protects against embryopathies.

(Parman *et al.*, 1998; Wells *et al.*, 2005); hydroxyl radical formation (Wells *et al.*, 2005); oxidation of embryonic cellular macromolecules, including DNA (Nicol *et al.*, 2000; Wells *et al.*, 2005; Winn and Wells, 1999); and embryo protection by antioxidative enzymes (superoxide dismutase, catalase, glutathione [GSH] reductase, GSH peroxidase, glucose-6-phosphate dehydrogenase [G6PD]) (Nicol *et al.*, 2000; Wells *et al.*, 2005; Winn and Wells, 1999), antioxidants, and free radical trapping agents (GSH, caffeic acid, vitamin E, phenylbutylnitrone) (Wells *et al.*, 2005).

Knockout mice deficient in p53, which contributes to DNA repair (Fig. 1), are also more susceptible to the embryopathic effects of several ROS-initiating, DNA-damaging teratogens (Moallem and Hales, 1998; Nicol *et al.*, 1995), including the ROS-initiating anticonvulsant drug phenytoin (Wong and Wells, 2002), suggesting the potential importance of DNA damage in teratogenesis. As might be expected, since ATM activates p53 expression (Saito *et al.*, 2002) (Fig. 1), pregnant *Atm*-deficient knockout mice are reported to be more susceptible to conceptal death caused by early gestational exposure to IR during early gastrulation (Heyer *et al.*, 2000). Furthermore, we recently have shown that with later gestational exposure to IR during organogenesis, *Atm*-deficient embryos are more susceptible to birth defects, *in utero* death, and postnatal lethality, indicating that Atm, like p53, has an important teratological suppressor function (Laposa *et al.*, 2004).

It has been postulated that ATM is important in the recognition and repair primarily of double-strand breaks caused by IR (Barlow *et al.*, 2000; Rotman and Shiloh, 1999; Suzuki *et al.*, 1999), which would imply a relatively limited protective importance. However, IR enhances the formation of ROS, including hydroxyl radicals, which can produce an array of oxidative damage to cellular macromolecules, not all of which result in DNA double-strand breaks. Furthermore, ROS-mediated oxidative damage resulting from even constitutive oxidative stress can have embryopathic effects when antioxidative pathways are compromised (Nicol *et al.*, 2000). Since Atm is an upstream regulator of p53, which is also a regulator in the genotoxic response pathway and a teratological suppressor gene (Nicol *et al.*, 1995) (Fig. 1), we hypothesized that Atm may have broader developmental importance in protecting the embryo from more subtle forms of oxidative DNA damage caused by constitutive and xenobiotic-enhanced ROS formation. Our embryo culture results herein, together with our *in vivo* results in the following paper (Bhuller and Wells, 2006), support this hypothesis and provide the first evidence that even heterozygous *Atm*-deficient embryos are more susceptible to both spontaneous and phenytoin-enhanced embryopathies, which may have clinical implications.

MATERIALS AND METHODS

Animals. Breeding pairs of heterozygous (+/−) *Atm*-deficient mice (129S6/SvEv Tac-Atm^{tm1Awb}, Jackson Laboratory, Bar Harbor, ME) were used to generate a colony. The animals were housed in plastic cages with ground corncob bedding (Beta Chip; Northeastern Products, Warrensburg, NY) and maintained in a temperature-controlled room with a 12-h light/dark cycle. Food (Laboratory Rodent Chow 5001; PMI Feeds, St Louis, MO) and tap water were provided *ad libitum*. Third-generation *Atm* +/+ and +/− females and +/− males were used for the study, since −/− males and females are infertile. One male was housed with three females from 5:00 P.M to 9:00 A.M. The presence of a vaginal plug in a female mouse was considered as gestational day (GD) 1, and these females were separated from the colony and housed together in groups of four or fewer animals per cage.

