Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway

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Received August 2, 2004; Revised and Accepted October 12, 2004

To date, the only reported genetic defect identified in the developmental disorder, Seckel syndrome, is a mutation in ataxia telangiectasia and Rad3-related protein (ATR). Seckel syndrome is clinically and genetically heterogeneous and whether defects in ATR significantly contribute to Seckel syndrome is unclear. Firstly, we characterize ATR-Seckel cells for their response to DNA damage. ATR-Seckel cells display impaired phosphorylation of ATR-dependent substrates, impaired G2/M checkpoint arrest and elevated micronucleus (MN) formation following exposure to UV and agents that cause replication stalling. We describe a novel phenotype, designated nuclear fragmentation (NF), that occurs following replication arrest. Finally, we report that ATR-Seckel cells have an endogenously increased number of centrosomes in mitotic cells demonstrating a novel role for ATR in regulating centrosome stability. We exploit these phenotypes to examine cell lines derived from additional unrelated Seckel syndrome patients. We show that impaired phosphorylation of ATR-dependent substrates is a common but not invariant feature of Seckel syndrome cell lines. In contrast, all cell lines displayed defective G2/M arrest, increased levels of NF and MN formation following exposure to agents that cause replication stalling. All the Seckel syndrome cell lines examined showed increased endogenous centrosome numbers. Though ATR cDNA can complement the defects in ATR-Seckel cells, it failed to complement any of the additional cell lines. We conclude that Seckel syndrome represents a further damage response disorder that is uniquely associated with defects in the ATR-signalling pathway resulting in failed checkpoint arrest following exposure to replication fork stalling.

INTRODUCTION

Two related phosphoinositol-3 kinases like protein kinases (PIKKs) play key roles in signal transduction pathways that regulate the response to DNA damage (1). Whereas ATM (ataxia telangiectasia mutated protein) is activated following DNA double-strand breakage, ATR (ataxia telangiectasia and Rad3-related protein) responds to single-strand regions of DNA exposed during stalling of replication forks and/or during repair of certain DNA adducts. ATR exists as a complex with ATRIP (ATR interacting protein) (2). It is thought that RPA coating of single-strand regions of DNA results in ATR/ATRIP complex recruitment (3,4). ATR/ATRIP phosphorylates a number of proteins that function in the DNA damage response including H2AX, 53BP1, p53, Nbs1 and Chk1 (5–8) (reviewed in 1,9). Phosphorylation of H2AX, a variant form of the histone H2A, is one of the earliest events in the damage response and appears to be required for the retention of additional damage response proteins at the site of the damage (10). Rad17/Rfc2-5 and Rad9/Rad1/Hus1 complexes are recruited to the site of damage independently of ATR but are phosphorylated in an
ATR-dependent manner (3,11). Available evidence, including studies using yeasts, indicates that their retention at the damage site facilitates ATR’s ability to phosphorylate its downstream substrates (11,12). Brc1 also facilitates ATR/ATRIP-dependent phosphorylation events (13). ATR-dependent signalling helps to stabilize stalled replication forks and establish arrest at critical cell cycle checkpoints, including intra S and G2/M phase checkpoints (9). Chk1, a substrate of ATR, plays a critical role in establishing arrest at these checkpoints (6,8). The ability of ATR to phosphorylate p53 indicates that ATR likely also regulates damage-induced apoptosis (7). ATR and ATM phosphorylate and activate many of the same substrates and thus represent overlapping pathways responding to distinct DNA lesions. Unlike ATM, however, ATR is essential for both embryonic development and somatic cell growth (2,14,15).

Mutations in ATM cause ataxia telangiectasia (A-T), a human condition conferring clinical and cellular radiosensitivity, progressive ataxia, cancer predisposition (mainly lymphoma) and immune dysfunction (16). Many of the mutations identified in A-T abolish ATM activity consistent with the finding that ATM is non-essential. Recently, we identified a hypomorphic, synonymous mutation (A2101G) in ATR that affects splicing in two related Pakistani Seckel syndrome families (SCKL1) (17). We have designated this class of Seckel syndrome as ATR-Seckel (18). As would be anticipated for an essential protein, the mutation in the ATR-Seckel family reduced but did not abolish ATR function.

Seckel syndrome is characterized by severe intrauterine growth retardation, proportionate dwarfism, microcephaly, with skeletal and brain abnormalities (OMIM 210600) (19,20). Although lymphoma has been reported in some Seckel patients, they tend not to present with ataxia or immunodeficiency (21–23). Strikingly, Seckel syndrome patients display features, including microcephaly and dysmorphic facies, commonly found in other syndromes associated with impaired responses to DNA damage (24). However, other investigations have failed to show any marked or consistent defects in the response to DNA damage in Seckel syndrome cells and the basis underlying Seckel syndrome remains largely unexplained (21,25). The identification of ATR-Seckel prompted us to re-examine the possibility that Seckel syndrome might be a DNA damage response disorder. An important function of ATR is the co-ordination of responses to replication fork stalling, which arises during normal replication possibly as a consequence of endogenous DNA damage and also following exposure to specific agents that prevent replication fork progression (9). Previous studies on Seckel syndrome have tended to focus on the response to DNA damage induced by ionizing radiation, which is handled by the ATM-dependent signal transduction pathway, or the response to cross-linking agents, which is dramatically impaired in Fanconi anaemia (FA) (21,25). Defects in ATR signalling only modestly impact upon the response to these agents. Seckel syndrome is genetically and clinically a heterogenous disorder with at least four susceptibility loci having been identified (26–29). Thus, it is unlikely that mutations in ATR can account for all instances of Seckel syndrome. In this study, we aimed to address whether Seckel syndrome might be specifically associated with an impaired response to replication fork stalling as a consequence of defects in the ATR signal transduction pathway.

