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Usp7 promotes medulloblastoma cell survival and metastasis by activating Shh pathway

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ABSTRACT

The ubiquitin-specific protease Usp7 plays roles in multiple cellular processes through deubiquitinating and stabilizing numerous substrates, including P53, Pten and Gli. Aberrant Usp7 activity has been implicated in many disorders and tumorigenesis, making it as a potential target for therapeutic intervention. Although it is clear that Usp7 is involved in many types of cancer, its role in regulating medulloblastoma (MB) is still unknown. In this study, we show that knockdown of Usp7 inhibits the proliferation and migration of MB cells, while Usp7 overexpression exerts an opposite effect. Furthermore, we establish Usp7 knockout MB cell line using the CRISPR/Cas9 system and further confirm that Usp7 knockout also blocks MB cell proliferation and metastasis. In addition, we reveal that knockdown of Usp7 compromises Shh pathway activity and decrease Gli protein levels, while P53 level and P53 target gene expression have no obvious changes. Finally, we find that Usp7 inhibitors apparently inhibit MB cell viability and migration. Taken together, our findings suggest that Usp7 is important for MB cell proliferation and metastasis by activating Shh pathway, and is a putative therapeutic target for MBs.

1. Introduction

Most cellular processes required proper levels of regulatory proteins. Precise protein turnover allows cells to rapidly adapt to internal and external stimuli [1]. Ubiquitin-mediated protein destabilization by the proteasome has emerged as a critical manner to govern protein turnover [2]. For ubiquitination, the proteins are targeted for degradation by covalent ligation to ubiquitin, a 76 amino acid residue protein [3]. Similar to other protein modifications, the process of ubiquitination is reversible due to the function of deubiquitinases, which remove ubiquitin chains from substrates [4]. Ubiquitin-specific protease (Usp7) is the first identified deubiquitinase isolated as a partner of the herpesvirus protein [5]. Increasing substrates of Usp7 have been identified, including P53 [6], Pten [7], Gli [8], and N-Myc [9], suggesting that Usp7 possibly plays both oncogenic and anti-cancer roles in distinct tissues. As a matter of fact, Usp7 has been implicated in many tumors, such as non-small cell lung cancer [10] and liver cancer [11]. Whether Usp7 is involved in regulating medulloblastoma (MB) formation is still unknown.

MB is the most common malignant tumor of central nervous system in children [12]. Multiple signaling pathways have been closely associated with MB formation, including Shh, Wnt [13]. Hyperactivation of the Shh pathway is a major reason, with 25% of human tumors harboring mutations in Patched, Sufu, Smo, or other genes in this pathway [14]. Since our previous data have demonstrated that Usp7 positively regulates Hh pathway in *Drosophila* and mammalian cells [8], it is fruitful to investigate whether Usp7 governs MB formation through triggering Shh signaling.

In this study, we find that knockdown of *usp7* inhibits the proliferation and metastasis of MB cells, while overexpression of *usp7* plays opposite roles. To remove the off-target effect, we knock out endogenous *usp7* using CRISPR/Cas9 method and demonstrate that loss of *usp7* also hampers MB cell proliferation. In addition, we

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figure out that *usp7* knockdown attenuates Shh pathway, while does not alter P53 protein level and P53 target gene expression. Finally, we further illustrate that Usp7 inhibitor treatment blocks MB cell proliferation and migration, suggesting that Usp7 inhibitor acts as a potential drug for MBs. Taken together, our findings show that Usp7 positively regulates MB formation through activating Shh signaling and provide Usp7 putative therapeutic target for MBs.

2. Methods and materials

2.1. Constructs

To generate Fg-Usp7 and Myc-Gli1 plasmids, we amplified the corresponding cDNA fragments using Vazyme DNA polymerase, and then cloned it into CMV-3 × Fg or pcDNA3.1-Myc vectors. Fg-Usp7-CA was generated using PCR-based site-directed mutagenesis at the background of Fg-Usp7.

2.2. Cell culture, transfection, and western blot

Daoy and D283 cells were purchased from ATCC and cultured in Eagle's Minimum Essential medium (Gibco) containing 10% FBS and 1% penicillin/streptomycin. Cells were transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 48 h s after transfection, cells were harvested for immunoprecipitation and western blot analysis with standard protocols as described [15]. The following antibodies were used for western blot: mouse anti-Fg (M2) (1:5000; Sigma); mouse anti-Actin (1:5000; Genscript); rabbit anti-Gli1 (1:1000; Santa Cruz); rabbit anti-Gli2 (1:1000; Santa Cruz); rabbit anti-Gli3 (1:1000; Santa Cruz); rabbit anti-Usp7 (1:1000; Santa Cruz); mouse anti-P53 (1:1000; Santa Cruz); goat anti-mouse HRP (1:10000; Abmax) and goat anti-rabbit HRP (1:10000; Abmax).