Embryo culture. Pregnant *Atm* +/+ or +/− dams were sacrificed on GD 9.5 by cervical dislocation, embryos were explanted, and embryo culture was performed as described previously (Winn and Wells, 1995). Briefly, the number of somite pairs (i.e., 4–6) at the beginning of the culture period was used to characterize the embryonic stage or baseline. After a 24-h exposure to either the drug vehicle (0.002 *N* NaOH) or a concentration of phenytoin (20 μg/ml, 80 μM) that is within the therapeutic range (10–20 μg/ml) (Sigma Chemical Co., St Louis, MO), the embryos were analyzed for developmental and morphological

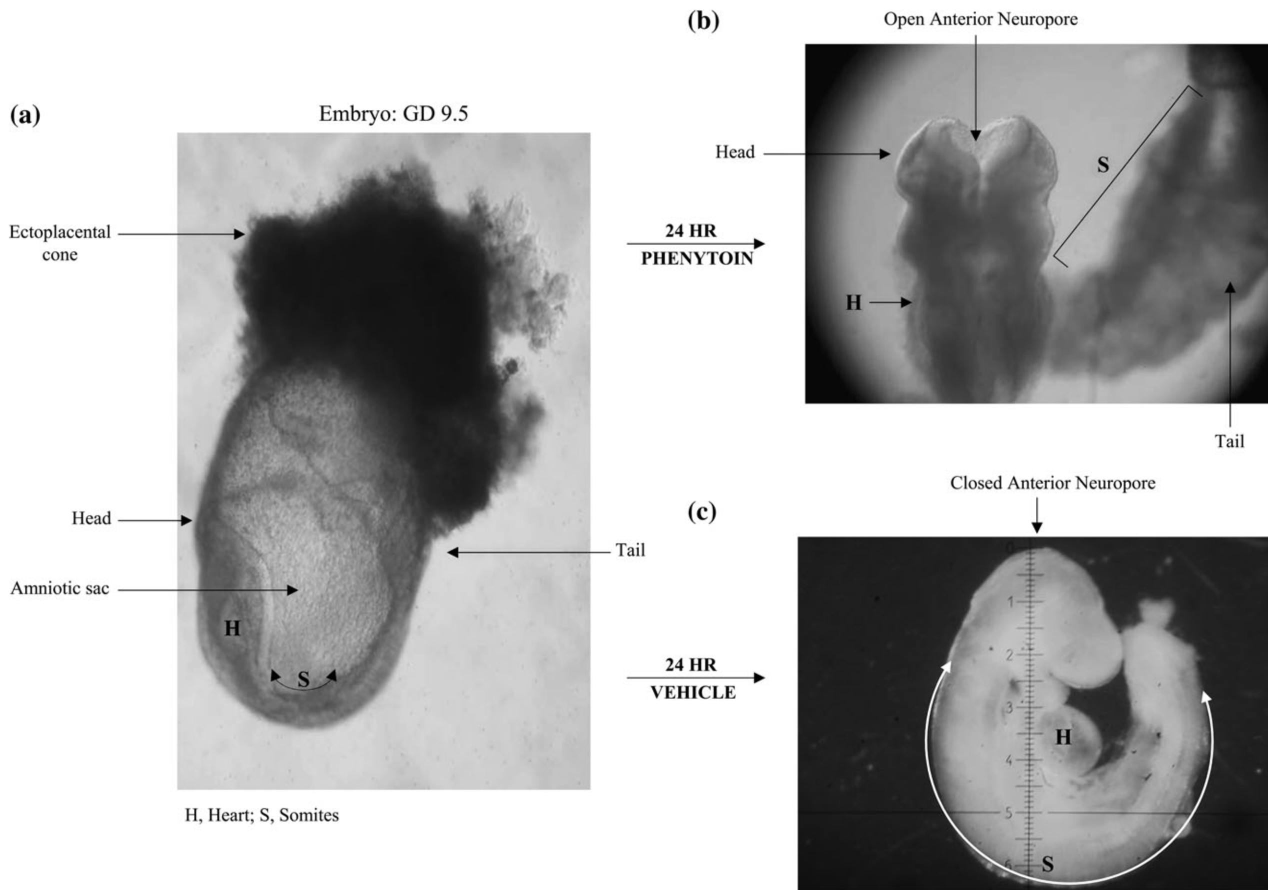

FIG. 2. Embryonic growth and development in culture. (a) The left hand panel shows the lateral view of an embryo within its membranes at the beginning of the culture on GD 9.5 (vaginal plug = GD 1). At this stage, the embryo exhibits four to six somite pairs (S) along its back or dorsal aspect, indicated by the double-headed arrow, and is oriented in a ''belly flop'' or ''squirrel'' position with the heart (H) on the ventral side. (b) The right upper panel shows the effect of a 24-h exposure to phenytoin, during which the embryo has failed to either turn or close its anterior neuropore. (c) The right lower panel shows the embryo 24 h later with its membranes removed, at the end of the culture period, during which the embryo has ''turned'' into the ''C'' or ''fetal'' position, closed its cranial anterior neuropore, and increased by up to 24 the number of somite pairs along its back, indicated by the extended double-headed arrow. This outcome is similar to that for an embryo developing *in vivo* over the same gestational period.

parameters (Fig. 2). Individual embryos with their yolk sacs were snap frozen in liquid nitrogen and used as a source of DNA for genotyping (see below).

Genotyping. Offspring of the mice in our breeding colony were genotyped by a PCR-based assay using mouse-tail DNA that was purified using a resin-based DNA extraction kit (Qiagen, Mississauga, ON). The resin-based DNA extraction kit was also used to purify genomic DNA from yolk sacs and embryos. PCR was also used to determine the embryonic genotype from cultured embryos. The PCR primers utilized were neo sense 013 (5′-CTTGGGTGGAGAGGCTA TTC-3′), neo antisense 014 (5′-AGGTGAGATGACAGGAGATC-3′), *Atm* sense 640 (5′-GCTGCCATACTTGATCCATG-3′), and *Atm* antisense 