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Melanoma antigen-D2 controls cell cycle progression and modulates the DNA damage response

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ABSTRACT

Overexpression of the ubiquitous type II melanoma antigen-D2 (MAGED2) in numerous types of cancer suggests that this protein contributes to carcinogenesis, a well-documented characteristic of other MAGE proteins. Modification of MAGED2 intracellular localization during cell cycle phases and following treatment with camptothecin (CPT) and phosphorylation by ATM/ATR following ionizing irradiation led us to investigate the molecular functions of MAGED2 in the cellular response to DNA damage. Cell cycle regulators, cell cycle progression, and bromodeoxyuridine (BrdU) incorporation were compared between MAGED2-sufficient and -depleted U2OS cells following exposure to CPT. At 24 h post-CPT removal, MAGED2-depleted cells had lower levels of p21 and p27, and there was an increase in S phase BrdU-positive cells with a concurrent decrease in cells in G2. These cell cycle modifications were p21-independent, but ATR-, SKP2-, and CDC20-dependent. Importantly, while MAGED2 depletion reduced CHK2 phosphorylation after 8 h of CPT treatment, it enhanced and prolonged CHK1 phosphorylation after a 24 h recovery period, indicating sustained ATR activation. MAGED2 depletion had no impact on cell survival under our experimental conditions. In summary, our data indicate that MAGED2 reduced CPT-related replicative stress, suggesting a role for this protein in genomic stability.

1. Introduction

Melanoma antigen-D2 (MAGED2) is a member of the melanoma antigen protein family [1], which consists of 55 members in humans, and can be subdivided into two types. Type I MAGE proteins (MAGEA to C), which are normally expressed during embryogenesis and in gamete tissues, are often aberrantly expressed in cancer cells, and are proposed to be oncogenic drivers. Type II MAGE proteins (MAGED to H, and L and Necdin) are ubiquitously expressed at tissue-specific levels [2,3]. Only certain type II MAGE proteins, e.g., MAGED2, are overexpressed in cancer cells. High *MAGED2* mRNA and protein levels have been detected in small intestine, gastric, breast, testicular, ovarian, lung, and thyroid cancers, as well as melanomas [4–10]. This overexpression supports MAGED2 as a potential tumor biomarker. All MAGE proteins contain a conserved 170 amino acid MAGE homology domain that binds to RING-containing E3 ubiquitin ligases [11–13]. MAGE proteins regulate E3 ubiquitin ligase activity, determine the nature of the substrates to be ubiquitinated by these ligases, and modulate subcellular localization of these ligases through "MAGE-RING ligase" interactions [11–14]. Therefore, MAGE proteins are important regulators of a large number of cellular processes, including cell cycle regulation, DNA damage response, proliferation, and apoptosis [15–20].

The molecular functions of MAGED2 have yet to be fully characterized and appear to be dependent on cell type and context. For example, MAGED2 depletion using small interfering RNAs (siRNAs) or methionine deprivation in melanoma and MDA-MB-231 cells increases TRAIL-induced apoptosis by enhancing TRAIL-R2 levels in a p53-dependent manner [21,22]. Several MAGED2 mutations, the majority nonsense mutants, lead to X-linked polyhydramnios during gestation and severe transient antenatal Bartter's syndrome [23]. As stated above, MAGED2 overexpression has been observed in numerous tumors, but its precise function in oncogenesis is unknown. In a previous publication, we reported on the subcellular localization of MAGED2 and identified nuclear and nucleolar localization signals [24]. We observed cell cycle-linked intracellular relocalization of MAGED2, and strikingly, drastic

Abbreviations: APC/C, anaphase-promoting complex or cyclosome; ATM, ataxia-telangiectasia mutated protein; ATR, ataxia-telangiectasia- and rad3-related protein; CDC20, cell division cycle protein 20; CDK, cyclin-dependent kinase; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; CPT, camptothecin; DDR, DNA damage response; DSB, double-strand break; MAGE, melanoma antigen; RPA, replication protein A; PI, propidium iodide; SCF, SKP1/CUL1/F-box protein complex; SKP2, S phase kinase-associated protein 2; Top1cc, DNA topoisomerase I cleavage complex

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nuclear accumulation of MAGED2 after the induction of DNA lesions using several genotoxic compounds. MAGED2 participation in the DNA damage response (DDR) has previously been inferred based on its ATM/ATR-dependent phosphorylation after exposure to ionizing radiation [25].