2.3. RNA interference

To silence *usp7* expression in MB cells, a mixture of two different siRNAs were used as described [8]. The siRNA sequences were as follows: *usp7*-siRNA-1 (5′-ACCCUUGGACAAUAUUCCUdTdT), *usp7*-siRNA-2 (5′-AGUCGUUCAGUCGUCGUAUdTdT), *gli1*-siRNA (GGCU-CAGCUUGUGTGUAAUdTdT) [16], *mock*-siRNA (5′-UUCU- CCGAAC-GUGUCACGUdTdT). All siRNAs were transfected at a final concentration of 100 nM using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. MTT assay

After treatments, log-phase cells were seeded onto 96-well plates (1×10^3–10×10^3 cells per well). At 12, 24, 48 and 72 h after transfection, 20 μL MTT (5 mg/ml) was added, followed by additional incubation at 37 °C for 4 h before discarding supernatants. Then, 150 μL DMSO was added to each well before shaking gently for 10 min to dissolve crystals. The absorbance of each well was measured at 570 nm using microplate reader. Viability of tumor cells = absorbance of treatment wells/absorbance of control well × 100%. Data are presented as means ± SD of values from at least three experiments.

2.5. RNA isolation, reverse transcription, and Real-time PCR

After different treatments, Cells were lysed in TRIzol (Invitrogen) for RNA isolation following standard protocols. 1 μg RNA was used for reverse transcription. Real-time PCR was performed on ABI Fast7500 with Maxima SYBR Green qPCR Master Mix (Vazyme). 2-ΔΔCt method was used for relative quantification. The primer pairs used were as follows: *usp7*, 5′-GGAAGCGGGAGATACAGATGA-3′ (forward) and 5′-AAGGACCGACTCACTCAGTCT-3′ (reverse); *gli1*, 5′-TGTGTAT- GAAACTGACTGCCG-3′ (forward) and 5′-CCCAGTGGCA-CACGA- ACTC-3′ (reverse); *hhip1*, 5′-ATTGCTTCCCTAATGTCCT-3′ (forward) and 5′-GGGAGGTAGACCCACACCA-3′ (reverse); *ptch1*, 5′-TGTGC- GCTGTCTTCCTTCTG-3′ (forward) and 5′-CACGGCACT-GAGCT- TGATTC-3′ (reverse); *bcl2*, 5′-CATGCCAAGAGGGAAA-CACCAG-3′ (forward) and 5′-GTGCTTTGCATTCTTGGATGAG-3′ (reverse); *p53*, 5′-ACTTGTCGCTCTTGAAGCTAC-3′ (forward) and 5′-GATGCGG- AGAATCTTTGGAAC-3′ (reverse); *p21*, 5′-TGCAACTAC-TACAGA- AACTG-3′ (forward) and 5′-CAAAGTGGTCGGTAGCCACA-3′ (reverse); *puma*, 5′-GCCAGATTTGTGAGACAAG-3′ (forward) and 5′-CAGGCACCTAATTGGGCTC-3′ (reverse); *bax*, 5′-CCCGAGAGGT-CTTTTTCCGA-3′ (forward) and 5′-CCAGCCCATGATGGTTCTGA-3′ (reverse); *actin*, 5′-AGAGCTACGAGCTGCCTGAC-3′ (forward) and 5′-AGCACTGTGTTGGCGTACAG-3′ (reverse). Data are presented as means ± SD of values from at least three experiments.

2.6. Transwell invasion assay

After treatments, 3×10^4 Daoy cells were seeded on top of a thick layer of Matrigel in transwell inserts (BD Biosciences) and cultured for another 24 h. Invasive cells adhered to the lower surface of filter were washed with PBS, fixed with 4% paraformaldehyde and stained with 0.05% crystal violet. The invasive cells were counted under a light microscope.

2.7. CRISPR/Cas9 genome editing

The following sgRNA targeting *usp7* was designed using the CHOPCHOP online tool: TGATGGACACAACACCGCGG. It was annealed to the complementary oligo and cloned into the pSpCas9(BB)-2A-GFP (Addgene). Daoy cells were transfected with this plasmid. Cells were continued incubating for 24 h post-transfection and sorted by flow cytometry for GFP. GFP-positive single cells were isolated and re-grown into small colonies in 96-well plates. Genomic DNA from target cells is amplified by PCR. Putative mutants were further validated by Sanger sequencing.

3. Results

3.1. Knockdown of usp7 attenuates the proliferation and migration of MB cells

To investigate whether Usp7 is involved in regulating MB formation and metastasis, we knocked down endogenous *usp7* in Daoy cells using siRNA. Western blot result showed that *usp7*-RNAi effectively blocked *usp7* expression (Fig. 1A). Compared with control cells, *usp7* knockdown apparently inhibited the proliferation of Daoy cells (Fig. 1A). On the other hand, we found that overexpression of wild type Usp7 (Fg-Usp7) promoted Daoy cell proliferation, while the dominant-negative form of Usp7 (Fg-Usp7-CA) played an opposite role (Fig. 1B). To validate our result, we chose another MB cell line, D283, to repeat our experiments. Consistently, knockdown of *usp7* inhibited D283 cell proliferation (Fig. 1C). Overexpression of Usp7 increased, while Usp7-CA decreased the proliferation of D283 cells (Fig. 1D). These observations strong suggest that Usp7 plays a possible role for the proliferation of MB cells.

The metastasis process is important for tumorigenesis. We next wanted to assess whether Usp7 regulates the metastasis of MB cells. Given D283 cell line is suspended, we only examined the migration of Daoy cells using transwell assay. Compared with *mock*-siRNA, *usp7*-siRNA treatment obviously inhibited the migration of Daoy cells (Fig. 1E). Conversely, overexpression of Usp7, not Usp7-