641 (5′-TCCGAATTTG CAGGAGTTG-3′) (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, Canada). The reaction conditions were: 2 µl per reaction (5–20 ng) genomic DNA, 1.2 µl per sample of 10× PCR buffer (Perkin Elmer, Branchburg, NJ), 1.2 µl per sample of 25 mM $MgCl_2$ (Perkin Elmer), 0.024 µl per sample of 5 U/µl *Taq* polymerase (Perkin Elmer), 0.96 µl per sample of 2.5 mM dNTP, 0.3 µl per sample of 20 µM of each primer, 1.66 µl per sample of DNA loading dye (cresol red [sodium salt], Sigma), and 3.756 µl per sample of ddH_20 for a final volume of 12 µl. Cycling conditions were—94°C for 3 min; 12 cycles of: 94°C, 20 s; 64°C, 30 s; 72°C, 35 s, with the annealing temperature decreasing by 0.5°C per cycle, followed by 25 cycles of: 94°C, 20 s; 58°C, 30 s; 72°C, 35 s, and completed with a final extension at 72°C for 2 min and then placed on hold at 4°C. The PCR products were separated on a gel consisting of 1.5% (w/v) agarose, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA.

Immunoprecipitation/western blotting. Maternal cerebellum and ''whole'' brain (without the cerebellum) from *Atm* +/+, +/−, and −/− dams and approximately 20 *Atm* +/+ GD 9.5 embryos were immediately snap frozen in liquid nitrogen. Cerebellum from +/+ mice was used as a positive control (Kuljis *et al.*, 1997; Oka and Takashima, 1998), and wild-type whole brain (without the cerebellum) and *Atm* −/− cerebellum were used as negative controls. The samples were homogenized (T8-Turrax, IKA Scientific, Wilmington, NC) for 1 min on ice in RIPA buffer (1× phosphate-buffered saline, 1% Nonidet P-40 [Amaresco, Solon, Ohio], 0.5% sodium deoxycholate, and 0.1% SDS), which was supplemented immediately prior to use with a mammalian protease inhibitor cocktail (Sigma). Cellular debris was removed by centrifugation at 10,000 × g for 5 min in a microcentrifuge (10 min at 4°C). The supernatant was removed and total cellular protein quantified using a high-sensitivity adaptation of the Lowry assay. Total cellular protein (500 µg) was incubated with 1 µl of normal rabbit IgG (control), 20 µl of resuspended Protein G-Agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and adjusted to 500 µl with RIPA buffer. The tubes were incubated at 4°C for 30 min, with end over end rotation, and the samples were centrifuged at 1000 × g for 5 min to pellet the beads. The supernatant (cell lysate) was transferred to a 1.5-ml