To this end, we characterized an ATR-Seckel (SCKL1) cell line for its response to DNA damage and established robust assays that monitor impaired ATR function. We show that the ATR-Seckel cells exhibit a range of aberrant responses to agents that cause replication stalling. They also exhibit supernumery centrosomes in mitotic cells, demonstrating a novel role for ATR in the maintenance of centrosome stability. To examine additional Seckel syndrome patients, we established cell lines from severely clinically affected individuals with features meeting the more recently refined requirements for Seckel syndrome (28). We describe remarkable overlapping phenotypes between this panel of Seckel syndrome cell lines and the ATR-Seckel line. The characteristic cellular features of Seckel syndrome include aberrant responses to replication stalling and elevated numbers of centrosomes in mitotic cells. We conclude that Seckel syndrome is commonly caused by defects in the ATR-signalling pathway, although the defective protein frequently lies downstream of ATR.

RESULTS

Characterization of ATR-Seckel cells

ATR-Seckel cells show reduced phosphorylation of H2AX and Chk1 following treatment with replication inhibitors. Previously, we have shown that an ATR-Seckel fibroblast cell line is impaired in H2AX phosphorylation following exposure to UV (17). Here, using immunofluorescence (IF) we show that DK0064 (ATRA2101G), a lymphoblastoid cell line (LBL) derived from the same patient, is impaired in H2AX phosphorylation at 1 h following treatment with the replication inhibitor, hydroxyurea (HU) (Fig. 1A and B). We also observed reduced phosphorylation of Chk1 (Ser317), an important ATR substrate regulating cell cycle checkpoint arrest, following exposure to aphidicolin (APH), a polymerase inhibitor, in DK0064 (ATRA2101G) by IF (Fig. 1C and E) and by western blotting (Fig. 1D). HU- and APH-induced phosphorylation of both proteins (H2AX and Chk1) 1 and 2 h, respectively, post-treatment is normal in an A-T LBL (GM03189D), consistent with previous findings that ATM does not play a significant role in phosphorylation following replication stalling (30). Introduction of ATR into DK0064 (ATRA2101G) cells by transfection of ATR cDNA in a mammalian expression vector (pcDNA3–ATR) fully restores the ability of these cells to phosphorylate Chk1 after treatment with APH (Fig. 1F). These data confirm that ATR is the principle kinase that phosphorylates H2AX and Chk1 in response to replicative inhibition and demonstrate that defective functioning of this pathway can be observed in ATR-Seckel LBLs.

ATR-Seckel cells are defective in UV-induced G2/M arrest. ATR is activated following exposure to UV-irradiation (5). An important consequence of ATR signalling is arrest at the G2/M checkpoint (6). This is observed as a decrease in the percentage of mitotic cells (histone H3-p-Ser10 positive) 24 h post-UV treatment of wild-type (WT) LBLs relative to untreated cells (Fig. 1G). In contrast, DK0064 (ATRA2101G) fail to show any reduction in mitotic index in
Figure 1. Damage response defects in ATR-Seckel cell. (A) ATR-defective LBLs are impaired in HU-induced phosphorylation of H2AX. WT, ATM (GM03189D) and ATR-defective (DK0064) LBLs were treated with 5 mM HU and incubated for 1 h. Phosphorylation of H2AX on Ser139 (p-H2AX) after HU was detected using IF with phosphospecific antibodies. Nuclei were counterstained with DAPI. A representative experiment is shown in (A). (B) The percentage of cells staining positively for H2AX (Ser139) phosphorylation after treatment with 5 mM HU for 1 h was determined for each cell line. This graph represents the mean of three separate experiments. (C) ATR-defective LBLs are impaired in APH-induced phosphorylation of Chk1. WT, ATM (GM03189D) and ATR-defective (DK0064) LBLs were treated with 0.1 mM APH and incubated for 2 h. Phosphorylation of Chk1 on Ser317 (p-Chk1) was detected by IF with nuclei counterstained using DAPI. (D) Western blot analysis of APH-induced phosphorylation of Chk1 (Ser317). Cell lines were treated as in (C). (E) The percentage of cells staining positively for Chk1 phosphorylation (Ser317) following treatment with 0.1 mM APH for 2 h was determined for each cell line. This graph represents the mean of three separate experiments. (F) The defect in APH-induced Chk1 phosphorylation in DK0064 is complemented by transient transfection with full length ATR cDNA (Mock, mock transfected; ATR, transfected with pcDNA 3-ATR; UNT, untreated; APH, exposed to 0.1 mM APH for 2 h). Graph shows the mean of three separate experiments. (G) ATR-defective LBLs fail to arrest at the G2/M checkpoint following exposure to UV. WT, ATM (GM03189D) and ATR-defective (DK0064) LBLs were untreated (UNT) or exposed to UV (2.5 J/m²) and the mitotic index estimated following 24 h incubation. Mitotic cells were identified as histone H3-p-Ser10 positive cells by IF. A decrease in mitotic index following UV-irradiation was taken to indicate a G2/M checkpoint arrest. Graph represents the mean of three separate experiments. (H) The UV-induced G2/M checkpoint defect seen in ATR-defective LBLs (DK0064) is complemented by transfection with ATR cDNA (Mock, mock transfected; ATR, transfected with pcDNA3-ATR; UNT, untreated; UV, treated with 2.5 J/m²). Graph shows the mean of three separate experiments. (I) Elevated MN formation following DNA damage in ATR-defective LBLs. WT, ATM (GM03189D) and ATR defective (DK0064) LBLs were treated with HU (1 mM) and examined for MN formation following 24 h culture in the presence of 5 μg/ml cytochalasin B. A 1.5–2-fold increase in MN formation was only observed in DK0064 cells. Graph shows the mean of three separate experiments. Inset shows a representative MN in a BN. (J) Transfection of ATR-defective LBLs (DK0064) with ATR cDNA complements the elevated HU-induced MN formation observed in this line (Mock, mock transfected; ATR, transfected with pcDNA3-ATR; UNT, untreated; HU, treated with 1 mM HU).
response to UV (Fig. 1G). A-T LBLs (GM03189D) exhibit a similar reduction in mitotic index to WT cells, demonstrating efficient G2/M checkpoint arrest consistent with the fact that ATM does not regulate G2/M arrest after UV-irradiation. Furthermore, transfection of DK0064 (ATR^{A2101G}) with ATR cDNA fully complements the UV-induced G2/M checkpoint defect (Fig. 1H).

