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Brief report

ATM-mediated response to DNA double strand breaks in human neurons derived from stem cells

Sharon Biton[a], *Michal Gropp*[b], *Pavel Itsykson*[b], *Yaron Pereg*[a], *Leonid Mittelman*[c], *Karl Johe*[d], *Benjamin Reubinoff*[b], *Yosef Shiloh*[a,*]

[a] *The David and Inez Myers Laboratory for Genetic Research, Department of Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*
[b] *Hadassah Human Embryonic Stem Cell Research Center, The Goldyne Savad Institute of Gene Therapy, Hadassah University Hospital, Jerusalem 91120, Israel*
[c] *Interdepartmental Core Facility, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*
[d] *Neuralstem, Inc., 9700 Great Seneca Highway, Rockville, MD 20850, USA*

ARTICLE INFO

Article history:
Received 24 August 2006
Accepted 19 October 2006

Keywords:
Ataxia-telangiectasia
ATM
DNA damage response
Embryonic stem cells
Neural stem cells
Neurons

ABSTRACT

Ataxia-telangiectasia (A-T) is a multi-system genomic instability syndrome that is caused by loss or inactivation of the ATM protein kinase. ATM is largely nuclear in proliferating cells, and activates an extensive network of pathways in response to double strand breaks (DSBs) in the DNA by phosphorylating key proteins in these pathways. The prominent symptom of A-T is neuronal degeneration, making the elucidation of ATM's functions in neurons essential to understanding the disease. It has been suggested that ATM is cytoplasmic in neurons and functions in processes that are not associated with the DNA damage response. Recently we showed that in human neuron-like cells obtained by *in vitro* differentiation of neuroblastomas, ATM was largely nuclear and mediated the DSB response as in proliferating cells. We have now extended these studies to two additional model systems: neurons derived from human embryonic stem cells, and cortical neurons derived from neural stem cells. The results substantiate the notion that ATM is nuclear in human neurons and mediates the DSB response, the same as it does in proliferating cells. We present here unique and powerful model systems to further study the ATM-mediated network in neurons.

1. Introduction

The nuclear protein kinase ATM is the chief activator of the massive cellular response to double strand breaks (DSBs) in the DNA [1–3]. ATM orchestrates an elaborate signaling network consisting of repair mechanisms, cell cycle checkpoints, apoptotic pathways, and many other stress responses that lead the cell to repair and survival, or apoptosis. Following the induction of DSBs, ATM is activated [4] and phosphorylates a multitude of downstream targets, each of which in turn modulates one or more response pathways [3].

Loss or inactivation of ATM due to *ATM* mutations leads to a prototype genomic instability syndrome, ataxia-telangiectasia (A-T). A-T is characterized by neuronal

* *Corresponding author*. Tel.: +972 3 6409760; fax: +972 3 640 7471.
E-mail address: yossih@post.tau.ac.il (Y. Shiloh).
Abbreviations: NLCs, neuron-like cells; NPs, neuroprecursors; hESC, human embryonic stem cells; NSCs, neural stem cells
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doi:10.1016/j.dnarep.2006.10.019

degeneration, immunodeficiency, genomic instability, sensitivity to ionizing radiation and cancer predisposition [5–7]. A-T's major feature is the cerebellar ataxia, which appears in early infancy and gradually develops into severe neuromotor dysfunction. The ataxia reflects progressive degeneration of the cerebellar cortex and gradual loss of Purkinje and granule cells; other parts of the nervous system may show degenerative changes at a later age [5–7].

Understanding the neuronal degeneration, A-T's prominent feature, requires elucidating the functions of ATM in neurons. While there is a wealth of data on ATM's mobilization of the DSB response in proliferating cells, it was suggested that ATM in neurons is cytoplasmic and functions in other capacities [8–10]. This notion severed ATM's well documented function from the major symptom caused by its inactivation and obscured the molecular basis of the neurodegeneration in A-T.