microcentrifuge tube, and 4 μl of primary antibody (ATM [H-248], Santa Cruz Biotechnology Inc.) was added. After incubation for 1 h at 4°C, using end over end rotation, 20 μl of resuspended Protein G-Agarose were added, and the tubes were incubated overnight. The samples were centrifuged at 1000 × g for 5 min at 4°C, washed four times with RIPA buffer, and after each wash the supernatant was carefully aspirated and discarded. The pellet was resuspended in 40 μl of 2× sample buffer (1.0 ml glycerol, 0.5 ml β-mercaptoethanol, 3.0 ml 10% SDS, 1.25 ml 1.0M Tris-HCl, pH 6.7, and 1–2 mg bromophenol blue). The samples were boiled for 2–3 min and 12 μl of each sample, along with 12 μl of prestained molecular weight standards (SeeBlue +2 Prestained Protein Standard, Invitrogen, Carlsbad, CA), were loaded on a 5% acrylamide gel. The gel was run at constant current (20 mA) until the 250-kDA marker migrated halfway into the resolving gel. Proteins were transferred to a polyvinylidene difluoride membrane (HyBond-P, Amersham Pharmacia Biotech, Piscataway, NJ), blocked in 5% skim milk/Tris-buffered saline Tween (TBST), and incubated overnight at 4°C on a rocker platform. The membrane was then washed for 5 min in TBST and incubated overnight at 4°C, with Atm (H-248) (Santa Cruz Biotechnology Inc.), diluted 1:200 in TBST/5% powdered milk. After washing in six 10-min changes of TBST with rocking, anti-rabbit IgG-horseradish peroxidase diluted 1:5000 (Santa Cruz Biotechnology Inc.) was used to incubate the membrane for 1 h at room temperature. The membrane was then washed for four 5-min changes of TBST, and antibody-bound proteins were visualized using the Amersham ECL chemiluminescent kit for 5 min and developed in an automatic processor (Kodak M35A X-OMAT processor).

Statistical analysis. The statistical significance of differences between treatment groups in each study group was determined using a standard computerized statistical program SigmaStat software, version 2.03 (Jandel Scientific, San Rafael, CA). Groups were compared using one-way analysis of variance, and the significance of differences between specific pairs was determined by Tukey's test. Binomial data were examined using the chi-square test or Fisher's exact test. A probability of $p < 0.05$ was chosen as the level for statistical significance.

RESULTS

Effect of Atm *Deficiency in Untreated Embryos*

Since the *Atm* strain had not been previously investigated using phenytoin, we carried out a preliminary validation study using CD-1 mice as a positive control. The CD-1 mouse is well characterized for its susceptibility to phenytoin embryopathies in culture (Winn and Wells, 1995, 1997), and both embryonic development in culture and the phenytoin-enhanced embryopathies observed herein were consistent with these published results (Fig. 3, left side).

For wild-type (+/+) *Atm*-normal embryos cultured in the absence of drug, developmental and morphological parameters were similar to those from cultured embryos from other mouse strains (Winn and Wells, 1995, 1997), including the CD-1 embryos examined herein (Fig. 3), and were considered normal (Figs. 2 and 3). In contrast, untreated *Atm*-null (−/−) embryos had reduced yolk sac diameter, crown-rump length, and somite development compared to their +/+ littermates ($p < 0.05$) (Fig. 3). Furthermore, even the untreated +/− *Atm*-deficient embryos had a reduction in these parameters compared to +/+ littermates ($p < 0.05$), although the severity of these embryopathies was less than that in their −/− *Atm*-deficient littermates ($p < 0.05$). There was no effect of embryonic *Atm* genotype on the parameters of anterior neuropore closure or turning.

Embryopathic Effects of Phenytoin in Atm-*Deficient Embryos*

In +/+ *Atm*-normal embryos incubated with the ROS-initiating teratogen phenytoin compared to vehicle controls, there were enhanced embryopathies reflected by reductions in yolk sac diameter, crown-rump length, and somite development ($p < 0.05$) (Fig. 3). There was also a trend for decreased anterior neuropore closure and turning, but unlike in other strains (Winn and Wells, 1995), this difference was not significant, suggesting that this strain is somewhat resistant to some but not all phenytoin embryopathies.

