Conditional expression of MCM7 increases tumor growth without altering DNA replication activity

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Abstract The minichromosome maintenance (MCM) 2–7 complex is a putative DNA helicase complex that facilitates the initiation of DNA replication. Here, we generated a cell line MCM7$^{+/-}$/MCM7-FLAG, in which one allele of MCM7 is mutated whereas a tetracycline-repressible promoter could manipulate the expression of exogenous MCM7 protein. Overexpressed MCM7 protein supports efficient DNA replication of Epstein–Barr virus oriP and rapid formation of tumors in nude mice without altering the activity of cellular DNA replication. This system provides a unique setting for studying the function of MCM7 and for screening for potential therapeutics for malignant tumors.

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Key words: MCM7; DNA replication; Homologous recombination; Tetracycline-repressible promoter; Tumor growth

1. Introduction

Minichromosome maintenance protein (MCM) is composed of six structurally related subunits (MCM2–7) and is essential for eukaryotic DNA replication initiation and the early stage of the elongation process [1–5]. MCM proteins are members of the AAA+ (ATPases associated with a variety of cellular activities) family of proteins [6]. The DNA-dependent ATPase motifs, including both Walker A and B motifs, are highly conserved in the central region of MCM proteins from yeast to mammalian cells suggesting its crucial roles in the functions of MCM [7]. Although helicase activity with all six MCM subunits has remained elusive, a subassembly of MCM, the MCM4/6/7 double trimeric complex, possesses DNA helicase activity as a presumed replicative helicase at replication forks in eukaryotic cells [8–13].

Recently it has been shown that MCM proteins are more frequently detected in cells from malignant tissues than in those from normal tissues, suggesting that MCM proteins could be a good indicator of cancer cells [14]. Immunohistochemical studies in a variety of tissues demonstrate the increased expression of MCM7 in solid tumors and premalignant proliferative states [15]. In this study, we directly assessed the in vivo role of MCM7 in human cells by genetic approaches capable of manipulating MCM7 protein level. Our results clearly showed that enhanced MCM7 protein induced the acceleration of oriP-dependent Epstein–Barr virus (EBV) replication and tumor mass formation whereas the activity of cellular DNA replication was not changed. This is a first experimental link between MCM7 and malignant cell proliferation.

2. Materials and methods

2.1. MCM7 targeting construct and screening for recombinants

A BAC clone (RP11-506M12; accession number AC073842) containing the human MCM7 gene was used as the source for homologous arms. The two polymerase chain reaction (PCR)-amplified fragments, 2.2 kb and 3.8 kb subclone, containing the region immediately 5′ and 3′ of KS (the highly conserved Lys^{387} and Ser^{388} residues in the Walker A motif located in exon 9), were assembled in the pKO plasmid (Stratagene) surrounding a promoterless neomycin (Neo)-resistant gene containing simian virus 40 polyadenylation signals (Neo-poly(A)) (Fig. 1A). G418-resistant clones were screened by PCR (Fig. 1B). The oligonucleotides 5′-cccaagcttgtgagtggttagagaacaatggcaa-3′ (M-4), 5′-caatggccgatcccattaataacttcgt-3′ (7-1), 5′-ggcatgctgggggatgcggtataacttcg-3′ (7-2), and 5′-cagtggaacgaggtcaggggacacccttgt-3′ (M-21) were used as primers.

2.2. Construction of inducible cell lines

Tetracycline-inducible FLAG-tagged MCM7 cell lines were produced using the Tet-off system (Clontech). pTet-Off (Clontech) in which the original Neo marker is replaced by a puromycin (Puro)-resistant gene was used. After Puro selection, the integrity and functioning of pTet-Off was verified by luciferase reporter assay using pTRE2hyg-Luc (Clontech). A FLAG tag was fused to the 3′-end of the human MCM7 cDNA coding region by PCR. The amplified fragment was inserted downstream of the tetO-CMV minimal promoter of pTRE2hyg (Clontech). The resulting plasmid expresses MCM7-FLAG protein under the control of tetR-VP16 [16]. After transfection and selection with hygromycin B, desirable clones were tested by Western blot using anti-FLAG antibody. Tet system approved fetal bovine serum (Clontech) was used.

