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Enhancer of zeste homolog 2 depletion induces cellular senescence via histone demethylation along the INK4/ARF locus

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ABSTRACT

Polycomb group proteins are epigenetic transcriptional repressors that function through recognition and modification of histone methylation and chromatin structure. As a member of PcG proteins, enhancer of zeste homolog 2 (EZH2) targets cell cycle regulatory proteins which govern cell cycle progression and cellular senescence. In previous work, we reported that EZH2 depletion functionally induced cellular senescence in human gastric cancer cells with mutant p53. However, whether EZH2 expression contributes to the change of key cell cycle regulators and the mechanism involved are still unclear. To address this issue, we investigated the effects of EZH2 depletion on alteration of histone methylation pattern. In gastric cancer cells, INK4/ARF locus was activated to certain extent in consequence of a decrease of H3K27me3 along it caused by EZH2 silence, which contributed substantially to an increase in the expression of $p15^{INK4b}$, $p14^{ARF}$ and $p16^{INK4a}$ and resulted in cellular senescence ultimately. Furthermore, MKN28 cells, which did not express $p16^{INK4a}$ and $p21^{cip}$, could be induced to senescence via $p15^{INK4b}$ activation and suppression of $p15^{INK4b}$ reversed senescence progression induced by EZH2 downregulated. These data unravel a crucial role of EZH2 in the regulation of INK4/ARF expression and senescence procedure in gastric cancer cells, and show that the cellular senescence could just depend on the activation of $p15^{INK4b}$/Rb pathway, suggesting the cell-type and species specificity involved in the mechanisms of senescence inducement.

1. Introduction

Senescence is a major anticancer barrier characterized by a permanent cell cycle arrest and triggered by telomere shortening, DNA damage, or excessive mitogenic signals due to oncogene activation (Bartkova et al., 2006), which accompanied by a set of characteristic morphological and physiological features distinguishing from proliferating cells, arrested quiescent and terminally differentiated cells (Campisi, 2011). Oncogene induced senescence acts as a potent tumor suppressor mechanism through the tumor suppressor pathways, $p14^{ARF}$/MDM2/p53 and $p16^{INK4a}$/retinoblastoma (Rb) (Halazonetis et al., 2008). It is also accepted that tumor cells can be forced to undergo senescence by genetic manipulations and by epigenetic factors, including anticancer drugs, radiation, and differentiating agents (Carnero, 2013). Thus pro-senescence approach has been proposed and developed as potential anti-cancer therapy strategy.

The INK4/ARF locus locates on the human chromosome 9p21 and encodes $p15^{INK4b}$, $p14^{ARF}$, and $p16^{INK4a}$, which are known as common key reprogramming regulators (Hirosue et al., 2012). $p14^{ARF}$ activates p53 by sequestering murine double minute 2 (MDM2), an E3 ubiquitin ligase, to the nucleolus, thereby preventing the MDM2-mediated targeting of p53 to proteolytic degradation (Kulju and Lehman, 1995). p53 subsequently activates $p21^{cip}$, which is the mediator of p53 dependent senescence (Brown et al., 1997). $p16^{INK4a}$ and $p15^{INK4b}$ are cyclin-dependent kinase inhibitors (CDKIs) that prevent CDK4 dependent phosphorylation

Abbreviations: PcG, polycomb group; EZH2, enhancer of zeste homolog 2; shRNA, short hairpin RNA; Rb, retinoblastoma; MDM2, murine double minute 2; CDKIs, cyclin-dependent kinase inhibitors; PRC1, polycomb repressive complex 1; PRC2, polycomb repressive complex 2; H3K27me3, histone H3 Lys27 trimethylation; q-RT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-poly-acrylamide gel electrophoresis; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; DMSO, dimethyl sulfoxide; SA-β-gal, senescence β-galactosidase; ChIP, chromatin immunoprecipitation; HSCs, hematopoietic stem cells.

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of Rb, thereby preventing E2F-mediated cell cycle progression (Hirosue et al., 2012). As a central role in the decision of cellular fate, INK4/ARF expression is controlled by various signal transduction pathways and patterns of expression vary depending on physiological circumstances (Kim and Sharpless, 2006).

Polycomb group (PcG) proteins are highly conserved epigenetic effectors that maintain, by posttranslational modification of histones, the silenced state of genes involved in critical biologic processes, including cellular development, stem cell plasticity, and tumor progression (Deb et al., 2014). PcG proteins operate as two classes of multimeric chromatin-binding complexes—polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) (Kuzmichev et al., 2004). Enhancer of zeste homolog 2 (EZH2), a component of PRC2, specially trimethylates lysine 27 of histone H3 (H3K27me3) via the SET domain, thereby regulates genes expression by epigenetic regulatory mechanism (Pal et al., 2013). For gastric cancer, EZH2 is more highly expressed in cancerous than non-cancerous tissues, and closely correlated with tumor size, depth of invasion, vessel invasion, lymph node metastasis and clinical stages (Choi et al., 2010). Additionally, EZH2 over-expression increases cell proliferation and tumorigenesis through repressing the expression of cell cycle CDKIs such as $p16^{INK4a}$, $p14^{ARF}$ and $p21^{cip}$ (Cakouros et al., 2012, Fan et al., 2011). These findings suggest that EZH2 is involved in the progression to advanced disease, and may be a key driver of cellular ectopic proliferation via repression of CDKIs expression to avoid senescence.

We previously reported that inhibiting expression of EZH2 conferred promotion to doxorubicin induced premature senescence partly dependent on p53-$p21^{cip}$ axis activated (Bai et al., 2014), but we provided little information available regarding the mechanism involved. In this study, we sought to determine the impact of EZH2 on senescence in gastric cancer cells and explore the mechanism whereby EZH2 may influence the activity of CDKIs.