ATR-Seckel cells exhibit increased HU-induced micronuclei. Micronucleus (MN) formation is a useful and simple assay to evaluate the response to DNA damage. Around 6% of untreated WT LBLs exhibit MN (Fig. 1I). Interestingly, ATR-Seckel LBLs have an endogenous frequency of MN which is double this level, suggesting elevated genomic instability. More strikingly, following exposure to a dose of HU that fails to enhance the frequency of MN in either WT or an A-T cell line (GM03189D), DK0064 (ATR^{A2101G}) cells show a significantly elevated frequency of MN (Fig. 1I). Transfection of DK0064 (ATR^{A2101G}) with ATR cDNA complements HU-induced MN formation (Fig. 1J).

ATR-Seckel cells exhibit increased DNA damage-induced nuclear fragmentation. After treatment of ATR-Seckel cells with a range of DNA damaging agents, we observed aberrant cells when examined 24 h post-treatment in the presence of nocodazole. We have called this phenomenon nuclear fragmentation (NF) as it resembles but is distinct from classical premature chromosome condensation (PCC) (see what follows and Discussion) (Fig. 2A) shows the quantification of %NF cells, Fig. 3A shows a representative NF cell. A representative field showing an NF cell in a background of normal cells is presented in Supplementary Material, Fig. S1D). Unlike PCCs, the DNA appears to be fragmented in NF cells as observed by DAPI staining. Although there is an increase in the endogenous level of such cells in ATR-Seckel, treatment with DNA damaging agents enhances the difference between ATR-Seckel and WT cells (Fig. 2A). HU, camptothecin (CPT) and low doses of APH, agents which cause replication stalling, are potent inducers of this response in ATR-Seckel cells (Fig. 2A). IR also induces NF but the increase in ATR-Seckel versus WT cells is not significantly different. This phenotype is not observed in cells derived from A-T, FA-D2 or LIG4 syndrome patients (Fig. 2B for A-T; data not shown). However, pre-treatment of A-T cells with a high concentration of the PI3-K inhibitor wortmannin, which inhibits ATR activity, induces the NF phenotype following HU treatment (Fig. 2B). In contrast, inhibition of DNA-PK following pre-treatment of A-T cells with LY294002, a potent DNA-PK inhibitor, did not affect HU-induced NF (Fig. 2B). We conclude that inactivation of ATR rather than ATM or DNA-PK causes this phenotype. Furthermore, transfection of ATR cDNA fully complemented the UV- and HU-induced NF phenotype of DK0064 (ATR^{A2102C}) cells whereas transfection of a dominant negative kinase dead version of ATR (pcDNA3-ATR-KD) had no impact on NF (Fig. 2C). Taken together, our findings demonstrate that inactivation of ATR results in a characteristic NF phenotype. As this phenotype appears to be specific for the ATR-Seckel cells, we considered it a potentially useful assay with which to monitor additional cell lines from Seckel patients. We, therefore, undertook further characterization of this phenotype to establish its basis.

As NF cells partially resemble apoptotic cells, we used the TUNEL assay to examine whether they represent cells undergoing apoptosis. HU-induced nuclear fragmented cells failed to stain positive in the TUNEL assay in contrast to other cells present in the population, indicating that NF cells are not apoptotic cells (Supplementary Material, Fig. S1A).

