Mechanisms of Ageing and Development 133 (2012) 549–555

Contents lists available at SciVerse ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

Two mechanisms underlying the loss of $p16^{Ink4a}$ function are associated with distinct tumorigenic consequences for WS MEFs escaping from senescence

Xiaoming Wu [1], Shuting Jia [1], Xiufeng Zhang, Xiaoyu Si, Wenru Tang *, Ying Luo *

Laboratory of Molecular Genetics of Aging & Tumor, Faculty of Medicine, Kunming University of Science & Technology, 727 Jing Ming Nan Road, Chenggong County, Kunming, 650500, Yunnan Province, China

ARTICLE INFO

Article history:
Received 31 January 2012
Received in revised form 22 June 2012
Accepted 8 July 2012
Available online 16 July 2012

Keywords:
Genomic DNA deletion
p53 mutation
$p16^{Ink4a}$ methylation
Senescence
Werner syndrome

ABSTRACT

Werner syndrome (WS) mouse embryonic fibroblasts (MEFs) can spontaneously escape from senescence and become immortalized, either tumorigenic or non-tumorigenic. Our data revealed a single $p53^{N236S}$ point mutation in the tumorigenic cell lines, which was correlated with the down-regulation of $p21^{Waf1/Cip1}$. $p16^{Ink4a}$ expression was significantly decreased in all immortalized cell lines.

Bisulfate sequencing indicated that the *$p16^{Ink4a}$* gene was methylated in the tumorigenic cells. Exogenous overexpression of $p21^{Waf1/Cip1}$ demethylated *$p16^{Ink4a}$* and restored its expression, which induced cell growth arrest and senescence. While in non-tumorigenic immortalized cells, the *Ink4a* loci and adjacent genomic DNA were found to be deleted.

These data suggest that the loss of $p16^{Ink4a}$ function by either genomic DNA deletion or methylation have been adopted by senescent WS MEFs escaping from senescence, with distinct tumorigenic consequences. The fact that cells that had escaped senescence *via* the spontaneous biallelic deletion of the *Ink4a* loci could not form tumors suggests that the functional loss of $p16^{Ink4a}$ *per se* might not be sufficient for tumorigenesis; most likely, it is a byproduct and passenger mutation. The mutations in factors regulating *$p16^{Ink4a}$* methylation might be the driver mutation. These findings shed light on the strategy of anti-aging by regulating $p16^{Ink4a}$ expression.

1. Introduction

Premature aging diseases, or progeroid syndromes, are rare genetic diseases; such diseases include Werner syndrome (WS), Hutchinson–Gilford progeria syndrome, Bloom syndrome, Cockayne syndrome and Rothmund–Thomson syndrome (Ramirez et al., 2007; Kipling et al., 2004). These diseases share features of cancer prone, therefore, despite their rarity, progeroid syndromes and their animal models have provided important tools for basic research aimed at understanding the crosstalk between cancer and aging. Werner syndrome (WS) is an autosomal recessive progeric disease that is characterized by premature atherosclerosis, ischemia heart disease, osteoporosis, cataracts, *etc.* (Epstein et al., 1966). Cellular senescence in human fibroblasts appears to be triggered by telomere erosion (Wright and Shay, 2002), and it appears that the telomeres of WS fibroblasts erode at a faster rate (Schulz et al., 1996). Fibroblasts from WS patients have a dramatically reduced cellular life span that can be rescued by the reconstitution of telomerase, suggesting that telomere dysfunction is essential for WS cellular senescence (Wyllie et al., 2000). Dysfunctional telomeres have been shown to initiate DNA damage checkpoint responses to induce cellular senescence (D'adda Di Fagagna et al., 2003). Further study found that WS cellular senescence was associated with hypophosphorylated pRb and high levels of $p16^{Ink4a}$ and $p21^{Waf1/Cip1}$, and the disruption of p53 function made cells re-enter the cell cycle (Davis et al., 2003). p53 deficiency has been shown *in vivo* to rescue cells from the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis (Chin et al., 1999). Therefore, the loss of p53 function is necessary for senescent cells to escape senescence, although this is associated with an increased risk of carcinogenesis.

