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RNA interference against mixed lineage leukemia 5 resulted in cell cycle arrest

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ABSTRACT

Mixed lineage leukemia 5 (MLL5) encodes a mammalian trithorax group (TrxG) protein located within chromosome band 7q22, which is a frequently deleted region found in acute myeloid malignancies. Trithorax and polycomb (PcG) group proteins are evolutionarily conserved transcriptional regulators that maintain the expression of *Homeobox* (*HOX*) genes at the epigenetic level during development. Recently, the emerging roles of TrxG and PcG group proteins in cell cycle regulation have begun to be elucidated. In this study, we demonstrated that the mammalian trxG protein MLL5 is involved in multiple cell cycle regulation. Knockdown of MLL5 by small interfering RNA resulted in the retarded cell growth and attenuated intake of BrdU in multiple tumor and normal diploid cells. The cell cycle arrest induced by knockdown of MLL5 took place at both the G1 and G2/M phases. This growth-inhibitory effect and dual-phase arrest were also found in p53-knockout cell lines, suggesting that the transactivation activity of p53 was dispensable for the MLL5-knockdown-mediated cell cycle arrest. In addition, up-regulation of cyclin-dependent kinase inhibitor p21 and dephosphorylation of retinoblastoma protein were observed in all cell lines tested regardless of their p53 status. Taken together, our data suggest that silencing of MLL5 leads to up-regulation of p21 and dephosphorylation of pRb, which at least partially contributes to the G1 phase and G2/M phase arrest. These findings provide evidence that MLL5 might be an important cell cycle regulator, participating in cell cycle regulatory network machinery at multiple cell cycle stages.

1. Introduction

Mixed lineage leukemia 5 (MLL5) is a mammalian *trithorax* group (*trxG*) gene located at chromosomal band 7q22, a frequently deleted region found in acute myeloid malignancies (Emerling et al., 2002; Fischer et al., 1997). TrxG proteins and their antagonistic counterpart, polycomb (PcG) proteins, function in specifying positional information such as antero-posterior patterning, through activating or repressing the stable state of *Hox* gene expression (Gould, 1997; Simon and Tamkun, 2002). TrxG and PcG proteins form multiprotein complexes, regulating transcription at the epigenetic level by mechanisms such as chromatin remodeling and histone modification (Simon and Tamkun, 2002; Schuettengruber et al., 2007). In addition to the well-established functions in embryonic development, some studies suggest that TrxG and PcG proteins may influence both Hox-dependent and independent downstream pathways that control cell proliferation. Both TrxG and PcG

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proteins have been reported to interact with retinoblastoma (Rb) protein, resulting in cell cycle alteration (Dahiya et al., 2001). In addition, deficiency of PcG group gene *bmi-1* resulted in a marked increase in tumor suppressors $p16^{INK4a}$ and $p19^{ARF}$ levels, and impaired progression into the S phase of the cell cycle (Jacobs et al., 1999). Knockdown of ALR, human homolog to *Drosophila* TRR (trithorax-related), has recently been reported to reduce growth kinetics and impair adhesion-related cytoskeletal activities (Issaeva et al., 2007).

The human mixed lineage leukemia (MLL) family consists of five members (Fig. 1), among which MLL1 (also known as ALL-1, HRX, and Htrx) is the best-characterized protein in the family (Gu et al., 1992; Djabali et al., 1992). Numerous studies have reported that MLL1 is associated with myeloid and lymphoid leukemia when fused to a wide variety of chromosomal partners (Ayton and Cleary, 2001; Ernst et al., 2002). Compared with other MLL family members, including MLL1, ALR (Prasad et al., 1997), MLL2 (FitzGerald and Diaz, 1999) and MLL3 (Ruault et al., 2002), MLL5 encodes a relatively smaller protein with 1858 amino acids. *MLL5* is homologous to *Drosophila CG9007* (Emerling et al., 2002), and is evolutionarily more distantly related to other family members (Fig. 1). MLL5 contains only one plant homeodomain (PHD) zinc finger instead of a cluster found in other family members. In addition, the Su(var)3-9, enhancer-of-zeste and trithorax (SET) domain found on MLL5 is located at the N-terminal region rather than at the carboxyl-terminus. It also lacks DNA binding motifs such as A-T hooks, bromodomain or HMG domains encoded by other family members.