To examine whether NF formation is a consequence of replication stalling in the absence of ATR, DK0064 (ATR^{A2101G}) cells were arrested and maintained at the G1/S boundary using double thymidine block and then exposed to UV. UV irradiation of these arrested cells failed to induce NF when examined 24 h post-treatment (Fig. 2D). Thus, we conclude that UV photolesions do not induce NF in the absence of DNA replication. To examine whether NF requires progression through S phase, DK0064 (ATR^{A2101G}) cells arrested at the G1/S boundary by double thymidine block were released into S phase following removal of thymidine, UV-irradiated at different times post-release and then incubated for 24 h in the presence of nocodazole prior to analysis for NF. Progression of the synchronized cells through S phase was monitored by incorporation of [3H] thymidine (inset Fig. 2E). DNA synthesis was maximal at 2 h and continued to 6 h post-release. By 8 h, most cells had progressed through S phase. Parallel irradiation and subsequent analysis of NF in the synchronized DK0064 (ATR^{A2101G}) cells showed that NF was maximally elevated in cells undergoing replication but decreased to background levels when cells are no longer replicating at the time of treatment (Fig. 2E). This strongly suggests that NF is a consequence of replication in the presence of DNA damage in ATR-defective cells. To gain further evidence that NF is derived from damaged S phase cells, DK0064 (ATR^{A2101G}) were pulse labelled with BrdU (30 min) prior to UV-irradiation. NF was only observed in BrdU-labelled cells, demonstrating that in DK0064 (ATR^{A2101G}) NF are derived from cells that have replicated in the presence of DNA damage (Supplementary Material, Fig. S1B).

To examine whether NF cells might represent those that have prematurely progressed to mitosis, we examined them for the presence of standard mitotic markers. It should be noted that NF is examined in the presence of nocodazole, thus cells cannot have progressed beyond mitosis. One event characteristic for DNA condensation during mitosis is phosphorylation of the histone H3 (H3-p-Ser10). UV-induced NF cells did not stain for H3-p-Ser10 although H3-p-Ser10 positive cells were observed amongst non-fragmented cells (Fig. 3A). This phenotype further distinguishes NF from PCCs, which stain positively for H3-p-Ser10 (31,32). Another feature of mitotic cells is the appearance of two distinct γ tubulin containing centrosomes. UV-induced NF do not contain detectable γ tubulin staining (Fig. 3A). As cells enter mitosis, cyclin B1 which is normally cytoplasmic, enters the nucleus. UV-induced NF cells also fail to stain for cyclin B1 (Fig. 3B). Finally and importantly, the lamin B1 containing nuclear envelope is normally specifically degraded during the prophase stage of mitosis. Most UV-induced NF cells have remnants of lamin B1 staining, indicating that the nuclear membrane is partially intact (Fig. 3C). These studies
show that NF cells have not progressed normally through mitosis. The extensive fragmentation of the DNA in NF cells precluded an assessment of whether they have undergone chromosome condensation. The presence of lamin staining suggests that they are probably derived from late S/G2 phase or early prophase cells. Hence, DNA damage-induced NF appears in the context of defective ATR and is dependent on replication in the presence of DNA damage. We propose that it represents a consequence of a failure to recover from replication stalling and/or to coordinate cell cycle checkpoint responses following replication of damaged DNA.

**Figure 2.** Characterization of NF, a novel phenotype of ATR-Seckel cells. (A) ATR-defective cells show increased nuclear fragmentation following exposure to DNA damaging agents. WT and ATR-defective (DK0064) LBLs were treated with various DNA damaging agents (APH 0.1 mM, HU 5 mM, CPT 3 mM, UV 10 J/m², IR 10 Gy) and examined after 24 h growth in the presence of 1.5 μM nocodazole. Drugs were maintained in the medium during this period. This phenotype is not observed in cells from A-T, LIG4 syndrome or FA-D2 patients (Fig. 2B and data not shown). Graph represents the mean of at least three separate determinations. (B) HU-induced NF formation correlates with inhibition of ATR kinase activity. HU-induced NF formation is neither observed in cells from A-T (GM03189D) nor in GM03189D cells pre-treated with 200 μM LY294002 (Ly) to inhibit DNA-kinase activity (Ly and HU + Ly). HU-induced NF is observed in GM03189D pre-treated with wortmannin (100 μM), which inhibits ATR (W and W + HU). Ctrl, untreated GM03189D; HU, GM03189D cells exposed to 5 mM HU for 24 h in the presence of 1.5 μM nocodazole; Ly, LY294002-treated GM03189D cells; HU + Ly, GM03189D cells treated with HU and Ly; W, wortmannin-treated GM03189D cells; HU + W, GM03189D cells treated with HU and wortmannin. (C) HU (5 mM) and UV (10 J/m²) induced NF can be complemented in DK0064 by transfection with full length ATR but not with a dominant negative kinase-dead ATR cDNA. Mock, mock transfected DK0064 cells either UNT, untreated; HU, exposed to 5 mM HU for 24 h or treated with UV 10 J/m² and examined 24 h post-irradiation; ATR, cells transfected with wild type human ATR cDNA and treated as described earlier. Graph represents the mean of at least three separate determinations. (D) NF formation requires active DNA replication. DK0064 (ATR-Seckel) cells were either untreated (UNT) or arrested and maintained at the G1/S border using a double thymidine block (DT) and analysed for 24 h following no further treatment (UNT) or after UV-irradiation (UV). Graph represents the mean of at least three separate determinations. (E) UV-induced NF formation in DK0064 (ATR- Seckel) occurs maximally during S phase. DK0064 (ATR-Seckel) cells synchronized by DT block were examined for thymidine incorporation at various times post-release (inset right panel) to monitor the kinetics of progression through S phase. At the same time, cells were irradiated with UV (5 J/m²) and examined for NF formation after 24 h growth in the presence of 1.5 μM nocodazole. NF formation occurs maximally when cells are actively replicating at the time of UV-irradiation. Graph represents the mean of three separate experiments.