2. Materials and methods

2.1. Cell culture

Human gastric cancer cell lines MKN28 (GeneChem), AGS and SGC-7901 (The Type Culture Collection of the Chinese Academy of Sciences) were maintained in RPMI 1640 (Hyclone), supplemented with 10% fetal bovine serum (Hyclone) in a humidified CO_2 (5%) incubator at 37 °C. Gastric cancer cells were split to 50% confluence 24 h before treatment. At the end of the treatment course, cells were harvested for analysis.

2.2. RNA interference

For RNA interference mediated suppression of human EZH2 and human $p15^{INK4b}$, two shRNA-expressing plasmid were constructed (Genechem) separately. The annealed oligos of EZH2 (GeneBank ID: NM_004456) and $p15^{INK4b}$ (GeneBank ID: NM_004936) (EZH2-shRNA 1: 5′ccggCCAACACAAGTCATCCCATTActcgag TAATGGGATGACTTGTGTTGGttttt3′ and 5′ aattcaaaaaCCAACACAAGTCATCCCATTActcgag TAATGGGATGACTTGTGTTGG3′; EZH2-shRNA 2: 5′ccggCGGAAATCTTAAACCAAGAATctcgagATTCTTGGTTTAAGATTTCCGttttt3′ and 5′aattcaaaaaCGGAAATCTTAAACCAAGAATctcgagATTCTTGGTTTAAGATTTCCG3′; $p15^{INK4b}$-shRNA 1: 5′ccggACTAGTGGAGAAGGTGCGACActcgagTGTCGCACCTTCTCCACTAGTttttt3′ and aattcaaaaaACTAGTGGAGAAGGTGCGACActcgagTGTCGCACCTTCTCCACTAGT3′; $p15^{INK4b}$-shRNA 2: 5′ccggCCCAACGGAGTCAACCGTTTCctcgagGAAACGGTTGACTCCGTTGGGttttt3′ and 5′ aattcaaaaaCCCAACGGAGTCAACCGTTTCctcgagGAAACGGTTGACTCCGTTGGG3′) were inserted into GV248 vector. Gastric cancer cells were transfected with plasmids using Lipofectamine 2000 (Invivogen). Stable pools were selected in media containing 2 μg/ml puromycin (Sigma), and individual drug-resistant clones were collected and expanded.

2.3. RNA isolation, cDNA synthesis, and q-RT-PCR

Total RNA was isolated by Trizol (Takara). cDNA was synthesized by using PrimeScript RT Reagent Kit (Takara) according to manufacturer's instructions. Quantitative real-time PCR (q-RT-PCR) was conducted using the Taq polymerase (Takara) and data are shown as fold change ($2^{-\Delta\Delta ct}$). q-RT-PCR amplification was performed using the following primers: EZH2 forward (F) 5′ GACGGCTTCCCAATAACA 3′, reverse (R) 5′ TGAGGCTTCAGCACCACT 3′; $p14^{ARF}$ F 5′ TGGAGGCGGCGAGAACAT 3′, R 5′ AGTAGCATCAGCACGAGGG 3′; $p15^{INK4b}$ F 5′ GTGGCTACGAATCTTCCG 3′, R 5′ GTCGCTTGCACATCCTCT 3′; $p16^{INK4a}$ F 5′ GAGGCCGATCCAGGTCAT 3′, R 5′ TCTAAGTTTCCCGAGGTTTCT 3′; GAPDH F 5′ AACGGATTTGGTCGTATTG 3′, R 5′ GGAAGATGGTGATGGGATT 3′.

2.4. Cell proliferation assay

For the determination of cell growth, 1×10^4 cells per well in 24-well plates were incubated on day 0. Cells were trypsinized briefly and cell number counted after 1, 2, 3 and 4 days.

2.5. Western blotting

Cell protein lysed in RIPA buffer containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Guge Biotechnology). Proteins were resolved by sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were incubated with the indicated primary antibodies and anti-rabbit or mouse secondary antibodies conjugated to horseradish peroxidase (HRP) (Pierce). After developed by the ECL system (Guge Biotechnology), the signal was detected by GelDoc XR System (Biorad). Antibodies used were as follows: rabbit anti-human EZH2 (Cell Signaling Technology), rabbit anti-human H3K27me3 (Cell Signaling Technology), mouse anti-human $p14^{ARF}$ (Cell Signaling Technology), rabbit anti-human $p15^{INK4b}$ (ORIGENE), rabbit anti-human $p16^{INK4a}$ (RabMAbs), rabbit anti-human pRb (pS780) (RabMAbs), rabbit anti-human Rb (Affinity Biosciences), rabbit anti-human MDM2 (RabMAbs), rabbit anti-human GAPDH (Sigma).

2.6. Cell cycle analysis

Cells washed with phosphate-buffered saline (PBS) and fixed with ice cold 75% ethanol at 4 °C overnight. Samples were then washed with PBS twice and stained with 100 μg/ml propidium iodide (Sigma) containing RNase A (Sigma) for 30 min at 37 °C. Cell cycle distribution in different phases was determined using flow cytometry (Becton Dickinson).

2.7. Colony formation assay

Cells seeded at a concentration of 1000 cells per well in 6-well plates. After incubation for 10 days, the cells were washed and fixed in 4% paraformaldehyde for 15 min at room temperature and stained with crystal violet. The colonies were then photographed and counted.

2.8. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT)

Cell viability was measured using MTT assay. Cells were plated in 96-well plates. At the end of treatment, 20 μL MTT (5 mg/mL)