Published in final edited form as:

Methods Mol Biol. 2017 ; 1534: 31–39. doi:10.1007/978-1-4939-6670-7_3.

Cellular Model of p21-Induced Senescence

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Abstract

Cellular senescence is a unique process of normal physiology, from embryonic development to aging, also known for its association with a broad range of pathological conditions. Therefore a reliable model of cellular senescence remains an indispensable tool for the investigation of senescence-associated changes and human disease. Here we describe a model of HT1080 fibrosarcoma cells with an inducible senescence phenotype. These cells are equipped with the lac repressor and exogenous p21 under the control of a lac repressor regulated promoter. The senescent phenotype is induced in these cells by isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible expression of senescence-associated cell cycle inhibitor p21^{Waf1/Cip1/Sdi1}.

Keywords

Senescence; p21^{Waf1/Cip1/Sdi1}; Lac-repressor; IPTG; HT1080-p21–9

1 Introduction

Cell senescence, originally defined as irreversible proliferative arrest that occurs in normal cells after a limited number of cell divisions, is now viewed more broadly as a general biological program of terminal growth arrest. Cellular senescence is associated with normal development and pathological conditions [1, 2]. Cells that underwent senescence cannot divide even if stimulated by mitogens, but they remain metabolically and synthetically active and show characteristic changes in morphology, such as enlarged and flattened cell shape and increased granularity [3]. The most widely used surrogate marker of senescent cells is the senescence-associated β -galactosidase activity (SA- β -gal), which is detectable by X-gal staining at pH 6.0 [4]. SA- β -gal appears to reflect increased activity of lysosomal acid β -galactosidase [5]. As elucidated primarily in the normal fibroblast models, growth arrest of senescent cells is initiated with the activation of p53. In the case of replicative senescence, p53 protein is stabilized through the involvement of p14^{ARF}, a tumor suppressor that sequesters the Mdm2 protein, which promotes p53 degradation. The activated p53 has

multiple effects on gene expression, the most relevant of which in regard to senescence is transcriptional activation of p21^{Wai1/Cip1/Sdi1}, a pleiotropic inhibitor of cyclin/cyclin-dependent kinase (CDK) complexes that mediate cell cycle progression [6]. p21 induction causes cell cycle arrest in senescent cells. The activation of p53 and p21 in senescent cells is only transient; protein levels of p53 and p21 decrease after the establishment of growth arrest. While p21 expression goes down, another CDK inhibitor, p16^{Ink4A}, becomes constitutively upregulated and maintains growth arrest in senescent cells [7, 8].

To examine the transition from growth arrest to senescence and the properties of senescent cells, we have used a cellular system based on inducible expression of p21^{WAF1/CIP1}, in human HT1080 fibrosarcoma cells. p21 was cloned in an IPTG-inducible retroviral vector (*see* Fig. 1a) and transduced into HT1080 cells expressing the lac I repressor. IPTG dose-dependent induction of p21 (*see* Fig. 2a, b) resulted in growth arrest in G1 (at low p21 levels) or in G1, S, and G2 (at high p21 levels) (*see* Fig. 2c). p21 induction also led to time- and dose-dependent expression of senescence-associated β -galactosidase (SA- β -gal) and morphologic features of senescent cells (*see* Fig. 2). After the removal of IPTG, most of the cells reentered the cell cycle, but many of them died or stopped growing after a small number of cell divisions. The dead or growth-arrested cells were predominantly in G2/M or polyploid. The failure to recover was directly correlated with the induced levels of p21 and the duration of p21 induction. Cells that were released from IPTG after 5 days of p21 induction (poor recovery) showed predominantly abnormal mitoses, in contrast to a high frequency of normal mitoses in cells that were released after 1 day of induction (significant recovery) [9]. Analysis of the effects of transient p21 induction on the expression of genes involved in the control of cell division suggests that p21-mediated inhibition of specific genes involved in mitosis control (such as CDC2 or cyclin A) may be responsible for abnormal mitosis after release from p21-induced growth arrest (*see* Fig. 3) [9, 10]. Hence, the failure of HT1080 p21–9 cells to recover after p21-induced growth arrest is due to mitotic catastrophe [9].