Previous work in our laboratory brought genetic-molecular evidence that the neurodegeneration in A-T does indeed result from defective DSB response [11]. Subsequently, we examined ATM's subcellular localization in human neuron-like cells (NLCs) obtained by neuronal differentiation of neuroblastoma cells, and found that in this model system of human neurons, ATM is largely nuclear. We further showed that, like with proliferating cells, treatment of NLCs with DSB-inducing agents activates nuclear ATM and subsequently the ATM-mediated network [12]. These results suggested that ATM in human neurons might be nuclear and carry out a similar function as in proliferating cells. In the present work we sought to substantiate this conclusion by examining ATM's subcellular localization and function in the DSB response in two additional and unique models of human neurons. The first one is obtained by *in vitro* differentiation of pluripotent human embryonic stem cells (hESC) into neural precursors that further differentiate into the three neural lineages, including mature neurons [13–15]. The second model is based on a stable line of neural stem cells (NSC), which was isolated from fetal cerebral cortex and differentiates in culture into mature neurons (K. Johe et al., unpublished results). Here, we show that ATM is nuclear in these two model systems and is in charge of activating the DSB response.

2. Materials and methods

2.1. *Neuronal cell systems and experimental protocols*

Human embryonic stem cells (HES-1 cell line) [13], with normal karyotype (46,XX) were cultured on human foreskin feeder layers [16], and differentiation into neural precursors was carried out as previously described [15]. Derivation and maintenance of human neural stem cells from embryonic cerebral cortex were performed according to published methods [17], as were immunofluorescence and immunoblotting analyses [12,14].

2.2. *Chemicals and antibodies*

Neocarzinostatin (NCS) was obtained from Kayaku Chemicals (Tokyo, Japan). The ATM inhibitor KU-55933 was a gift from Drs. Graeme Smith and Steve Jackson (KuDOS Pharmaceuticals Ltd. and Wellcome Trust/Cancer Research UK Gurdon Institute, Cambridge, UK, respectively). Antibodies were purchased from the following manufacturers—α-neurofilament 200 (NF 200) polyclonal antibody, α-MAP-2 monoclonal antibody and α-tubulin monoclonal antibody: Sigma–Aldrich (St. Louis, MO); α-MAP-2 polyclonal antibody: Chemicon (Temecula, CA); α-GFAP polyclonal antibody: DAKO (Denmark); α-pS139-H2AX: Upstate Biotechnology, Inc. (Waltham, MA); α-Tuj1 monoclonal antibody: Covance Research Products (Berkeley, CA); α-pS15-p53 polyclonal antibody, α-pT68-Chk2 polyclonal antibody, α-pSQ/pTQ polyclonal antibody and α-pS1981-ATM monoclonal antibody: Cell Signaling Technology (Beverly, MA). α-pS1981-ATM polyclonal antibody: Rockland (Gilbertsville, PA); α-pS957-SMC1 polyclonal anti-

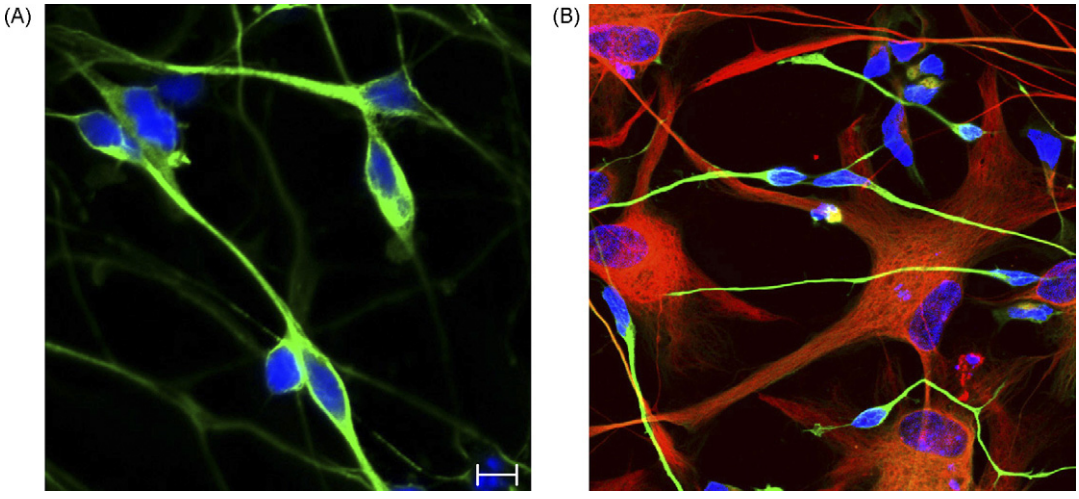

Fig. 1 – Cultures of neurons derived from hESC and NSC. (A) Neurons differentiated from hESC-derived NPs were immunostained with an antibody against neuronal microtubule associated protein, MAP2 (green). Bar = 5 μm. (B) Cultures of cortical neurons derived from neural stem cells were co-stained with antibodies against the neuron-specific class III β-tubulin TUJ1 (green) and the astrocyte marker GFAP (red). The DNA was stained with DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