**ATR-defective Seckel cells exhibit increased numbers of mitotic cells with supernumery centrosomes.** During the
course of these studies, we noticed that a significant percentage of mitotic (phospho-Ser10 histone H3 positive) DK0064 (ATRA2101G) cells exhibited more than two centrosomes/cell (Fig. 4A). Staining of centrosomes using anti-\(\alpha\) tubulin IF showed that 20–30% of mitotic DK0064 (ATRA2101G) cells contained more than two centrosomes/cell (mean 3–5). The number of mitotic cells with more than two centrosomes did not increase following treatment with various DNA damaging agents (Fig. 4A). Normal mitotic cells must have only 2 centrosomes/cell which are nucleated by microtubules to facilitate correct alignment of chromosomes prior to cytokinesis. Staining of microtubules in DK0064 (ATRA2101G) cells with aberrant centrosome numbers using anti-\(\alpha\) tubulin showed that all the centrosomes were nucleated by microtubules (Supplementary Material, Fig. 1C). Interphase (phospho-Ser10 histone H3 negative) DK0064 (ATRA2101G) cells did not exhibit an increased number of centrosomes (data not shown), suggesting that cells with elevated numbers of centrosomes fail to undergo cell cycle progression. Transfection of DK0064 (ATRA2101G) cells with pcDNA-ATR dramatically reduced the number of mitotic cells with more than two centrosomes (Fig. 4B). These findings provide the first demonstration that ATR regulates normal centrosome stability.

Analysis of additional Seckel syndrome cell lines. We next employed several of the assays characterized by using the DK0064 (ATRA2101G) cells (ATR-induced phosphorylation, G2/M checkpoint arrest, MN formation, NF and mitotic centrosome number) to investigate ATR pathway function in six additional cell lines established from unrelated Seckel syndrome patients.

Firstly, HU-induced H2AX phosphorylation was examined (Fig. 5A). Two of the Seckel lines (GM09703 and 00P-787) showed as dramatically an impaired response as DK0064 (ATRA2101G). Three lines exhibited a significantly impaired but intermediate response (1180-L, 1231-L and 01P-215) whilst one line (LT262) showed a similar level of HU-induced H2AX phosphorylation to WT cells. Thus, impaired HU-induced H2AX phosphorylation is a common but not invariant phenotype of Seckel syndrome patients.

Secondly, the Seckel syndrome cell lines were examined for their ability to effect UV-induced G2/M arrest. None of the lines tested exhibited a significant G2/M arrest following exposure to UV (Fig. 5B), similar to DK0064 (ATRA2101G) and unlike WT or A-T LBLs (Fig. 1G).

Thirdly, the panel of cell lines were examined for MN formation following treatment with HU. Five out of the six lines
showed an elevated endogenous level of cells with MN similar to or greater than that observed with the DK0064 (ATRA2101G) cell line. As the WT line exhibits around 6% of cells with MN, it is difficult to assess the significance of this finding although the level in three of the cell lines (GM09703, 00P-787 and 1231-L) was substantial (~20%) (Fig. 5C). Strikingly, however, HU treatment resulted in a further increase in four of the lines. Two lines, however (1231-L and 00P-787), failed to show an elevated response to HU treatment although the endogenous levels were already high in these lines. As this treatment did not result in any elevation in the MN frequency in WT or A-T cells (Fig. 1J), this provides strong evidence for an aberrant response to replication fork arrest in Seckel syndrome.

As DNA damage-induced NF appears to be a specific consequence of impaired ATR function (Fig. 2A and C), we also used this assay to identify potential defects in ATR function in our genetically uncharacterized Seckel cell lines. All of the Seckel lines tested exhibited an increased level of HU-induced NF compared with WT cells (Fig. 5D).

Finally, the unexpected endogenous increase in mitotic centrosome number in DK0064 (ATRA2101G) cells prompted us to examine this phenotype in our panel of Seckel syndrome cell lines. Strikingly, all the Seckel syndrome lines exhibited an endogenous increased level of mitotic cells with more than two centrosomes per cell (Fig. 5E). Thus, we conclude that Seckel syndrome is characterized by supernumery centrosomes in a significant percentage of mitotic cells.