The DDR network is critical to maintaining genomic stability. ATM and ATR kinases are the major upstream transducers of the DDR cascade and convey signals to mediators and effectors controlling cell cycle arrest, DNA repair, transcription factors, and apoptosis [26,27]. DNA double-strand breaks (DSB) activate ATM, while replication protein A (RPA)-coated stretches of single-stranded DNA adjacent to double-stranded DNA are indispensable for ATR activation [28–30]. These RPA-coated stretches arise when replication forks are stalled by an obstacle, which creates replicative stress, or during homologous recombination [30]. CHK1 and CHK2 kinases, which are activated by ATR and ATM, respectively, regulate cell cycle checkpoints by inhibiting cyclin-dependent kinase (CDK)/cyclin complexes [31,32]. Both ATR/CHK1/p53/p21 and ATM/CHK2/p53/p21 axes delay S phase and block cells in G2/M, whereas the latter also blocks cells at the G1/S interface [33]. In addition, ATR activates replication checkpoints, which leads to a global slowdown of replication forks, stabilization of stalled replication forks that facilitates their recovery, and inhibition of the firing of late-licensed replication origins.

Two E3 ubiquitin ligase complexes, SKP1/CUL1/F-box protein (SCF) and anaphase-promoting complex or cyclosome (APC/C), operate at distinct stages of the cell cycle, are important in cell cycle regulation, and mediate ubiquitin-proteasome-dependent degradation of key cell cycle proteins. SCF is active during all cell-cycle stages and plays a prominent role in the G1/S transition. APC/C activity is restricted to progression through mitosis and G1. In conjunction with S-phase kinase-associated protein 2 (SKP2), the SCF complex targets p21, p27, p57, cyclin E1, and cyclin D1 [34,35]. The main substrates of APC/C associated with cell division cycle 20 (CDC20) are SKP2 and mitotic cyclins such as cyclin A2 and cyclin B1 [36].

Camptothecin (CPT) stabilizes the DNA/DNA topoisomerase I cleavage complex (Top1cc) [37]. Collision of DNA replication forks with Top1cc leads to stalling of these forks and replication-associated DSBs. Collision of RNA polymerase with Top1cc arrests transcription forks and leads to the formation of RNA loops that can later be converted into DSBs [38]. These collisions activate ATM and/or ATR kinases, thus triggering the cell cycle and replication checkpoints. When CPT is removed, transcription resumes rapidly, DNA topoisomerase 1 is degraded, and DSBs are repaired by homologous recombination or non-homologous end-joining [39,40]. Replication stress can be relieved by restarting stalled replication forks, by initiating new replication forks downstream of the stalled ones, or through homologous recombination of the collapsed forks [41].

In this present study, we characterized the role of MAGED2 protein in cell cycle progression and DDR activation after exposure to CPT-based chemotherapy. MAGED2 was found to play a role in cell cycle progression and DDR based on its depletion leading to (i) an accumulation in BrdU-positive cells with a concurrent decrease in cells in G2, (ii) enhancement of ATR activity, and (iii) downregulation of numerous cell cycle regulators, i.e., p21, p27, and cyclin D1. These cell cycle modifications are ATR-, SKP2-, or CDC20-dependent. Therefore, MAGED2 can be considered a new cell cycle regulator in the DDR network.

2. Materials and methods

2.1. Cell lines, chemical reagents, and antibodies

The HeLa cervical cancer cell line was obtained from the American Type Culture Collection, the p53-positive U2OS human osteosarcoma cell line was provided by A. Decottignies (Genetic and Epigenetic Alterations of Genomes, de Duve Institute, Catholic University of Louvain, Brussels, Belgium), and the p53-negative Saos human osteosarcoma cell line was provided by E. Dejardin (Laboratory of Molecular Immunology and Signal Transduction, GIGA-Research, University of Liège, Liège, Belgium). These cell lines were cultured in Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; GIBCO, Carlsbad, Ca, USA) and 100 U/mL penicillin/streptavidin (Lonza) at 37 °C in a humidified atmosphere of 5% CO_2. The absence of mycoplasma in cell supernatants was regularly confirmed using the MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, LT07-710).

