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Mini Review

Visualizing the dynamics of senescence stress response in living animals

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Although the role of *p16*^{INK4a} tumor suppressor gene expression is well documented in various cell culture studies, its *in vivo* roles are poorly understood. To gain further insight into the roles and mechanisms regulating *p16*^{INK4a} gene expression *in vivo*, we attempted to visualize the dynamics of *p16*^{INK4a} gene expression using bioluminescence *in vivo* imaging technique in living mice. By monitoring and quantifying the *p16*^{INK4a} gene expression repeatedly in the same mouse throughout its entire lifespan, we were able to unveil the dynamics of *p16*^{INK4a} gene expression in the aging process. This system can also be applied to chemically- or genetically- induced carcinogenesis. Here, we introduce you a novel approach to study senescence stress response *in vivo* and its potential towards understanding molecular link between aging and cancer.

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Introduction

Oncogenic proliferative signals are coupled to a variety of growth inhibitory processes[1)]. In cultured primary human fibroblasts, for example, ectopic expression of oncogenic Ras or its downstream mediator initiates cellular senescence, the state of irreversible cell cycle arrest, through up-regulation of cyclin-dependent kinase (CDK) inhibitors, such as p16^{INK4a}. To date, much of our current knowledge of how human *p16*^{INK4a} gene expression is induced by oncogenic stimuli derives from studies undertaken in cultured primary cells[2)]. However, since human *p16*^{INK4a} gene expression is also induced by tissue culture imposed stress[3-5)], it remains unclear whether the induction of human *p16*^{INK4a} gene expression in tissue cultured cells truly reflects an anti-cancer process or an artifact of tissue culture imposed stress. To eliminate any potential problems arising from tissue culture imposed stress, we have recently developed a bioluminescence imaging (BLI) system for non-invasive and real-time analysis of human *p16*^{INK4a} gene expression in the context of a living animal[6)].

Real-time imaging of *p16*^{INK4a} gene expression in living animals

In order to monitor human *p16*^{INK4a} gene expression as accurately as possible, we used a large genomic DNA segment of human chromosome that contains entire *INK4a/ARF* gene locus[6)] (Fig.1). Furthermore, this human chromosome

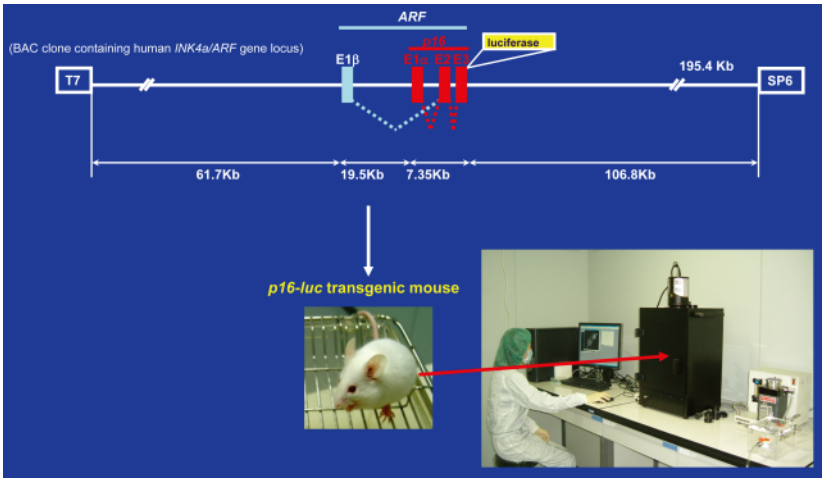

Fig.1 Strategy for *in vivo* imaging of *p16^{INK4a}* gene expression

A large genomic DNA segment (195.4 kb) of human chromosome that contains the entire *INK4a/ARF* gene locus and surrounding sequences was engineered to express luciferase-tagged p16^{Ink4a}. FISH technique reveals that the transgenic mice line (p16-luc) contanins a single copy of the human chromosome segment. The arrow shows the transgene. The p16-luc mouse was anesthetized and subjected to *in vivo* bioluminescence imaging after injection of luciferin.