Not all Seckel syndrome cell lines are defective in ATR. The overlapping phenotype of these additional Seckel syndrome cell lines to the DK0064 (ATRA2101G) cell line raised the possibility that some of these lines may also harbour mutations in ATR. We used a combination of western blot analysis, complementation assays and direct sequencing to examine whether the defects in any of the genetically uncharacterized Seckel lines could be attributed to mutations in ATR. Western blotting revealed normal expression levels of ATR and its partner protein, ATRIP, in all of the Seckel LBLs (data not shown). Mapping studies have localized the genetic defect in LT262 to chromosome 18p11.31–q11.2. As ATR is located on chromosome 3q22.1–q24, mutations in ATR are unlikely to be responsible for Seckel syndrome in this patient and it was not included in the ensuing analysis. Transfection with pcDNA3-ATR fully complements the APH-induced Chk1 phosphorylation (Fig. 1F), the UV-induced G2/M (Fig. 1H), and the HU-induced MN formation defects (Fig. 1J), along with the HU-induced NF (Fig. 2C) and the mitotic centrosome (Fig. 4B) defects of DK0064 (ATRA2101G). We examined the impact of ATR expression following transfection with pcDNA3-ATR on NF in the panel of cell lines (Fig. 6). In all cases, HU treatment resulted in a marked increase in NF independently of ATR expression in sharp contrast to the marked complementation of this phenotype when ATR was transfected into DK0064 cells (compare Figs 6 and 2C). Finally, ATR was also sequenced in GM09703, a line that closely resembles ATR-Seckel in all assays, and no mutations were observed. Thus, we conclude that the aberrant response to DNA damage described for the additional Seckel syndrome cell lines cannot be attributed to mutations in ATR and therefore must be attributed to mutations in other components of the ATR pathway.

**DISCUSSION**

Previously, we identified a mutational change in ATR in two related Seckel syndrome families, in which the defective gene localized to 3q21–24 (17,29). As Seckel syndrome is clinically and genetically heterogeneous with at least four susceptibility loci contributing to the disorder, the importance of a defect in ATR in two related families to a broad-based understanding of Seckel syndrome was unclear (26–29). Here, we provide important insight into the cellular defect underlying Seckel syndrome by showing that additional Seckel syndrome cell lines are impaired in the ATR-dependent damage response pathway. Thus, we argue that defective ATR
signalling makes a significant contribution to Seckel syndrome.

In the first part of our study, we extend the characterization of the ATR-Seckel cells, revealing further insight into ATR function. Previously, we examined ATR-Seckel fibroblasts, which owing to their slow cycling time, are unsuitable for studying the response to replication fork stalling. Thus, our previous analysis focused on aberrant responses to UV damage in G1 phase cells, which likely reflects the role of ATR in the response to bulky DNA lesions (17,18). Here, we have used more rapidly cycling LBLs, which are informative for studying the response to replication fork stalling. We observe evidence for elevated endogenous damage in ATR-Seckel cells in the MN assay. As anticipated for the known role of ATR in the response to DNA damage, we observe decreased phosphorylation of H2AX and Chk1 phosphorylation after replication fork stalling. We also show that ATR-Seckel cells fail to execute a G2/M checkpoint arrest after
Results in PCC \((33,34)\). Furthermore, Nghiem et al. of ATR kinase activity using adenosine or 2-deoxyadenosine ATR function. Studies using the Syrian hamster fibroblast notype of impaired ATR function. More interestingly, we UV, another anticipated but previously undemonstrated phe-

ocytes (31,32). Not only is the NF phenotype described here morphologically distinct from PCC but also NF cells fail to stain for phosphohistone H3 and retain remnants of a nuclear envelope. As NF cells do not express mitotic markers but retain a nuclear membrane, they appear to represent cells that have failed to progress through mitosis correctly. We propose that NF arises as a consequence of a failure to recover from replication stalling possibly due to irreversibly collapsed replication forks. Alternatively, they may represent cells that have prematurely entered prophase because of lack of a G2/M checkpoint in the presence of damage and/or collapsed replication forks. Interestingly, NF resembles a phenotype described as mitotic catastrophe \((36–38)\). It is important to note that the concentrations of HU and/or UV used to examine NF are substantially greater than those used to examine G2/M checkpoint arrest. Our studies have also revealed a further novel consequence of defective ATR function, namely supernumery centrosomes in mitotic cells. Interestingly, all the centrosomes in ATR- Seckel were nucleated with microtubules. A previous study has shown that centrosome splitting and multipolar spindle formation occurs in the presence of incompletely replicated or damaged DNA \((39)\). Thus, the elevated centrosome numbers in ATR-Seckel may be a consequence of an impaired ability to recover from endogenous replication fork stalling. Defects in BRCA1, BRCA2, p53, XRCC2 and XRCC3 in mammalian cells also confer centrosome instability \((40–43)\).

The function of many of these proteins in homologous recombination and thus in recovery from replication fork stalling further suggests a correlation between impaired recovery from replication stalling and centrosome duplication. Polo-like kinase 1 (Plk1) phosphorylates cyclin B1/cdk1 on centrosomes, which potentially regulates its nuclear localization \((44)\). ATR has been shown to inhibit Plk1 activity following DNA damage \((45)\). Surprisingly, however, this supernumery centrosome phenotype was not enhanced by DNA damage (UV and IR) or by agents that induce replication fork stalling (APH and HU) in ATR-Seckel cells. Thus an alternative explanation is that ATR may play a direct role in centrosome regulation.