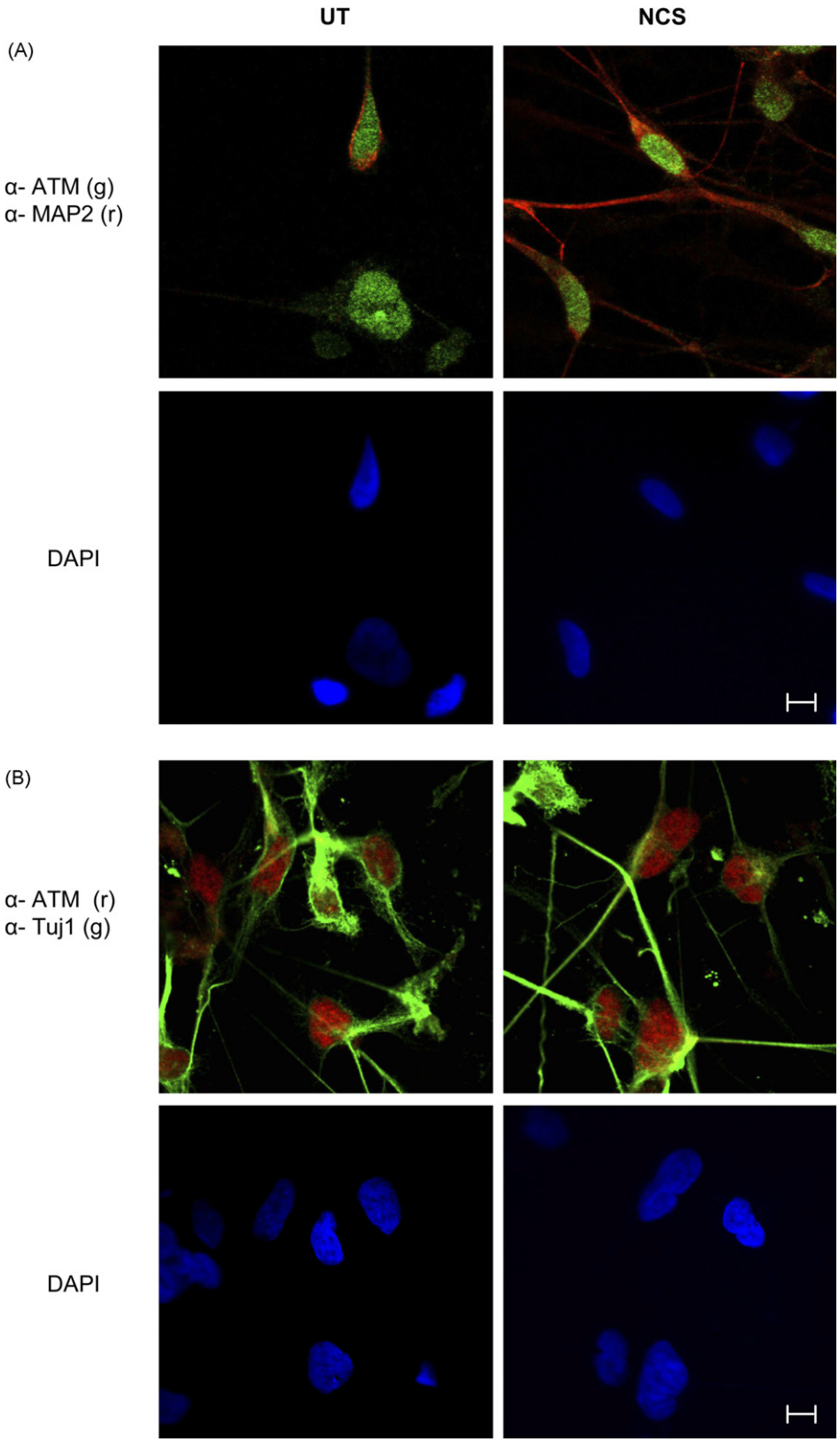

Fig. 2 – Nuclear ATM in neurons differentiated from hESC-derived NPs and in human cortical neurons. (A) Neurons differentiated from NPs derived from hESC were untreated or treated with 200 ng/ml of NCS for 30 min and subsequently immunostained with the anti-ATM antibody 5C2 (green). MAP2 (red) served as neuronal marker. Note the nuclear localization of ATM in both untreated and NCS-treated neurons. (B) Cultures of cortical neurons derived from neural stem cells were immunostained with the anti-ATM antibody Y170 (red). Tuj1 (green) served as a neuronal marker. The DNA was stained with DAPI (blue). Bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

body: Novus Biologicals, Inc. (Littleton, USA); α-pS824-KAP-1 polyclonal antibody: Bethyl Laboratories, Inc. (Montgomery, TX); α-ATM 5C2—from Dr. Eva Lee. α-ATM monoclonal antibody MAT3 was produced in our laboratory in collaboration with N. Smorodinsky; α-ATM Y170 rabbit monoclonal antibody: Epitomics, Inc. (Burlington, CA); secondary antibodies α-mouse IgG and α-rabbit IgG: Molecular Probes (Leiden, The Netherlands); HRP-conjugated α-rabbit IgG and