In −/− *Atm*-deficient embryos, phenytoin was highly embryopathic, with substantial reductions in anterior neuropore closure and turning, as well as reductions in yolk sac diameter, crown-rump length, and somite development ($p < 0.05$). These reductions were compared to both control −/− *Atm*-deficient littermates exposed to vehicle and control +/+ *Atm*-normal littermates exposed to phenytoin. The most dramatic embryopathic enhancement by phenytoin was for reduced anterior neuropore closure and turning, which were not affected by *Atm* deficiency in the absence of drug exposure.

Heterozygous *Atm*-deficient embryos demonstrated intermediary susceptibility to phenytoin embryopathies compared to drug-exposed +/+ *Atm*-normal and −/− *Atm*-deficient littermates, as well as to vehicle-exposed control embryos of the identical genotype. In particular, phenytoin-exposed +/− *Atm*-deficient embryos demonstrated intermediary susceptibility to reduced yolk sac diameter, crown-rump length, and somite development compared to the other phenytoin-exposed *Atm* genotypes and were more affected than control embryos of the same +/− genotype exposed only to vehicle ($p < 0.05$). A similar trend was evident with anterior neuropore closure and turning, but these differences were not significant.

Embryonic Atm Protein Expression during Organogenesis

Immunochemical detection was used to determine the expression of Atm in +/+ *Atm*-normal embryos during organogenesis and particularly during the gestational period of our embryo culture studies. Atm was highly expressed in GD 9.5 embryos compared to several positive and negative controls using tissues from adult animals (Fig. 4). As a positive control, Atm was expressed in adult *Atm* +/+ cerebellum, while in negative controls, Atm was neither expressed in adult *Atm* +/+ whole brain (without cerebellum) nor in adult *Atm*-deficient −/− cerebellum (Fig. 4).

Embryonic Atm Expression during Organogenesis

PCR was used to determine the genotype of the embryos during organogenesis. The wild-type allele produced a band at 147 bp, and the null allele produced a band at 280 bp (Fig. 5).