In the second part of our work, we use assays that monitor the characteristic defects conferred by ATR deficiency to examine cell lines derived from six additional Seckel syndrome patients. Our assays cover phenotypes that range from defects in direct, upstream functions of ATR \((e.g.\, sub-

strate phosphorylation)\) to downstream consequences of impaired ATR signalling \((e.g.\, G2/M arrest)\) to more general assays that monitor an impaired damage response \((e.g.\, the MN and NF assay)\). Aberrant ATR-dependent phosphorylation of H2AX is a common but not invariant feature of the Seckel syndrome cell lines. More strikingly, in the remaining assays that monitor a failed consequence of ATR signalling in response to replication fork arrest \((MN formation, ability to induce G2/M checkpoint arrest and NF)\), we observed an impaired response similar to that seen with the ATR-Seckel cell line in all the Seckel syndrome cell lines examined \((with\, the\, exception\, of\, two\, cell\, lines\, that\, did\, not\, show\, increased\, HU-induced\, MN\, formation)\). The G2/M checkpoint arrest phenotype is particularly revealing in this context because it demonstrates a specific defect in ATR signalling rather than a more general damage response defect. Thus, cell lines from six genetically uncharacterized, clinically severe, unrelated Seckel syndrome patients exhibit features consistent with aberrant ATR pathway function. Additionally, an increase in mitotic cells with more than two centrosomes is observed in all the Seckel syndrome cell lines examined. It is striking that whilst all the lines show defects in downstream effects of ATR signalling, they differ in the extent of their defect in the upstream steps. In assessing the significance of these findings, it has to be considered that ATR is essential and that any mutational changes are likely to be hypomorphic. Hypomorphic changes can impact upon a specific aspect of a protein’s function, which can result in separation of function phenotypes \((46,47)\). However, an alternative possibility is that ATR might not always be the mutated component. The genetic heterogeneity of Seckel syndrome and the fact that one of the lines likely has a defect mapping to chromosome 18 reinforced this possibility \((ATR\, maps\, to\, chromosome\, 3q22.1–q24)\). Our complementation analysis confirmed this and demonstrated that none of our panel of lines was defective in ATR. This was confirmed by sequence analysis of GM09703, a line with features closely resembling ATR-Seckel. We conclude that Seckel syndrome is commonly caused by defects in the ATR-signalling pathway but the defect may not always, and indeed frequently does not, arise from mutations in ATR itself. Some potential candidate genes for Seckel, in addition to ATR, include ATRIP, RPA1-3,
components of the Rad17/RFC2-5 complex, the Rad9/Rad1/Hus1 and the MCM complexes, TopBP1, Claspin and Chk1. We are currently attempting to identify the specific gene defects in our panel of Seckel cell lines.

Several other damage response disorders such as LIG4 syndrome, Nijmegen breakage syndrome and FA, are characterized by similarly impaired development, suggesting that the ability to respond to endogenously arising DNA damage is crucial for normal development. The ATR-signalling pathway may be particularly important in this context because it plays a vital role in the response to replication fork stalling, which has been estimated to occur multiple times per replication (48,49). It is noteworthy that defective ATR signalling may not significantly enhance the sensitivity to all DNA damaging agents but rather appears to have a greater impact in handling endogenous replication stalling. Thus, previous assays, such as radiosensitivity assays, to monitor DNA damage response defects in Seckel syndrome might have failed to observe any marked defects. In this context, it is noteworthy that ATR has recently been shown to be essential for maintaining common fragile site stability and that fragile site instability is observed in DK0064 (ATR<sup>A231T242</sup>) cells (49,50). Common fragile sites are slow replicating genomic regions that exhibit gaps and breaks under conditions of replicative stress (51). Additionally, Mec1, the budding yeast homologue of ATR, promotes fork progression and in the absence of Mec1 breaks occur in replication slow zones (48). The establishment of assays identifying Seckel syndrome will be important for diagnosis. In this context, the NF assay is particularly striking because we have found this to be a phenotype common to all the Seckel syndrome cell lines examined. The elevated numbers of mitotic cells with more than two centrosomes in Seckel syndrome is another striking finding which could potentially contribute to the clinical features characteristic of this disorder because centrosome and spindle pole orientation play crucial roles during brain development. Premature cell death during development as a consequence of supernumery centrosomes could thus contribute to the particular clinical features of this condition (severe growth retardation, microcephaly and cerebral cortical developmental abnormalities). Interestingly, mutations in Aspm (abnormal spindle in mammalian), which encodes another protein involved in spindle organization, were identified in additional patients displaying microcephaly (52). Further work is required to address the role of centrosome stability during brain development and its potential contribution to human disease.

In summary, we have characterized additional phenotypes associated with deficiency of ATR. Most strikingly, we describe NF as an aberrant response to replication stalling and show that ATR is required for efficient regulation of centrosome duplication. Importantly, we report a range of assays that serve to characterize and identify Seckel syndrome. Our findings demonstrate that Seckel syndrome is a further DNA damage response disorder and we provide strong evidence that it is commonly caused by defects in the ATR-signalling pathway. In contrast to A-T and FA, which are defective in the response to DSBs and cross-linking agents, respectively, Seckel syndrome is unusually sensitive to agents that cause replication stalling. A striking but unexpected finding is that Seckel syndrome is associated with supernumery centrosomes. Our findings provide novel insight into the basis underlying Seckel syndrome and suggest important avenues for future studies including the analysis of candidate genes.