CPT (C9911), BrdU (B5002), and paclitaxel (T7402) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Primary anti-MAGED2 (15252-1-AP) antibody was purchased from Proteintech, anti-p21 (OP64) antibody was from Merck Millipore, and anti-p53 (sc-126), anti-p27 (sc-528), anti-CHK2 (sc-17747), and anti-HSP90 antibodies (sc-7947) were from Santa Cruz. Anti-pp53 ser15 (9284), anti-pCHK1 ser317 (2344), anti-pCHK1 ser345 (2348), anti-CHK1 (2360), anti-pCHK2 thr68 (2661), anti-pRB ser807/811 (8516), anti-RB (9309), anti-cyclin A2 (4656), anti-cyclin E1 (4129), anti-cyclin D1 (2978), anti-RBX1 (11922), anti-ATR (13934), and anti-phosphohistone H3 ser10 antibodies (3377) were purchased from Cell Signaling Technology. Anti-BrdU (B2531) and anti-β-actin antibodies (A4700) were from Sigma-Aldrich, anti-cyclin B1 antibody (554177) was from BD Pharmingen, anti-pRPA32 ser33 antibody (A300-246A) was from Bethyl Laboratories, and anti-GAPDH antibody (AM4300) was from Ambion. Normal rabbit IgG (2729), used as a control during immunoprecipitation assays, and secondary HRP-coupled antibodies were purchased from Cell Signaling Technology and Alexa Fluor-conjugated antibodies were purchased from Invitrogen.

2.2. Transient knockdown with siRNAs

A pool of four siRNA duplexes (smartpool) targeting human MAGED2 (M-017284-00-0010) was purchased from Dharmacon and designated siMAGED2 #1. A second siRNA targeting MAGED2 (siMAGED2 #2) was purchased from Eurogentec (siMAGED2 #2 sequence: GAAGGUAUUUGGGAUUCAA). The siRNAs targeting SKP2 and CDC20 were purchased from Eurogentec (siSKP2 sequence: AAGGGA GUGACAAAGACUUUG and siCDC20 sequence: CGGCAGGACUCC GGGCCGA). Nonspecific siRNA (Silencer® Select Negative Control #2 siRNA) and an siRNA targeting ATR (siATR sequence: CCUCCGUGAUGUUGCUUA) were purchased from Ambion [42]. All siRNAs were transfected into cells using ProFection Mammalian Transfection System-Calcium Phosphate (Promega, Madison, USA).

2.3. Stable knockdown using short hairpin RNAs

To stably silence p21, U2OS cells were transduced with lentiviral particules allowing the expression of short hairpin RNA (shRNA). Lentiviral transfer plasmids encoding human CDKN1A (p21) targeted shRNAs (TRCN0000294474, shp21#1 and TRCN0000040123, shp21#2) and non-targeted control shRNA (SHC005, shCTR) were purchased from Sigma-Aldrich. Lentiviral vectors were generated by the GIGA Viral Vectors platform. Briefly, Lenti-X 293 T cells (Clontech® #632180) were cotransfected with shRNA transfer lentiviral plasmid, pSPAX2 (Addgene® #12260; Cambridge, MA, USA), and vesicular stomatitis virus G-protein-encoding vector [43]. Vesicular stomatitis virus G protein pseudotyped lentiviral vectors carrying the gene transfer lentiviral plasmid were formed. Viral supernatants were collected 48, 72, and 96 h post-transfection, filtered, and concentrated 100-fold by ultracentrifugation. The lentiviral vectors were then titrated with a qPCR Lentivirus Titration (Titer) Kit (ABM®, #LV900; Richmond, BC, Canada). U2OS cells were transduced with shRNA lentiviral vectors and selected with 10 μg/mL puromycin (Invivogen, ant-pr-1). The absence of replication-competent lentivirus and mycoplasma from cell supernatants was confirmed using qPCR Lentivirus Titration and MycoAlert™ PLUS Mycoplasma Detection Kits, respectively.