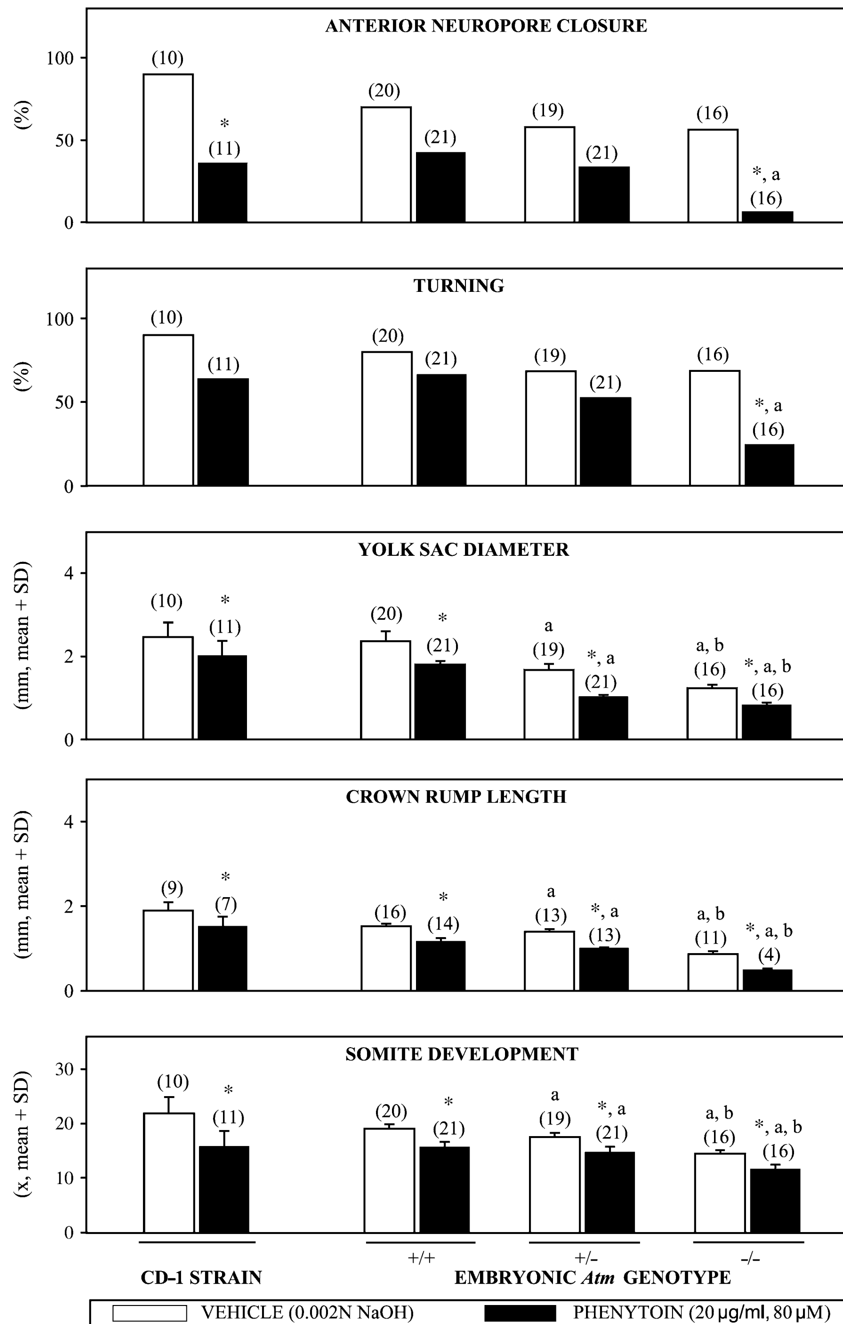

FIG. 3. Gene dose effect of *Atm* on spontaneous and phenytoin-enhanced embryopathies. On GD 9.5, *Atm* wild-type (+/+) and +/− and −/− knockout embryos were explanted and incubated for 24 h with either a therapeutic concentration of phenytoin (20 μg/ml, 80 μM) or its vehicle (0.002 *N* NaOH). The number of embryos is given in parentheses. Asterisk indicates a significant difference from vehicle control embryos of the same genotype, "a" indicates a significant difference from +/+ embryos with the same treatment, and "b" indicates a significant difference from +/− embryos with the same treatment ($p < 0.05$).

DISCUSSION

Atm has been shown previously to be embryoprotective against even low doses of IR during organogenesis (Laposa *et al.*, 2004), against earlier gestational exposure to IR during gastrulation (Heyer *et al.*, 2000), and against postnatal exposure of the CNS to high IR doses (Herzog *et al.*, 1998). While some investigators have speculated that *Atm*-mediated protection against IR is elicited primarily by DNA double-strand breaks (Barlow *et al.*, 2000; Rotman and Shiloh, 1999; Suzuki *et al.*, 1999), our results suggest that this gene has a broad developmental role in protecting the embryo from less severe forms of oxidative DNA damage, including that initiated by even constitutive embryonic oxidative stress.

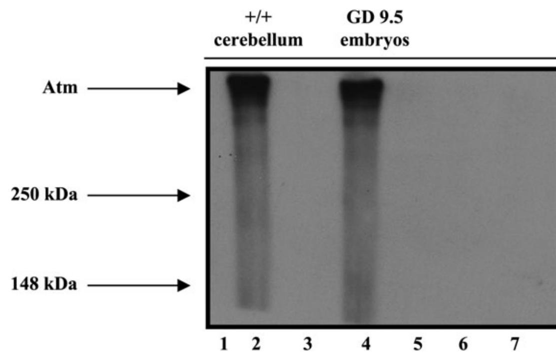

FIG. 4. Expression of Atm in GD 9.5 wild-type embryos compared to positive and negative controls from wild-type and null adult brain. Pregnant dams were sacrificed on GD 9.5 (vaginal plug = GD 1), Atm protein from embryonic and maternal tissues was separated using SDS-PAGE, and bands were detected by immunochemistry as described in Materials and Methods. Lane 1: blank; lane 2: adult cerebellum from *Atm* +/+ dam (positive control); lane 3: adult cerebellum from *Atm* −/− dam (negative control); lane 4: *Atm* +/+ embryos; lane 5: position of prestained molecular weight standards; lane 6: adult *Atm* −/− brain without cerebellum (negative control); and lane 7: adult *Atm* +/+ brain without cerebellum (negative control).