**MATERIALS AND METHODS**

**Cell lines**

EBV transformed lymphoblastoid cell lines (LBLs) were cultured in RPMI with 15% fetal calf serum and 5% CO<sub>2</sub>. The WT control lines used were Sweig and GM02188 (Coriell Cell Repository, NJ, USA). The A-T line (GM03189D) was also obtained from Coriell. Seckel syndrome LBLs were obtained from various sources: DK0064 is the ATR-defective Seckel line described previously (SCKL1) (17); LT262 is from the Danish Seckel mapping study (SCKL2; OMIM 606744) and was obtained from Anders Borglum; 1180-L and 1231-L were obtained from Hans Joenje; 01P-215 and 00P-787 were obtained from Karl Sperling and GM09703 was from Coriell. Mutations in NBS1, LIGIV, MRE11, RAD50 and H2AX were excluded in cell line 01P-215, and mutations in NBS1, LIGIV and RAD50 were excluded in cell line 00P-787.

**Antibodies and plasmids**

Antibodies for γH2AX and Chk1-pSer317 were from Upstate and Cell signalling, respectively. Antibodies against BrdU, γ tubulin and β tubulin were obtained from Sigma, whereas those for phospho-Ser10 histone H3, cyclin B1 and lamin B1 were obtained from Santa Cruz. The pcDNA3-ATR and pcDNA3-ATR-KD (kinase dead) plasmids were gifts from Prof. A. Carr.

**Immunofluorescence and western blotting**

For antibody staining, following treatment with DNA damaging agents, LBLs were swollen in 75 mM KCl for 10 min, then fixed in 3.7% paraformaldehyde prior to cytopinning onto poly-L-lysine coated slides. Cells were permeabilized (0.2% Triton X-100 for 1 min), washed extensively with phosphate-buffered saline (PBS) and then incubated with the antibody under analysis. Secondary antibodies used were FITC or Cy3 conjugate (Sigma) and nuclei were counterstained with DAPI. Images were captured using a Zeiss axioplan fluorescence microscope with simple PCI software. For western blotting, whole cell extracts were resolved using 8% SDS–PAGE and transferred onto nitrocellulose. Membranes were blocked in 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline (TBS) and probed with anti-Chk1-pSer317 (1:2000 dilution) in 5% BSA/TBS/Tween-20 (0.1% Tween-20) followed by probing with HRP-conjugated secondary antibody (Dako).

**NF and MN formation**

Cells were treated with DNA damaging agents in the presence of 1.5 μM nocodazole for 24 h. Nocodazole limits the cells to a single round of replication. Following treatment, cells were...
swollen in 75 mM KCl followed by fixation in Carnoy’s fixative (3:1 methanol–acetic acid) and immobilized onto poly-L-lysine coated slides by cytospin. Nuclei were stained with DAPI and visualized under fluorescence. To inhibit ATR and/or DNA-PK activity, cells were pre-treated with wortmannin (100 µM) for 15 min and/or LY294002 (200 µM) for 30 min prior to HU-induced NF analysis. For MN formation, cells were treated with 1 mM HU for 4 h then incubated with cytochalasin B (5 µg/ml) for 24 h. MN were visualized following swelling in KCl and fixation in Carnoy’s fixative by staining DNA with DAPI, whilst the cytoplasm was differentially stained using acridine orange (2 µg/ml). MN were scored in binucleated cells (BNs) and data are presented as the percentage of BNs containing MN.

**Double thymidine block**

Exponentially growing cells were treated with 2 mM thymidine and cultured for ~20 h. Cells were pelleted and washed with complete medium to remove excess thymidine. Cells were grown in the absence of exogenous thymidine for ~20 h before a second treatment with 2 mM thymidine. Cells were cultured in this thymidine containing medium for another 20 h. Blocked cells were released by washing out the excess thymidine.

**UV-induced G2/M arrest analysed by monitoring the mitotic index**

Cells were UV-irradiated with 2.5 J/m² UV in PBS followed by further culturing in complete medium for 24 h in the presence of 1.5 µM nocodazole. Cells were swollen in 75 mM KCl, and fixed in Carnoy’s fixative. Mitotic cells were visualized and the percent of mitotic cells was counted using DAPI from at least 500 interphase cells.

**Apoptosis**

Cells were treated as for NF and apoptotic cells detected using the FragEL TUNEL assay kit (Oncogene research products) according to the manufacturers protocol.

**Transfection**

Lymphoid lines (3 x 10⁵ cells/ml in 3 ml) were transiently transfected with 2 µg of pcDNA3-ATR or pcDNA3-ATR-KD using Genejuice (Novagen) according to manufacturers instructions then incubated for 24 or 48 h. For complementation of the G2/M mitotic index, cells were retransfected after 48 h. For the supernumerary centrosome phenotype of DK0064, cells were retransfected after 48 h and again after 72 h.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**ACKNOWLEDGEMENTS**

We are very grateful to the primary clinicians who are the first line in identifying Seckel syndrome patients from which our cell lines are derived. Special thanks to Margit Koenig, Krystyna Chrzanowska, Jens Michael Hertz, Thomas Balslev and Niels Birkebaek. The P.A.J. laboratory is supported by the Medical Research council, the Human Frontiers Science Programme, the Primary Immunodeficiency Association, the Leukaemia Research Fund, the Department of Health and EU grant (FIGH-CT-200200207). M.O.’D. is supported by the Leukaemia Research Fund.

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