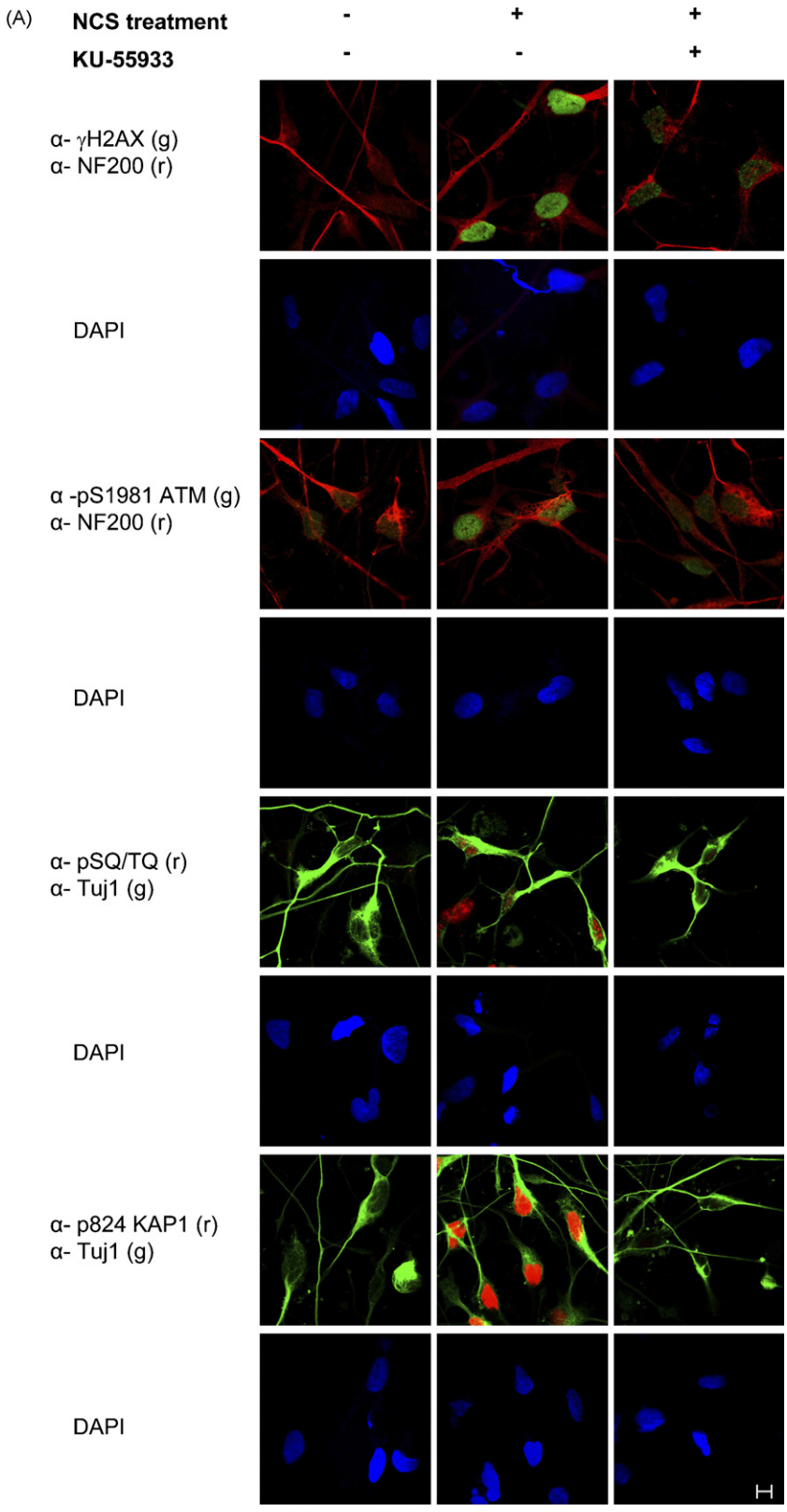

Fig. 3 – (*Continued*).