1.1 Applications of p21-Inducible HT1080-p21–9 Cell Line

Initially, p21-inducible cell system had been developed to recapitulate effects of DNA damaging drugs and to investigate the mechanisms of drug-induced senescence [11]. However, this system turned out to be very suitable for the analysis of many aspects of cellular senescence. These cells were extensively used for understanding p21-dependent transcriptional regulation and regulation of stability of tumor suppressors [12–15]. p21–9 cells were utilized for the investigation of the function of cell cycle controlling proteins in the regulation of intracellular localization of human papillomaviruses and mechanisms of regulation of cellular senescence by mTOR pathway and hypoxia [15–18]. The cells were used as a model for studies of abnormal mitosis and of p21-dependent regulation of ROS production [9, 19]. The inducible cells allow for easy comparison between proliferating and arrested (p21-induced) states. The system was also used for comparative analysis of the effects of growth inhibitory siRNAs on dividing and arrested cells and kinetics of shRNA silencing in proliferating and halted cells [20]. Additionally, it was suggested that protein biosynthesis is increased in senescent cells; therefore, p21-induced cells were used for enhanced production of exogenous proteins [21]. p21–9 cells were utilized for developing

high-throughput screening systems used for identification of chemical inhibitors of cyclin-dependent kinases [22–24].

1.2 Development of p21-Inducible Cellular System

The inducible system is based on a derivative of HT1080 human fibrosarcoma cells. HT1080 cells containing ecotropic retroviral receptor were transfected with p3'SS plasmid, expressing a modified lac I repressor and carrying a hygromycin-resistance gene [25, 26]. Transfected cells were selected with 100–120 µg/mL of hygromycin, and individual colonies were picked and screened for optimal repressor activity. Modified LNCX vector, containing tri-meric lac operator downstream of CMV early promoter (LNXCO3) and carrying firefly luciferase, was transduced into the colonies and induction of luciferase activity by treatment with IPTG was measured. Based on the screening, the best clone (HT1080 3'SS6) was identified, where luciferase activity was induced up to 15-fold with 1.25 mM of IPTG [27].

HT1080-p21–9 cells were generated by transduction of a p21-expressing retrovirus into HT1080 3'ss6 cells [11]. The inducible retroviral vector LNp21CO3 was constructed by cloning of 492 bp p21 coding sequence into the IPTG-inducible retroviral vector LNXCO3 [28]. LNp21CO3 retroviral vector was transduced into HT1080 3'SS6 cells, and the transduced population was selected with 200 µg/mL G418. Clonal line p21–9 was derived from the LNp21CO3-transduced population of HT1080 3'SS6 cells by end-point dilution followed by screening of individual colonies for the strongest induction of p21 expression by IPTG. Like the parental HT1080 cell line, p21–9 cells express the wild-type pRb and p53 [11, 29].

2 Materials

Prepare all solutions using ultrapure deionized water (18 MΩ at 25 °C) and cell culture grade reagents. All solutions applied to cultured cells have to be filtered through sterile 0.22 µM filter (EMD-Millipore Polyethersulfone (PES) filter or equivalent) and kept sterile.

1. DMEM tissue culture medium: 200 mM L-glutamine, fetal bovine serum.
2. G418 (Thermo Fisher Scientific or equivalent). To prepare 50 mg/mL stock solution, weigh out 500 mg G418 and transfer to 10 mL of deionized water and mix until fully dissolved. Sterilize by filtration and store in 1 mL aliquots at –20 °C.
3. IPTG (isopropyl-β-D-thiogalactopyranoside, EMD-Millipore #5800 or equivalent). To prepare 10 mM stock solution, transfer 238 mg of IPTG to 10 mL of deionized water and mix until fully dissolved. Sterilize by filtration and store in 1 mL aliquots at –20 °C.
4. Anti-p21 antibodies: Mouse antihuman CIP1 monoclonal antibody (Clone 70/CIP1/WAF1).

3 Methods

3.1 Induction of p21 and Monitoring of p21-Induced Senescence

1. Cells need to be plated at a relatively low density, approximately 40,000 cells per 60 mm tissue culture plate (*see* Notes 1 and 2).
2. At least 12 h cell reattachment should be allowed before treatment.
3. IPTG at 50 μ M should be applied to achieve full induction of p21 expression. There is direct dose dependence of p21 induction by IPTG up to 50 μ M. Higher concentrations do not affect the level of p21 expression.
4. Cells are undergoing growth arrest and fully develop the senescent phenotype 72 h post induction. Example of induction of p21 expression and accumulation of senescent phenotype (*see* Fig. 1b).
5. Senescent phenotype can be verified by SA- β -gal staining at pH 6.0 (*see* refs. 4, 30) (*see* Fig. 1c).

3.2 Release from p21

1. Cells need to be plated at a relatively low density, approximately 40,000 cells per 60 mm tissue culture plate or 5000 cells per cover slip (for microscopy).
2. At least 12 h cell reattachment should be allowed before treatment.
3. IPTG at 50 μ M should be applied to achieve full induction of p21 expression.
4. Cells are undergoing growth arrest and fully develop the senescent phenotype 72 h post induction. There is direct correlation between the severity of p21-related recovery problems and the length of p21 induction and IPTG concentration.
5. To release cells from p21 induction, IPTG-containing medium has to be thoroughly removed, cells washed twice with PBS, and fresh medium added.
6. Senescent phenotype can be verified by SA- β -gal staining; at pH 6.0, mitotic abnormalities and cell death can be followed by time-lapse video microscopy and FACS analysis (*see* refs. 4, 9, 30) (*see* Fig. 3).