2.3. Cell culture and transfections

HCT116 cells and their derivatives were cultured in McCoy's 5A medium (Invitrogen) containing 10% (v/v) fetal bovine serum (Hyclone) and antibiotics (penicillin and streptomycin) at 37°C under 5% CO_2. Cells were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer's instruction. Stable transformants were selected with each drug at the following concentrations: 400 μg/ml Geneticin (Invitrogen), 1 μg/ml Puro (Invitrogen), and 100 μg/ml hygromycin B (Invitrogen).

2.4. Cell proliferation and cell cycle analysis

Cells were counted after trypan blue treatment. DNA replication activity was measured by bromodeoxyuridine (BrdU) incorporation

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Abbreviations: EBV, Epstein–Barr virus; Dox, doxycycline; MCM, minichromosome maintenance; Neo, neomycin; Puro, puromycin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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into newly synthesized DNA using Cell Proliferation ELISA (Roche). BrdU labeling was conducted by adding BrdU to the tissue culture medium before harvest for a period of 2 h.

2.5. Antibodies, Western blot analysis, and chromatin fraction preparation

Anti-human MCM7, anti-human ORC2, anti-human MCM3 (Santa Cruz Biotechnology), anti-neomycin phosphotransferase II (Upstate), anti-FLAG M2 monoclonal antibody-peroxidase conjugate (Sigma), horseradish peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat IgG antibodies (Amersham Biosciences) were purchased. Cells were lysed in RIPA lysis buffer and Western blotting and immunoprecipitation were performed as described before [17]. For preparation of the chromatin fraction, cells were treated prior to lysis as described [18]. For quantifying immunoblotting, Scion Image 4.02 software (http://www.scioncorp.com) was used.

2.6. Assay for viral plasmid replication

Cells (2.5×10^5 per 35-mm dish) were transfected with a plasmid (1 μg), then washed extensively 1 day after transfection and 10% of the cells were returned to culture. Forty-eight hours after transfection, zeocin (400 μg/ml) was added and cells were selected for up to 2 weeks. Colonies were stained by crystal violet. The plasmid, pCEP4 (Invitrogen), contains the minimal replicator of EBV oriP and the EBNA-1 gene. The hygromycin-resistant gene was replaced by a zeocin-resistant gene derived from pcDNA3.1/Zeo (Invitrogen).

2.7. Tumorigenicity in nude mice

Cells were harvested before inoculation and resuspended in serum-free medium at a concentration of 1.5×10^7 cells/ml. Cells (3×10^6 cells in 0.2 ml) were then inoculated s.c. at the proximal dorsal midline into 4-week-old female athymic BALB/c-*nu/nu* mice (Japan Clea). Tumor volumes were calculated with the formula $L\times W^2$, where L is length and W is width. Mice were housed in barrier environments, with food and water with or without doxycycline (Dox; 2 μg/ml) provided ad libitum.

3. Results and discussion

3.1. MCM7 heterozygous cells

To examine the cellular roles of MCM7 in human cells, we first attempted to minimize the endogenous MCM7 protein level. The targeting vector was designed to create a non-functional allele by replacing the KS amino acids (Lys^{387} and Ser^{388} residues) located within the Walker A motif with the Neo-resistant gene by homologous recombination (Fig. 1A).