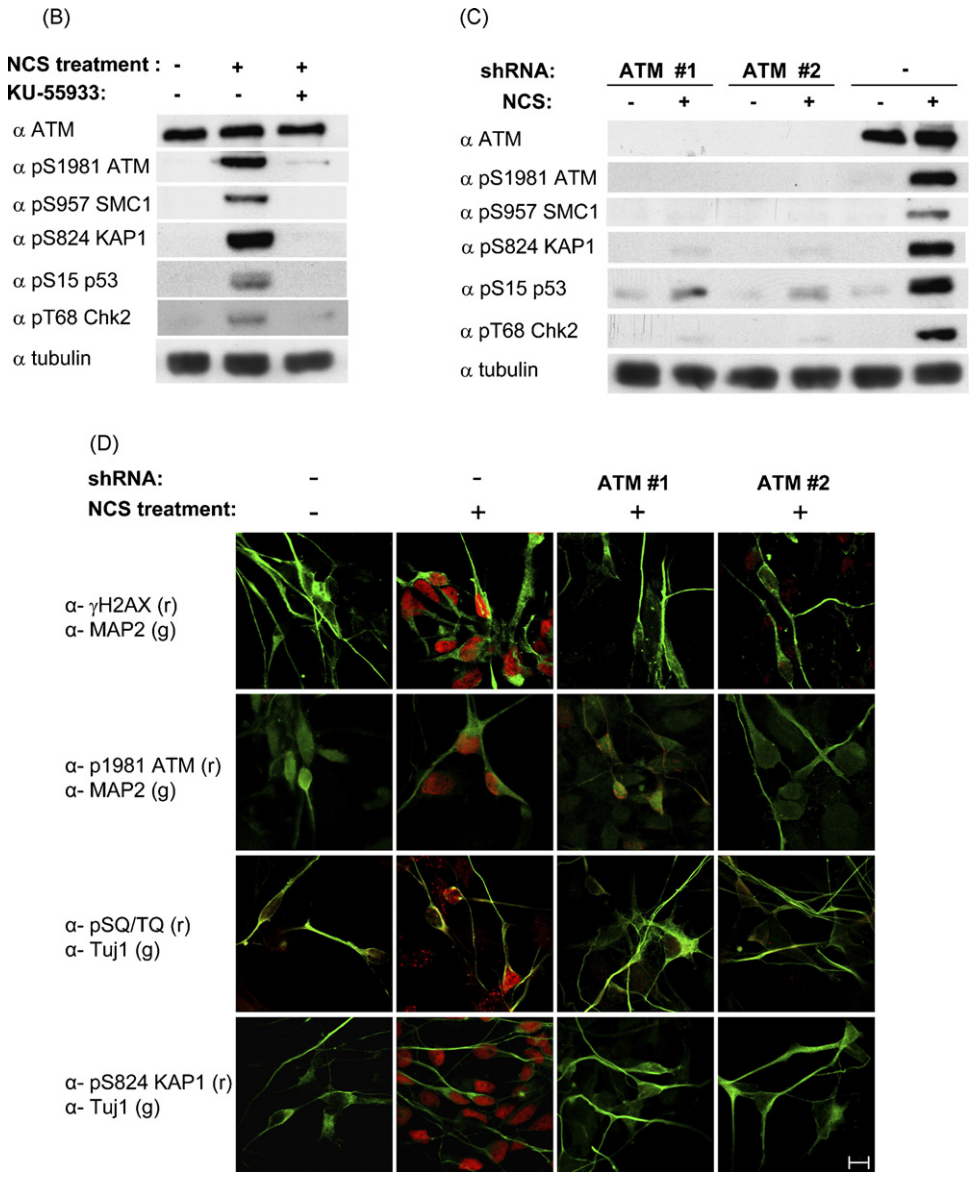

Fig. 3 – ATM activation and ATM-mediated DNA damage responses in human neurons. (A) Cortical neurons were pre-incubated with 10 μM of ATM inhibitor KU-55933 [19], subsequently treated with 100 ng/ml NCS for 30 min, and the following DSB responses were monitored using immunostaining with phospho-specific antibodies: phosphorylation of histone H2AX on Ser139 at the DSB sites (γH2AX); ATM autophosphorylation on Ser1981 [4]; phosphorylation of several ATM substrates detected collectively by an antibody against phosphorylated SQ or TQ motifs (α-pSQ/TQ); phosphorylation of the KAP-1 protein on Ser824 [24]. Bar = 5 μm. Note the nuclear responses and the effect of the ATM inhibitor on the phosphorylation of ATM targets. Similar results were obtained in neurons derived from hESC (not shown). (B) NPs derived from hESC were pre-incubated for 1 h with 10 μM of KU-55933 and subsequently treated with 100 ng/ml of the radiomimetic drug neocarzinostatin (NCS) for 30 min. Cellular lysates were subjected to immunoblotting analysis with the indicated antibodies. The following DSB-induced phosphorylations were examined: ATM autophosphorylation on Ser1981 [4], phosphorylation of the cohesin subunit Smc1 on Ser957 [25,26], phosphorylation of the KAP-1 protein on Ser824 [24], phosphorylation of p53 on Ser15 [27,28], and phosphorylation of the Chk2 protein kinase on Thr68 [29]. (C) ATM was stably knocked down in hESC using shRNA against ATM transcript [12]. Neural precursors derived from ATM-proficient and deficient hESC were treated with 100 ng/ml NCS for 30 min, and Western blotting