To mimic human WS, mutant mice bearing a deletion of the murine WRN helicase domain were generated. The mutant mice developed T-cell lymphoma; however, they failed to manifest an aging phenotype (Lebel and Leder, 1998). Another mutation that eliminated the expression of the C-terminal domain of the WRN helicase was introduced to generate the WS mouse model. However, the mutant mice again did not show any overt histologic signs of accelerated aging (Lombard et al., 2000). The fact that

* Corresponding authors at: Laboratory of Molecular Genetics of Aging & Tumor, Faculty of Life Science and Technology & Faculty of Environmental Science and Engineering, Kunming University of Science & Technology, 727 Jing Ming Nan Road, Chenggong County, Kunming, 650500, Yunnan Province, China.
Tel.: +86 871 5920753, fax: +86 871 5920753.
E-mail addresses: twr@sina.com (W. Tang), yingluo@kmust.edu.cn (Y. Luo).
[1] These authors contributed equally to the work.

0047-6374/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mad.2012.07.002

fibroblasts from WS patients show accelerated telomere erosion and senescence that can be rescued by the overexpression of TERT suggested that telomere dysfunction may play an essential role in the manifestation of the WS phenotype (Wyllie et al., 2000). To address this hypothesis, a mouse model of WS with a double knockout of *mTerc* and *Wrn* was generated, which faithfully replicated the human WS phenotype (Chang et al., 2004; Chang, 2005).

The previous study demonstrated that mouse embryonic fibroblasts (MEFs) from late-generation mice (G5 $mTR^{-/-}Wrn^{-/-}$) rapidly become senescent. Interestingly, the senescent MEFs are prone to escaping senescence and spontaneously becoming immortalized at a frequency of 1 in 1.4×10^5. Xenograft assays using immortalized cell lines revealed two distinct categories: one set of immortalized cell lines formed tumors (tumorigenic cell lines), whereas the second set of cell lines did not form tumors within 6 months (non-tumorigenic immortalized cell lines) (Laud et al., 2005). Investigating the molecular differences between these two types of cell lines might help elucidate how some cells could escape from senescence without paying the price of carcinogenesis.

In this study using non-tumorigenic and tumorigenic immortalized MEF cell lines derived from late generations of Werner Syndrome (*G5* $mTR^{-/-}Wrn^{-/-}$) mice, we revealed that different modifications of $p16^{Ink4a}$ play important roles in the molecular pathways involved in the consequences of escaping senescence.

2. Materials and methods

2.1. Cell lines, constructs and antibodies

The cell lines used were established as described (Laud et al., 2005). All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum in 3% oxygen and 5% CO_2 at 37 °C.

The $p21^{Waf1/Cip1}$ cDNA fragment was cloned into the retroviral vector PQCXIP (Clontech, CA). The P53S cDNA fragment was cloned from the immortalized cell line 395-3B-1 by RT-PCR and put into the retroviral vectors PQCXIP and PQCXIH (Clontech, CA), tagged with cMyc. Overexpression of the constructed proteins was confirmed by western blotting.

The antibodies used for western blotting were anti-phospho-ATM (Ser1981) (1:1,000, Cell Signaling, MA), anti-Chk2 (1:500, BD Transduction Laboratories, CA), anti-cMyc (9E10) (1:500, Santa Cruz, CA), anti-$p16^{Ink4a}$ (M-156) (1:500, Santa Cruz, CA), anti-$p21^{Waf1/Cip1}$ (F-5) (1:500, Santa Cruz, CA), anti-p53 Ab-1 (clone PAb240) (1:250, Neomarker, CA), anti-phospho-p53 (Ser15) (1:500, Cell Signaling, MA), and anti-γ-tubulin (1:5000, Sigma, MO).

2.2. SA-β-Gal staining and BrdU incorporation

S-A-β-Gal staining was performed as described previously (Dimri et al., 1995). Briefly, cultured cells were fixed and stained for SA-β-galactosidase activity in 37 °C for 4 h. BrdU incorporation was performed with the *in situ* cell proliferation kit FLUOS (Roche, Germany) according to the manufacturer's instructions. Briefly, cells were labeled with BrdU (final concentration: 10 μM) for 1 h and immunostained with anti-BrdU-FLUOS antibody. The BrdU incorporation rate was measured by counting cells labeled with anti-BrdU antibody.

2.3. Bisulfate sequencing PCR

DNA methylation of the p16 promoter region was determined by the bisulfate modification of genomic DNA and subsequent sequencing. Bisulfate modification was performed with the DNA methylation kit (EZ DNA Methylation-Gold kit, ZYMO Research, CA) according to the manufacturer's instructions. Briefly, 2 μg of genomic DNA was treated with CT conversion reagent and desulfonated with M-Desulfonation buffer. The modified DNA was recovered and the p16 promoter region was amplified using specific primers (forward primer: AGGAAGGAGG-GATTTATTGGTTATA, reverse primer: AAAATATTATCTCTTCCTAAACAAAAATTA). The PCR products were separated by gel electrophoresis, and the desired bands were purified (QIAquick Gel Extraction kit, QIAGEN, Germany) and cloned into the TA vector (pEASY-T1 Cloning Kit, TransGen, China). For each cell line, approximately 10 clones were sequenced using the M13 primer.