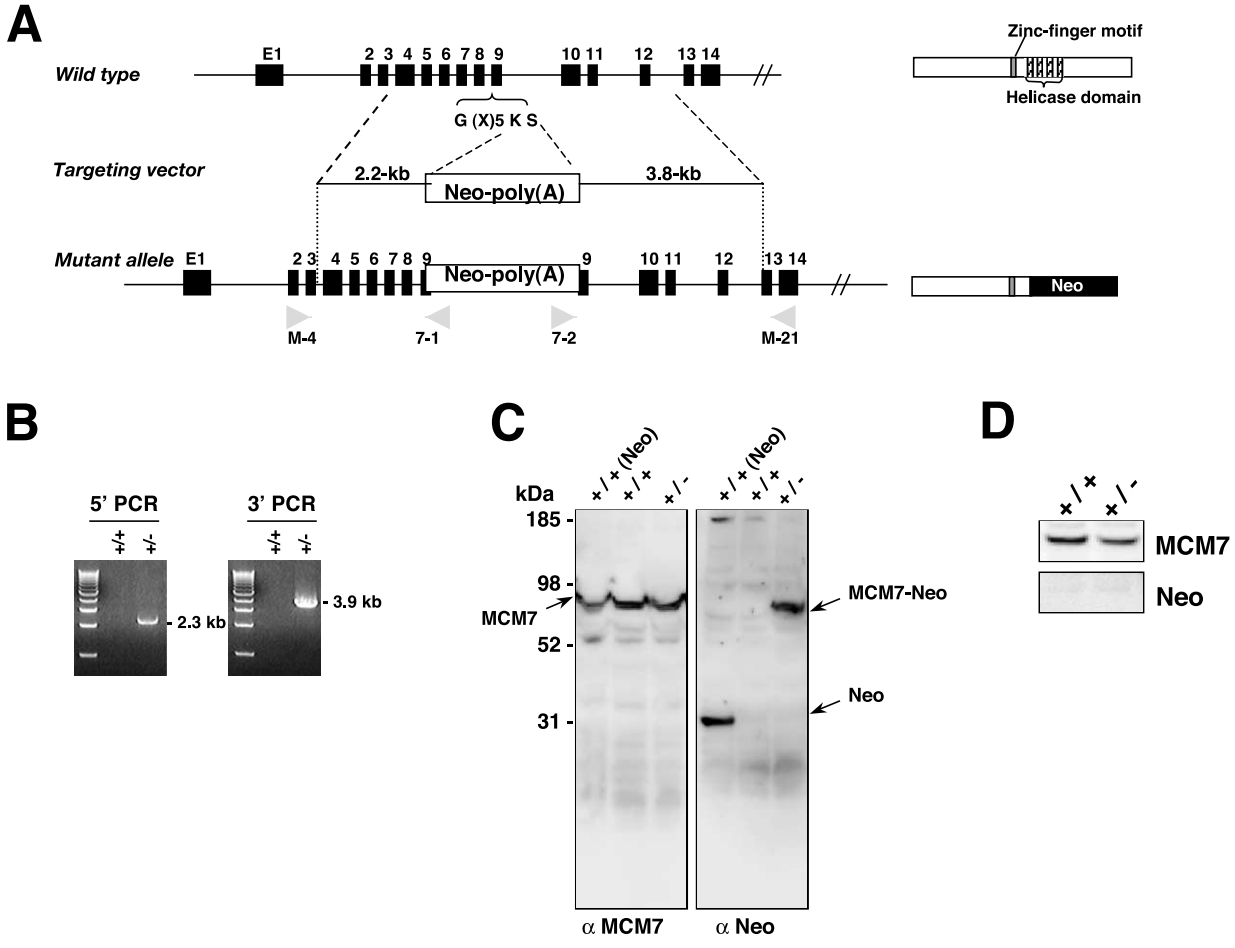

Fig. 1. MCM7 heterozygous HCT116 cells. A: Schematic of the human MCM7 gene locus and gene targeting strategy. The positions of exons (E) 1–14 of the human MCM7 gene are indicated as closed boxes. The Neo-resistant gene with poly(A) is indicated as an open box. Primers used for PCR screening are indicated. Recombination between the targeting vector and endogenous locus deletes the conserved lysine (K) and serine (S) of the Walker A motif G(X)5KS located in exon 9. A schematic representation of wild-type protein and C-terminally Neo-fused MCM7 protein is shown on the right side. Characteristic zinc-finger motif and helicase domain are indicated. B: Genomic DNA prepared from a parental +/+ clone and from a +/− clone was subjected to PCR with primer pairs shown in A. Gel electrophoresis and ethidium bromide stain to visualize the PCR products. C: Western blot analysis of total cell lysates using anti-MCM7 (left) and anti-Neo phosphotransferase II (right) antibody. Total cell lysates of +/+, pcDNA3/Neo-transfected +/+ cells (+/+(Neo)), and +/− cells loaded per lane. Note the wild-type Neo was detected as a 32 kDa protein. The positions of MCM7 and MCM7-Neo chimeric protein are also indicated. D: Western blots analysis of the chromatin fraction prepared from +/+ and +/− cells using anti-MCM7 (upper) and anti-Neo (lower) antibody.