analysis was carried out as in (B). Note ATM-dependence of all responses. (D) Immunofluorescence analysis of DNA damage responses in neurons derived from ATM-deficient and proficient NPs obtained from hESC. The cells were treated with 100 ng/ml NCS for 30 min and DNA damage responses were monitored by immunofluorescence using the indicated antibodies. Bar = 5 μm. MAP2, NF200 and Tuj1 served as neuronal markers.

α-mouse IgG: Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

2.3. *ATM knock-down in hESCs*

Generation and characterization of small hairpin RNA (shRNA) against ATM in our laboratory was described previously [12]. The shRNA cassete was cloned into a modified self inactivating HIV-based vector with green fluorescence protein (GFP) serving as a selection marker [18]. Transduction of hESCs by the HIV-1-based vector carrying the ATM shRNA cassette and GFP was carried out as previously reported [16]. Two different clones of ATM knock-down cells (#1, #2) were isolated based on GFP expression and the ATM levels.

3. Results

3.1. *Nuclear ATM in hESC-derived neurons and human neurons of cerebral origin*

The *in vitro* differentiation of hESC into neural precursors (NPs) and their subsequent differentiation into mature neurons, astrocytes and oligodendrocytes has been described [15], as have the protocols for differentiation of neural stem cells into neurons [17]. We characterized the neurons in the resultant cultures using various neuronal markers (Fig. 1). In both cell systems, immuno-localization of ATM using a highly specific antibodies [12] indicated that it was largely nuclear (Fig. 2).