Several lines of evidence suggest that the protective mechanism may involve Atm-dependent repair of oxidative DNA damage resulting from constitutive or phenytoin-enhanced formation of ROS. This evidence includes (1) the known role of ATM in DNA repair, (2) the substantial levels of embryonic Atm protein observed herein, (3) the enhanced embryonic oxidative DNA damage reported in published studies of several ROS-initiating teratogens including phenytoin (Wells *et al.*, 2005), and (4) the enhanced oxidative DNA damage in phenytoin-exposed, −/− *Atm*-deficient embryos *in vivo*, reported in the paper by Bhuller *et al.* (2006). This interpretation is consistent with a similar pattern of gene dose dependency for embryopathic susceptibility to the DNA-damaging teratogens phenytoin (Wong and Wells, 2002),

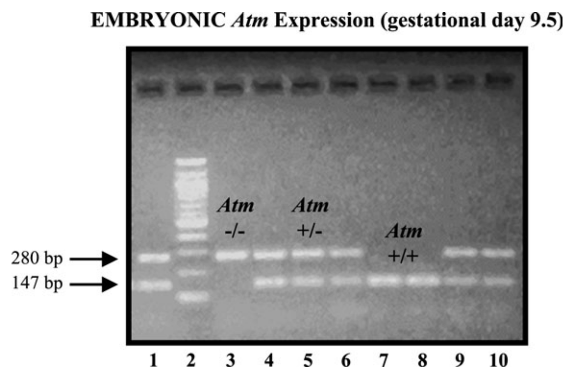

FIG. 5. Characterization of embryonic *Atm* genotype. Embryonic genotype was determined using PCR, as described in Materials and Methods. The wild-type allele produced a band at 147 bp and the null allele produced a band at 280 bp. Lane 1: *Atm* +/− embryo; lane 2: DNA ladder; lane 3: *Atm* −/− embryo; lanes 4–6: *Atm* +/− embryos; lanes 7–8: *Atm* +/+ embryos; and lanes 9–10: *Atm* +/− embryos.

benzo[*a*]pyrene (Nicol *et al.*, 1995), and cyclophosphamide (Moallem and Hales, 1998) in knockout mice deficient in *p53*, another teratological suppressor gene involved in the detection and repair of DNA damage (Komarova *et al.*, 1997).

The gene dose-dependent effect of *Atm* observed herein in embryo culture together with the enhanced DNA oxidation observed in phenytoin-exposed −/− Atm-deficient embryos *in vivo* (Bhuller *et al.*, 2006) also provide further evidence for embryopathic effects of phenytoin-enhanced ROS being mediated via oxidative DNA damage. However, the apparent embryopathic importance of oxidative DNA damage does not preclude a concomitant teratological contribution from other ROS-dependent mechanisms, as evidenced by recent studies in mouse models showing that Ras- (Winn and Wells, 2002) and NF-kB–mediated (Kennedy *et al.*, 2004) signal transduction pathways may also contribute to phenytoin embryopathies.

ATM can interact with multiple pathways involved in maintaining DNA integrity (Fig. 1) and has been particularly well characterized in response to double-strand breaks caused by IR, a classical free radical injury. However, several recent reports suggest a broader role for ATM in activating other ‘‘stress response systems’’ because (1) other DNA repair systems are preserved in A-T patients, (2) there is little evidence of elevated levels of DNA damage double-strand breaks (in the absence of IR) in A-T children, and (3) it has been difficult to explain how loss of the known function of ATM leads to degeneration of a specific population of postmitotic neurons (Quick and Dugan, 2001). The role of oxidative stress in the pathogenesis and progression of A-T was initially proposed due to the fact that cells from A-T patients appeared to be in a continuous state of oxidative stress as suggested by biomarkers, such as NF-kB, that were chronically elevated in lymphoblasts and fibroblasts from A-T patients (Rotman and Shiloh, 1999). Furthermore, another study of putative hydroxyl radical formation based upon the oxidative hydroxylation of salicylate indicated elevated ROS levels in *Atm*-deficient (−/−) cerebellum at 8 and 18 weeks of age, along with elevated superoxide levels in *Atm*-deficient (−/−) cerebellar Purkinje cells, which correlated well with the elevated levels of salicylate oxidation (Quick and Dugan, 2001). Elevated levels of GSH in *Atm*-deficient (−/−) mice was also proposed to reflect a compensatory mechanism to deal with overproduction of ROS, as were an increase in Mn-SOD activity and a decrease in catalase activity (Kamsler *et al.*, 2001). The pathological potential of constitutive oxidative stress is evident in the developing embryo, exemplified by the increase in *in utero* embryopathies in untreated mutant mice deficient in the antioxidative enzyme G6PD (Nicol *et al.*, 2000), and the increased embryopathies in the *Atm*-deficient mice observed in this study.