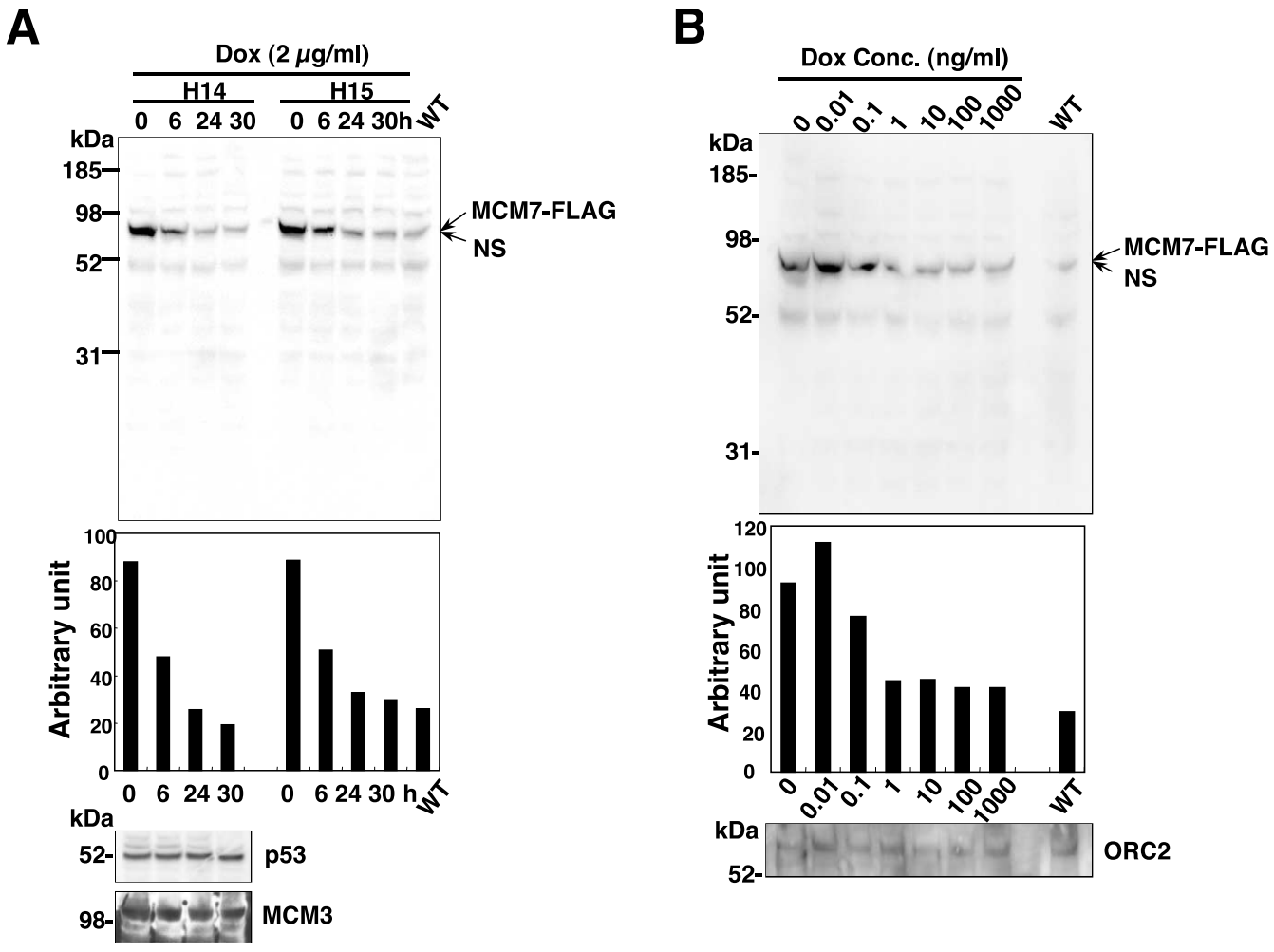

Fig. 2. Dox-regulated MCM7-FLAG expression in MCM7$^{+/-}$HCT116 cells. A: MCM7$^{+/-}$/MCM7-FLAG cells were grown in medium containing 2 μg/ml of Dox for the time period indicated at the top. Lysates of independent clones (H14 and H15) and wild-type cells (WT) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to Western blotting using an anti-FLAG antibody. MCM7-FLAG protein and non-specific (NS) band are indicated. Histograms of a representative blot are calculated (Scion Image Beta 4.02) and shown below. As loading controls, anti-p53 and MCM3 antibodies were used (bottom panel). B: Cells were treated with Dox at a concentration indicated at the top for 30 h. Lysates were resolved by SDS–PAGE and subjected to Western blotting using an anti-FLAG antibody. As a loading control, anti-ORC2 antibody was used (bottom panel). C: Cell proliferation rate of MCM7$^{+/-}$/MCM7-FLAG cells treated with (+) or without (−) Dox. Means and standard deviation of four measurements are indicated. D: BrdU incorporation of MCM7$^{+/-}$/MCM7-FLAG cells treated with (+) or without (−) Dox. Comparison of BrdU incorporation of asynchronous cells growing 2 h in culture, with the incorporation in +/+ cells held at 1. Means and standard deviation of four measurements are indicated. E: Western blot analysis of the chromatin fraction prepared from MCM7$^{+/-}$/MCM7-FLAG cells treated with (+) or without (−) Dox for 30 h. Chromatin fractions were directly subjected to immunoblotting (IB) using anti-MCM7 (upper) and anti-FLAG (middle) antibodies, or immunoprecipitation (IP) using anti-FLAG antibody, and then subjected to IB using anti-MCM3 (lower) antibody. F: MCM7 increases the DNA replication from oriP of EBV. Establishment of