3. Results

3.1. The molecular differences between tumorigenic and non-tumorigenic immortalized cell lines

Spontaneous immortalized foci have been shown to arise from senescent G5 $mTR^{-/-}Wrn^{-/-}$ MEFs at a frequency of 1 in 1.4×10^5

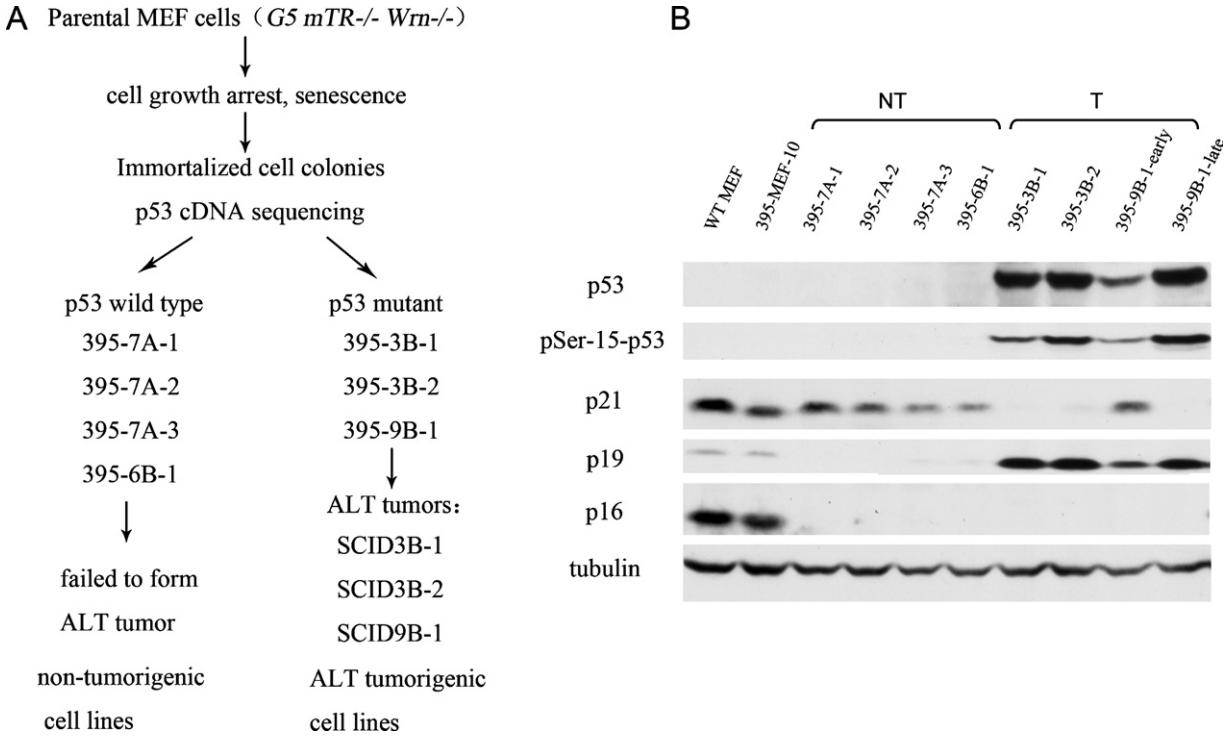

Fig. 1. The identification of two categories of cells that escape from senescence and the molecular differences between them. (A) Two categories of cell lines escape from senescence: tumorigenic and non-tumorigenic immortalized cell lines. (B) Western blot analysis showed that p53 was stabilized in tumorigenic (T) cell lines, and $p21^{Waf1/Cip1}$ was down-regulated. However, $p19^{Arf}$ was up-regulated. The $p21^{Waf1/Cip1}$ protein levels in non-tumorigenic (NT) cell lines were comparable to those in wild-type MEFs. Both $p19^{Arf}$ and the $p16^{Ink4a}$ protein levels were down-regulated in non-tumorigenic cell lines. Wild-type MEFs (WT-MEF) and parental WS MEFs (395-MEF-10) were used as controls. In tumorigenic cell lines, the p53 protein was stabilized, and $p21^{Waf1/Cip1}$ was down-regulated.