4 Notes

1. Cells need to be propagated in standard DMEM medium supplemented with 10 % FBS. Due to negative selection against p21, cells shouldn't be propagated in culture more than 2 weeks.
2. If p21 inducibility is diminished, cells could be reselected with G418 (200 μ g/mL) followed by subclone analysis. For each selected subclone, p21 expression has to be shown by western blot with anti-p21 antibodies, and the presence of senescence phenotype has to be confirmed.

Acknowledgments

This work was supported by NIH grant P20GM109091 (M.S., G.S., E.V.B.) and ACS grant IRG-13-043-01 (E.V.B.).

References

1. Munoz-Espin D, Serrano M (2014) Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol* 15(7):482–496. doi:10.1038/nrm3823 [PubMed: 24954210]
2. Munoz-Espin D, Canamero M, Maraver A, Gomez-Lopez G, Contreras J, Murillo-Cuesta S, Rodriguez-Baeza A, Varela-Nieto I, Ruberte J, Collado M, Serrano M (2013) Programmed cell senescence during mammalian embryonic development. *Cell* 155(5):1104–1118. doi:10.1016/j.cell.2013.10.019 [PubMed: 24238962]
3. Campisi J, d'Adda di Fagnana F (2007) Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 8(9):729–740 [PubMed: 17667954]
4. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* 92(20):9363–9367 [PubMed: 7568133]
5. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, Kleijer WJ, DiMaio D, Hwang ES (2006) Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* 5(2):187–195 [PubMed: 16626397]
6. Dotto GP (2000) p21(WAF1/Cip1): more than a break to the cell cycle? *Biochim Biophys Acta* 1471(1):M43–M56 [PubMed: 10967424]
7. Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC (1996) Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc Natl Acad Sci U S A* 93(24):13742–13747 [PubMed: 8943005]
8. Stein GH, Drullinger LF, Souillard A, Dulic V (1999) Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol* 19(3):2109–2117 [PubMed: 10022898]
9. Chang BD, Broude EV, Fang J, Kalinichenko TV, Abdryashitov R, Poole JC, Roninson IB (1999) p21^{Waf1/Cip1/Sdi1}-induced growth arrest is associated with depletion of mitosis-control proteins and leads to abnormal mitosis and endoreduplication in recovering cells. *Oncogene* 19(17):2165–2170
10. Muller WU, Nusse M, Miller BM, Slavotinek A, Viaggi S, Streffer C (1996) Micronuclei: a biological indicator of radiation damage. *Mutat Res* 366(2):163–169 [PubMed: 9001583]
11. Chang BD, Xuan Y, Broude EV, Zhu H, Schott B, Fang J, Roninson IB (1999) Role of p53 and p21^{waf1/cip1} in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. *Oncogene* 18(34):4808–4818 [PubMed: 10490814]
12. Vigneron A, Cherier J, Barre B, Gamelin E, Coqueret O (2006) The cell cycle inhibitor p21waf1 binds to the myc and cdc25A promoters upon DNA damage and induces transcriptional repression. *J Biol Chem* 281(46):34742–34750. doi:10.1074/jbc.M602492200
13. Tapias A, Ciudad CJ, Roninson IB, Noe V (2008) Regulation of Sp1 by cell cycle related proteins. *Cell Cycle* 7(18):2856–2867 [PubMed: 18769160]
14. Zhu H, Chang BD, Uchiumi T, Roninson IB (2002) Identification of promoter elements responsible for transcriptional inhibition of Polo-like kinase 1 and Topoisomerase II genes by p21^{WAF1/CIP1/SDI1}. *Cell Cycle* 1(1):59–66 [PubMed: 12429910]
15. Broude EV, Swift ME, Vivo C, Chang BD, Davis BM, Kalurupalle S, Blagosklonny MV, Roninson IB (2007) p21(Waf1/Cip1/sdi1) mediates retinoblastoma protein degradation. *Oncogene* 26(48):6954–6958 [PubMed: 17486059]
16. Deng W, Lin BY, Jin G, Wheeler CG, Ma T, Harper JW, Broker TR, Chow LT (2004) Cyclin/CDK regulates the nucleocytoplasmic localization of the human papillomavirus E1 DNA helicase. *J Virol* 78(24):13954–13965. doi:10.1128/jvi.78.24.13954-13965.2004