The PHD fingers are found in a large number of chromatin-associated proteins and were reported to associate with specific nuclear protein partners or nucleosomes (Aasland et al., 1995). The PHD fingers of MLL1 have been reported to mediate homodimerization and are required for the interactions with cyclophilin Cyp33 (Fair et al., 2001). Emerging roles for the SET methyltransferase activity in epigenetic regulation have been highlighted in other MLL protein members, which are able to methylate lysine residue at histone H3 and thereby influence the transcriptional state of genes (Milne et al., 2002; Nakamura et al., 2002; Lee et al., 2006; Demers et al., 2007). However, such methyltransferase activity has yet to be reported in MLL5. It has been reported that the sequences within the SET domain might not be sufficient for enzymatic activity since methylation may require an additional post-SET domain (Kouzarides, 2002), which is not found in MLL5. The physiological function of MLL5 remains largely unknown. It was previously reported to localize to nuclear speckles and ectopic overexpression of MLL5 protein inhibited cell cycle progression (Deng et al., 2004). A recent study on the genome-based RNA interference (RNAi) profiling in cell division proposed that MLL5 might function in cytokinesis and mitosis (Kittler et al., 2007).

In this study, we report that knockdown of MLL5 caused growth-inhibitory effects in both tumor and normal diploid cells. The cell cycle arrest took place at both G1 and G2/M phase and such cell cycle arrest was unlikely to be p53-dependent as similar effects were also noted in p53-knockout cells. Up-regulation of CDK inhibitor p21 and de-phosphorylation of pRb might be responsible for MLL5-knockdown-induced cell cycle arrest.

2. Materials and methods

2.1. Cell culture and synchronization

Human diploid lung fibroblasts (IMR-90 and WI-38), osteosacoma U2OS and cervical carcinoma HeLa cells were purchased from ATCC. Human colorectal carcinoma cell lines HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ were kind gifts from Dr. Bert Vogelstein (Bunz et al., 1998). Synchronization of HeLa and HCT116 cells at G2/M boundary was done as described previously (Deng et al., 2004; Kim et al., 2005). To synchronize HeLa cells at G1/S boundary, cells were incubated in 2 mM thymidine for 18 h before being released for 9 h. Cells were then again incubated in 2 mM thymidine for 17 h to reach G1/S boundary.

2.2. RNA interference

BLOCK iT™ RNAi designer software (Invitrogen) were used to identify potential siRNA targeting sites within *MLL5* mRNA sequence. Four MLL5 specific siRNA duplexes (#1, #2, #3 and #4) targeting nucleotide positions at 432, 1063, 5215 and 6807, respectively, from the transcription starting point, were synthesized by 1st BASE (Singapore). Scrambled siRNA (sense-5′-**UUCUCCGAACGUGUCACGUdTdT**, antisense-5′-**ACGUCACACGUUCGGAGAAdTdT**) was used as a control. The siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. For growth rate comparison, cells were seeded at day 1 and the transfection was performed at either day 2 and day 3 for HeLa and U2OS cells, or day 2 and day 4 for IMR-90 and WI-38 fibroblasts.

2.3. Growth curve and BrdU proliferation assay

Live cell populations after siRNA transfection at each time point were collected and counted in triplicates by a hematocytometer via trypan blue dye exclusion. For cell proliferation assay, BrdU was used as a marker. Seventy-two hours after transfection with scrambled or MLL5 siRNA, HeLa cells were grown on the coverslips with complete DMEM containing 10 μg/mL BrdU for 16 h, followed by being fixed in 100% methanol at −20 °C for 10 min and re-hydrated in PBS for 10 min. After denaturing DNA with 2N HCl for 30 min at RT, the acid was neutralized with 0.1 M borate buffer (pH 8.0) and the cells were blocked with 5% BSA for 30 min and incubated with mouse anti-BrdU IgG antibody conjugated with Alexa Fluor 594 (Molecular Probe) for 1 h at RT. Cells were then washed with PBS containing 0.05% Tween20 before co-staining with 4′-6-diamidino-2-phenylindole (Sigma) for 5 min. Immunofluorescence was visualized by Olympus FV500, and analyzed by Olympus Fluoview software.

2.4. Western blot and antibodies

Cells were lysed in Laemmlli sample buffer (62.5 mM Tris–HCl pH6.8, 2.5% SDS, 10% glycerol, 0.01% bromophe-