drug-resistant colonies of MCM7$^{+/-}$/MCM7-FLAG cells after transfection of pCEP4/Zeo treated with (+) or without (−) Dox. Following selection with zeocin for up to 2 weeks, colonies were visualized by staining with crystal violet. Histograms of a representative colony blot are calculated (Scion Image Beta 4.02) and shown at the bottom. Asterisk represents statistical significance ($P < 0.01$). Means and standard deviation of four measurements are shown.

The heterozygous clone (+/−) was identified by PCR screening in human colon cancer cells HCT116. For the 5′ and 3′ homologous regions, primer set M-4 and 7-1 produces a 2.3-kb and primer set 7-2 and M-21 produces a 3.9-kb product from the mutated allele (Fig. 1B). Western blot experiments revealed that the heterozygous clone (+/−) produced C-terminally Neo-fused protein (MCM7-Neo) along with wild-type protein, they have too close molecular weights to separate them (Fig. 1C). As a control, wild-type Neo protein transiently transfected into wild-type cells was detected by anti-Neo antibody as a 32-kDa protein (Fig. 1C, right panel). Chromatin fractions recovered from +/+ and +/− cells showed that both cells contained equal amounts of MCM7 protein (Fig. 1D, upper panel). There was no appearance of MCM7-Neo protein in the chromatin fraction of +/− cells (Fig. 1D, lower panel).

To determine whether one copy of MCM7 is sufficient to confer the initiation of DNA replication in HCT116 cells, cell growth and BrdU incorporation were measured. Cell proliferation rate and DNA replication activity were not changed (data not shown). These results are not surprising, because MCM proteins are 100–1000-fold more abundant than the estimated number of origins in *Saccharomyces cerevisiae* and mammalian cells [18–23].