17. Leontieva OV, Demidenko ZN, Gudkov AV, Blagosklonny MV (2011) Elimination of proliferating cells unmasks the shift from senescence to quiescence caused by rapamycin. *PLoS One* 6(10), e26126. doi:10.1371/journal.pone.0026126
18. Leontieva OV, Natarajan V, Demidenko ZN, Burdelya LG, Gudkov AV, Blagosklonny MV (2012) Hypoxia suppresses conversion from proliferative arrest to cellular senescence. *Proc Natl Acad Sci U S A* 109(33):13314–13318
19. Masgras I, Carrera S, de Verdier PJ, Brennan P, Majid A, Makhtar W, Tulchinsky E, Jones GD, Roninson IB, Macip S (2012) Reactive oxygen species and mitochondrial sensitivity to oxidative stress determine induction of cancer cell death by p21. *J Biol Chem* 287(13):9845–9854. doi: 10.1074/jbc.M111.250357 [PubMed: 22311974]
20. Maliyekkel A, Davis BA, Roninson IB (2006) Cell cycle arrest drastically extends the duration of gene silencing after transient expression of short hairpin RNA. *Cell Cycle* 5(20): 2390–2395 [PubMed: 17102616]
21. Bucciarelli T, Levenson V, Primiano T (2003). Culture product; using promoters; accumulation proteins. Patent No.: US 6,635,448 B2
22. Shtutman M, Baig M, Levina E, Hurteau G, Lim CU, Broude E, Nikiforov M, Harkins TT, Carmack CS, Ding Y, Wieland F, Buttyan R, Roninson IB (2011) Tumor-specific silencing of COPZ2 gene encoding coatomer protein complex subunit zeta 2 renders tumor cells dependent on its paralogous gene COPZ1. *Proc Natl Acad Sci U S A* 108(30):12449–12454. doi:10.1073/pnas.1103842108, 1103842108 [pii] [PubMed: 21746916]
23. Chang BD, Roninson IB (2006) High-content screening for drugs against cancer and age-related diseases. Patent Nos.: US 8,592,147 B2
24. Chang BD, Roninson IB, Porter D (2008) Cdk1 pathway inhibitors and uses thereof. Patent No.: US20090281129 A1
25. Albritton LM, Tseng L, Scadden D, Cunningham JM (1989) A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. *Cell* 57(4):659–666 [PubMed: 2541919]
26. Fieck A, Wyborski DL, Short JM (1992) Modifications of the E. coli Lac repressor for expression in eukaryotic cells: effects of nuclear signal sequences on protein activity and nuclear accumulation. *Nucleic Acids Res* 20(7): 1785–1791 [PubMed: 1315957]
27. Chang BD, Roninson IB (1996) Inducible retroviral vectors regulated by lac repressor in mammalian cells. *Gene* 183(1–2):137–142 [PubMed: 8996098]
28. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent J, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* 75(4):817–825 [PubMed: 8242752]
29. Chang BD, Watanabe K, Broude EV, Fang J, Poole JC, Kalinichenko TV, Roninson IB (2000) Effects of p21^{Waf1/Cip1/Sdi1} on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proc Natl Acad Sci U S A* 97(8):4291–4296 [PubMed: 10760295]
30. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O (2009) Protocols to detect senescence-associated beta-galactosidase (SA- β gal) activity, a biomarker of senescent cells in culture and in vivo. *Nat Protoc* 4(12):1798–1806. doi:10.1038/nprot.2009.191 [PubMed: 20010931]

Fig. 1. induction of p21 expression and cell senescence by IPTG. **(a)** Scheme of LNp21C03 retroviral vector. **(b)** HT1080-p21-9 cells were treated with 50 μM of IPTG for 8–72 h. Merged image of DIC and staining with p21 antibodies is shown. **(c)** Cells were treated with 50 μM IPTG for 72 h. Senescent phenotype was determined by staining for SA-β-gal activity with X-gal at pH 6.0. SA-β-gal expression in untreated (*left*) and IPTG-treated (*right*) p21-9 cells

Fig. 2. Dose-dependent induction of cell cycle arrest (**a**), p21 protein, and SA-β-gal by IPTG (**b**, **c**). HT1080-p21-9 cells were treated for 48 h with IPTG. ELISA measurement using WAF1 ELISA kit (Oncogene Research) (**b**) and western blot (**c**) of p21 protein expression. The percentage of SA-β-gal-positive cells was determined by X-gal staining at pH 6.0 following scoring of 100–400 cells per sample.

Fig. 3. Release from p21. **(a)** FACS profile of DNA content of attached cells at different time points after release from 1-day treatment with 50 μ M of IPTG. **(b)** Examples of normal (*left*) and abnormal (*right*) mitotic figures observed in p21–9 cells after release from IPTG (visualized with DAPI staining).