3.2. *ATM-mediated DNA damage response*

We treated the cells with the radiomimetic chemical drug neocarzinostatin (NCS) and monitored their DSB responses by immunoblotting or immunofluorescence analysis using a variety of anti-phospho antibodies. One of these antibodies detects ATM autophosphorylation on Ser1981, a hallmark of its activation [4], and the rest detect the phosphorylation of several ATM targets. We used two methods to examine ATM-dependence of these phosphorylations: we treated the cells with the ATM inhibitor KU-55933 [19], which normally abolishes ATM-dependent responses; we stably knocked down ATM in hESC, induced them to differentiate, and used these ATM-deficient neurons as negative controls. The results (Fig. 3) indicated that in both cell systems, nuclear ATM was activated in response to NCS treatment, and ATM-mediated phosphorylations were induced, similar to these responses in proliferating cells.

4. Discussion

Examination of dynamic stress responses in human neurons requires the use of tissue culture-based model systems. In our previous [12] and present study we examined ATM localization and function in three such models, each one based on induced neuronal differentiation in culture. In the course of these studies we noted that knocking down ATM in hESC did not affect their neuronal differentiation—the same observed lack of effect of ATM loss on neuronal differentiation of neuroblastomas [12]. These results suggest that ATM may not have a critical role in neuronal differentiation.

In all three systems ATM was found to be largely nuclear, ATM-mediated DSB responses previously identified in proliferating cells were induced in these cells as well, and the responses were ATM-dependent. Recently we collaborated with Barzilai and colleagues to show that ATM was nuclear and mediated the DSB response in murine cerebellar tissues [20]. Collectively, the data strongly suggest that nuclear ATM mediates the DSB response in neurons as it does in proliferating cells. The accumulating data suggest that the neuronal degeneration in A-T is due to the defective DSB response that is caused by lack of ATM. The experimental systems described here are expected to be highly useful for further studies of ATM's mode of action in neuronal cells. In view of increasing attempts to use stem cells for cell replacement therapy, especially in neurodegenerative disorders [21–23], further understanding of the ATM-mediated DNA damage response in neurons should ultimately point the way to effective treatment for A-T.

Acknowledgements

This study was supported by research grants from the A-T Children's Project, The A-T Medical Research Foundation, the National Institutes of Health (NS31763), the A-T Medical Research Trust and the A-T Ease Foundation. The work was carried out in partial fulfillment of the requirements for the Ph.D. degree of SB.

REFERENCES

[1] Y. Shiloh, ATM and related protein kinases: safeguarding genome integrity, Nat. Rev. Cancer 3 (2003) 155–168.
[2] C.J. Bakkenist, M.B. Kastan, Initiating cellular stress responses, Cell 118 (2004) 9–17.
[3] Y. Shiloh, The ATM-mediated DNA-damage response: taking shape, Trends Biochem. Sci. 31 (2006) 402–410.
[4] C.J. Bakkenist, M.B. Kastan, DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation, Nature 421 (2003) 499–506.
[5] T. Crawford, Ataxia telangiectasia, Pediatr. Neurol. 5 (1998) 287–294.
[6] H.H. Chun, R.A. Gatti, Ataxia-telangiectasia, an evolving phenotype, DNA Repair (Amst.) 3 (2004) 1187–1196.
[7] A.M. Taylor, P.J. Byrd, Molecular pathology of ataxia telangiectasia, J. Clin. Pathol. 58 (2005) 1009–1015.
[8] A. Oka, S. Takashima, Expression of the ataxia-telangiectasia gene (ATM) product in human cerebellar neurons during development, Neurosci. Lett. 252 (1998) 195–198.
[9] R.O. Kuljis, G. Chen, E.Y. Lee, M.C. Aguila, Y. Xu, ATM immunolocalization in mouse neuronal endosomes: implications for ataxia-telangiectasia, Brain Res. 842 (1999) 351–358.
[10] C. Barlow, C. Ribaut-Barassin, T.A. Zwingman, A.J. Pope, K.D. Brown, J.W. Owens, D. Larson, E.A. Harrington, A.M. Haeberle, J. Mariani, M. Eckhaus, K. Herrup, Y. Bailly, A. Wynshaw-Boris, ATM is a cytoplasmic protein in mouse brain required to prevent lysosomal accumulation, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 871–876.