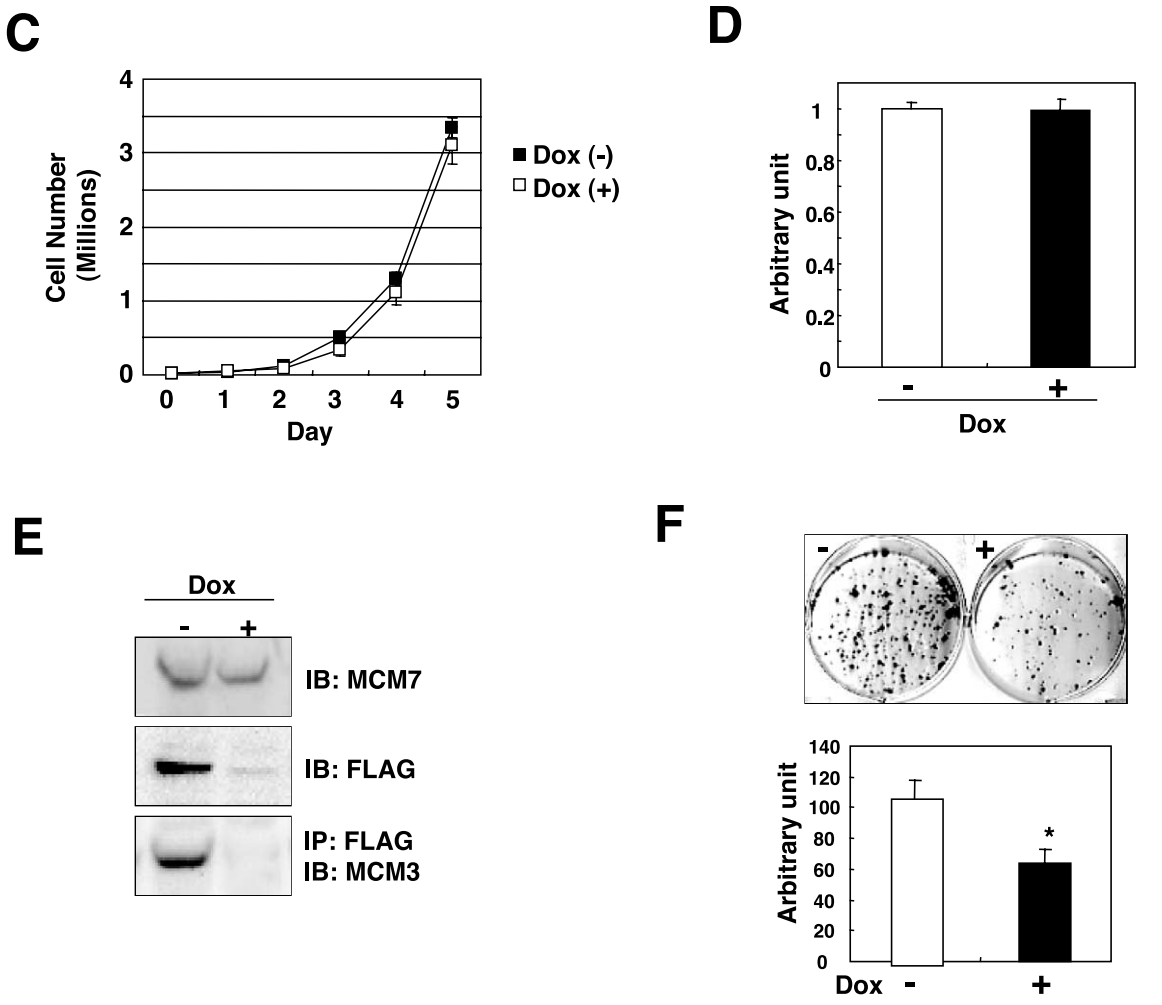

Fig. 2 (*Continued*).

3.2. Conditional MCM7 expression

To establish a cell line in which the exogenous MCM7 protein level is tightly regulated, we transfected first the tetracycline transactivator regulator protein expression vector and next the tetracycline-regulatable MCM7-FLAG expression vector, in which the expression of MCM7-FLAG could be shut off by Dox treatment. We used MCM7+/− cells because of their lower background of endogenous MCM7 protein than wild-type cells. We then tested the effect of the Dox treatment on MCM7-FLAG expression in MCM7$^{+/-}$/MCM7-FLAG cells. To follow the depletion of MCM7-FLAG, cell samples were taken at time points 0, 6, 24, and 30 h, after cells were split and placed in fresh medium containing 2 μg/ml of Dox, and analyzed by Western blotting with an anti-FLAG antibody (Fig. 2A). Upon addition of Dox, MCM7-FLAG protein levels decreased, and after 24 h the protein level of MCM7-FLAG was equal to the basal level of wild-type cells. Wild-type cells have no FLAG-tagged protein indicating the band seen in the same position of MCM7-FLAG is a non-specific signal (Fig. 2A). p53 and MCM3 remained unchanged at all time points (Fig. 2A). We also checked the MCM7-FLAG level after 30 h of treatment in culture medium containing different concentrations of Dox (Fig. 2B). Cell culture in medium containing 1 ng/ml Dox was sufficient to shut off the expression of MCM7-FLAG (Fig. 2B). ORC2 was consistently detected at all Dox concentrations (Fig. 2B).

To determine the effect of overexpressed MCM7 on cell growth and DNA replication, we compared the number of live cells and BrdU incorporation of MCM7$^{+/-}$/MCM7-FLAG cells in the presence or absence of Dox. No difference in growth rate and DNA replication activity was seen with or without Dox treatment (Fig. 2C,D). We then tested the functionality of MCM7-FLAG protein. Western blots of the chromatin fraction of MCM7$^{+/-}$/MCM7-FLAG cells treated with or without Dox showed equal amounts of MCM7 loaded on the chromatin fraction (Fig. 2E, upper panel). Specific chromatin loading of MCM7-FLAG was observed in the Dox-untreated MCM7$^{+/-}$/MCM7-FLAG cells and the functionality of this protein was further characterized by its co-immunoprecipitation with MCM3 protein (Fig. 2E, middle and lower panels).