The protective importance of antioxidative pathways is particularly critical for this study because, although mature cells have developed various enzymatic systems to detoxify ROS and repair damaged DNA, embryos are deficient in many

of these pathways. Although embryonic activity of G6PD is similar to that in adults (Nicol *et al.*, 2000), activities of other antioxidative enzymes like SOD, catalase, and GSH peroxidases during organogenesis are generally about 5% or less of adult activity (Wells *et al.*, 2005). Thus, compared to adult animals, this relative developmental deficiency in antioxidative activity makes *Atm*-deficient −/− and +/− embryos dramatically more susceptible than *Atm* +/+ embryos to both constitutive and phenytoin-enhanced ROS and subsequent damage, as reported in this study. Furthermore, the developmental importance of ATM deficiencies observed herein may have clinical implications. The rare, 1 in 40,000–100,000, frequency of ATM-null people (Meyn, 1999) may be due in part to ROS-mediated *in utero* conceptal death, which would be substantially exacerbated in embryos also exhibiting a common deficiency in G6PD, the major conceptal antioxidative enzyme during organogenesis (Nicol *et al.*, 2000). Perhaps more importantly, there is a 1–2% incidence of people with a heterozygous deficiency (Smilenow *et al.*, 2001), suggesting a potentially substantial population at risk.

From a developmental perspective, our results show for the first time that Atm is expressed in the developing embryo during organogenesis, the critical period for susceptibility to phenytoin teratogenicity and the time of greatest risk for various spontaneous embryopathies including *in utero* death. The protein levels were evaluated to determine whether wild-type embryos expressed Atm during the developmental period encompassing the embryo culture model, which was essential to the use of this mouse model, and proved to be true. The results are also consistent with the report of increased Atm mRNA, albeit not protein activity, in rat embryos (Vinson and Hales, 2003). Atm has also been reported to be colocalized with the peroxisomal matrix protein catalase, suggesting a role for Atm in regulating catalase activity (Kamlser *et al.*, 2001), which we have found to be protective against ROS-initiating teratogens (Wells *et al.*, 2005; Winn and Wells, 1999). Accordingly, the presence of Atm during organogenesis and the extranuclear pools of Atm may also contribute to its observed protection against ROS by modulating cellular responses to oxidative stress in cerebellar neurons and in the developing embryo.

In conclusion, this study showed that Atm-deficient embryos were more sensitive to even spontaneous embryopathies, with heterozygotes and null embryos progressively more susceptible than their wild-type littermates. Embryopathies were further enhanced by the ROS-initiating teratogen phenytoin, with the same gene dose dependency, indicating that *Atm*, like *p53*, may have a broader developmental role. In this role, Atm may function as an upstream "sensor" of ROS and/or oxidative DNA damage, triggering signal transduction pathways that protect the cell from constitutive or xenobiotic-enhanced oxidative stress. The potential significance of this broader role for Atm is further enhanced by the increased embryopathic risk observed with even a heterozygous deficiency, which is relatively common in humans. Several lines of evidence suggest that the mechanism underlying this developmental protection involves Atm-dependent repair of embryonic oxidative DNA damage, and further studies are warranted to determine whether the embryonic *Atm* genotype modulates oxidative DNA damage in culture similar to its effect *in vivo* (Bhuller *et al.*, 2006). In summary, these studies provide the first evidence that Atm may serve a broader developmental role in protecting embryos from agents that are less energetic than IR, including constitutive and drug-enhanced ROS, and that even heterozygous *Atm*-deficient embryos are vulnerable.

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