[11] T. Uziel, Y. Lerenthal, L. Moyal, Y. Andegeko, L. Mittelman, Y. Shiloh, Requirement of the MRN complex for ATM activation by DNA damage, EMBO J. 22 (2003) 5612–5621.
[12] S. Biton, I. Dar, L. Mittelman, Y. Pereg, A. Barzilai, Y. Shiloh, Nuclear ataxia-telangiectasia mutated (ATM) mediates the cellular response to DNA double strand breaks in human neuron-like cells, J. Biol. Chem. 281 (2006) 17482–17491.
[13] B.E. Reubinoff, M.F. Pera, C.Y. Fong, A. Trounson, A. Bongso, Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro, Nat. Biotechnol. 18 (2000) 399–404.
[14] B.E. Reubinoff, P. Itsykson, T. Turetsky, M.F. Pera, E. Reinhartz, A. Itzik, T. Ben-Hur, Neural progenitors from human embryonic stem cells, Nat. Biotechnol. 19 (2001) 1134–1140.
[15] P. Itsykson, N. Ilouz, T. Turetsky, R.S. Goldstein, M.F. Pera, I. Fishbein, M. Segal, B.E. Reubinoff, Derivation of neural precursors from human embryonic stem cells in the presence of noggin, Mol. Cell. Neurosci. 30 (2005) 24–36.
[16] I. Ben-Dor, P. Itsykson, D. Goldenberg, E. Galun, B.E. Reubinoff, Lentiviral vectors harboring a dual-gene system allow high and homogeneous transgene expression in selected polyclonal human embryonic stem cells, Mol. Ther. 14 (2006) 255–267.
[17] K.K. Johe, T.G. Hazel, T. Muller, M.M. Dugich-Djordjevic, R.D. McKay, Single factors direct the differentiation of stem cells from the fetal and adult central nervous system, Genes Dev. 10 (1996) 3129–3140.
[18] M. Gropp, P. Itsykson, O. Singer, T. Ben-Hur, E. Reinhartz, E. Galun, B.E. Reubinoff, Stable genetic modification of human embryonic stem cells by lentiviral vectors, Mol. Ther. 7 (2003) 281–287.
[19] I. Hickson, Y. Zhao, C.J. Richardson, S.J. Green, N.M. Martin, A.I. Orr, P.M. Reaper, S.P. Jackson, N.J. Curtin, G.C. Smith, Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM, Cancer Res. 64 (2004) 9152–9159.
[20] I. Dar, S. Biton, Y. Shiloh, A. Barzilai, Analysis of the ataxia telangiectasia mutated-mediated DNA damage response in murine cerebellar neurons, J. Neurosci. 26 (2006) 7767–7774.
[21] O. Lindvall, Z. Kokaia, Stem cells for the treatment of neurological disorders, Nature 441 (2006) 1094–1096.
[22] F.J. Muller, E.Y. Snyder, J.F. Loring, Gene therapy: can neural stem cells deliver? Nat. Rev. Neurosci. 7 (2006) 75–84.
[23] Y. Shufaro, B.E. Reubinoff, Therapeutic applications of embryonic stem cells, Best Pract. Res. Clin. Obstet. Gynaecol. 18 (2004) 909–927.
[24] Y. Ziv, D. Bielopolski, Y. Galanty, C. Lukas, Y. Taya, D.C. Schultz, J. Lukas, S. Bekker-Jensen, J. Bartek, Y. Shiloh, Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway, Nat. Cell Biol. 8 (2006) 870–876.
[25] P.T. Yazdi, Y. Wang, S. Zhao, N. Patel, E.Y. Lee, J. Qin, SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint, Genes Dev. 16 (2002) 571–582.
[26] S.T. Kim, B. Xu, M.B. Kastan, Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage, Genes Dev. 16 (2002) 560–570.
[27] S. Banin, L. Moyal, S. Shieh, Y. Taya, C.W. Anderson, L. Chessa, N.I. Smorodinsky, C. Prives, Y. Reiss, Y. Shiloh, Y. Ziv, Enhanced phosphorylation of p53 by ATM in response to DNA damage, Science 281 (1998) 1674–1677.
[28] C.E. Canman, D.S. Lim, K.A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M.B. Kastan, J.D. Siliciano, Activation of the ATM kinase by ionizing radiation and phosphorylation of p53, Science 281 (1998) 1677–1679.
[29] S. Matsuoka, G. Rotman, A. Ogawa, Y. Shiloh, K. Tamai, S.J. Elledge, Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 10389–10394.