3.3. Increased MCM7 expression supports the acceleration of EBV oriP-dependent DNA replication and tumor proliferation

A possible involvement of MCM proteins in the DNA replication from oriP of EBV has been recently documented [24,25]. Therefore, MCM7$^{+/-}$/MCM7-FLAG cells are a suitable assay system to check whether the DNA replication from

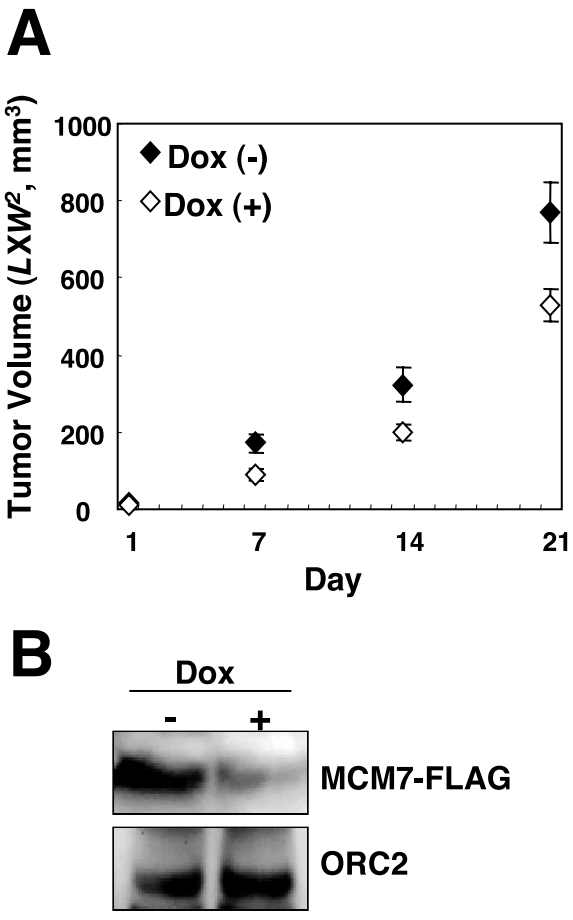

Fig. 3. Increased expression of MCM7 supports efficient tumorigenicity. A: Growth curves of MCM7$^{+/-}$/MCM7-FLAG cells treated with (+) or without (−) Dox xenografts in nude mice. The Dox (+) mice were maintained on 2 mg Dox/ml water for the duration of the experiments. Error bars represent the standard deviation ($n=4$ mice per group). B: Western blot analysis of xenograft lysates treated with (+) or without (−) Dox using anti-FLAG and anti-ORC2 antibodies.

oriP of EBV is dependent on a cellular licensing factor. A plasmid carrying oriP, EBNA-1 and a zeocin resistance gene (pCEP4/Zeo) was transfected into MCM7$^{+/-}$/MCM7-FLAG cells treated with or without Dox and the transfected cells were selected in zeocin for up to 2 weeks (Fig. 2F). Replication of pCEP4/Zeo allowed robust zeocin-resistant colonies to emerge in the Dox-untreated cells but a relatively small number of zeocin-resistant colonies was seen in the Dox-treated cells. pcDNA3.1/Zeo, a mammalian expression vector, produced almost the same number of colonies with or without Dox (data not shown). Since episomes contain only one origin of replication while chromosomes contain multiple origins, it is likely that viral episomes would be far more sensitive to the MCM7 protein level.

By global gene expression analysis, MCM7 expression was detected at high levels in metastatic colorectal cancers but at lower levels in non-metastatic tumors [26]. We, therefore, examined whether the presence or absence of Dox had any effect on tumorigenesis. MCM7$^{+/-}$/MCM7-FLAG cells were injected s.c. and grown as xenografts in nude mice for up to 3 weeks. There was an increase in tumor establishment of the Dox-untreated cells compared with Dox-treated cells (Fig. 3A). To check whether the regulation of MCM7-FLAG expression is maintained in xenografts, we performed immunoblotting using recovered tumors. We could observe that the expression of MCM7-FLAG protein was still regulated in xenografts (Fig. 3B). ORC2, a loading control, was equally detected. Taken together, these results indicate the increased expression level of MCM7 is responsible for the tumor growth as well as EBV oriP replication. This cell line should provide a unique system for the characterization of MCM7 function and for screening for potential therapeutic drugs for malignant tumors.

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