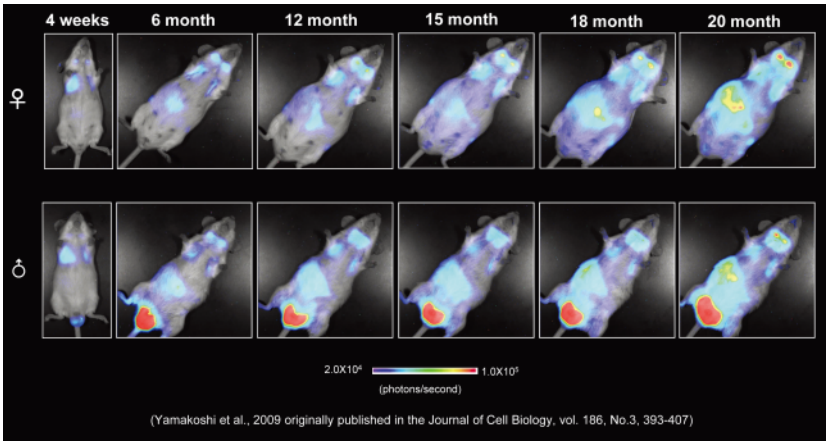

Fig.2 Real-time bioluminescence imaging of *p16^{INK4a}* gene expression during aging process *in vivo*

The same p16-luc mice were subjected to noninvasive BLI throughout their entire life span. The level of bioluminescent signals is significantly increased throughout the body during aging. (Yamakoshi et al., 2009 originally published in the Journal of Cell Biology, Vol.186, No.3 393-407.)

segment was engineered to express a fusion protein of human p16^{INK4a} and firefly luciferase (p16-luc fusion protein) without deleting any genomic DNA sequences of the *INK4a/ARF* gene locus[6] (Fig.1). This is very important because BMI-1, which is a negative regulator of *p16^{INK4a}* gene expression, has been shown to bind not only to the promoter region but also to the intron region of the *p16^{INK4a}* gene locus. Moreover, the expression of p16-luc fusion protein enables us to specify *p16^{INK4a}* gene expression but not ARF gene expression from this overlapping gene locus.

A transgenic mouse line that carries p16-luc DNA (*p16-luc* mouse) was established. By monitoring and quantifying the bioluminescent signal repeatedly in the same *p16-luc* mouse throughout its entire lifespan, we were able to unveil the dynamics of human *p16^{INK4a}* gene expression in the aging process of the transgenic mouse (Fig.2). Importantly moreover, the levels of bioluminescence signal were well correlated with not only exogenous (human) but also endogenous (mouse) *p16^{INK4a}* gene expression, indicating that overall regulation of human *p16^{INK4a}* gene expression is very similar to that of mouse *p16^{INK4a}* gene expression, at least in mouse cells[6]. This is well consistent with previous notion that the levels of *p16^{INK4a}* gene expression were increased during aging process of both rodents and primates[7, 8]. These results illustrate the potential of the p16-luc mice for analysis of *p16^{INK4a}* gene expression against oncogenic stimuli *in vivo*.

The response of *p16^{Ink4a}* gene expression against oncogenic stimuli *in vivo*

Although ectopic expression of oncogenic Ras initiates

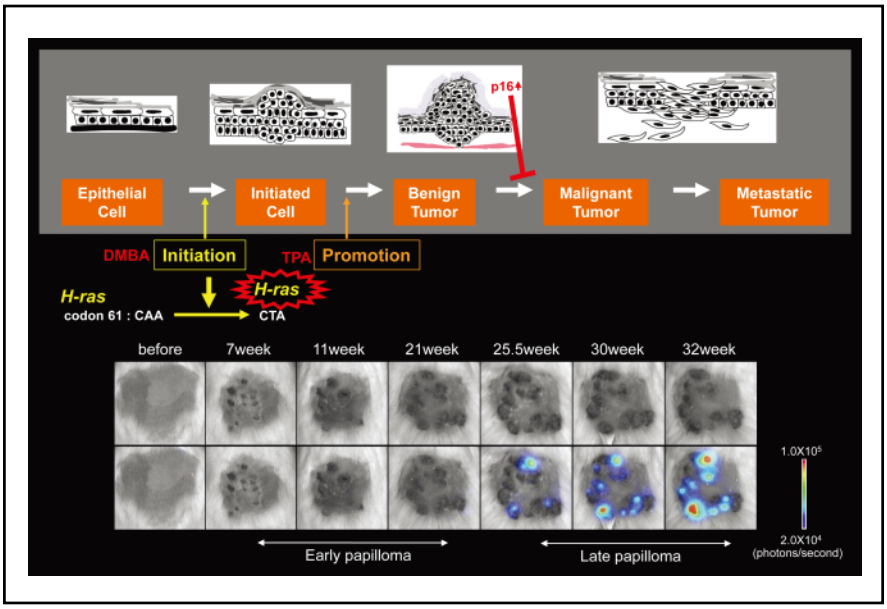

Fig.3 Real-time imaging of p16^{INK4a} expression during skin papilloma development

The *p16-luc* mice were subjected to a conventional chemically induced skin papilloma protocol with a single dose of DMBA followed by multiple treatments with TPA. This protocol causes an oncogenic mutation in the *H-ras* gene. Benign skin papillomas began to appear after 7weeks of DMBA treatment, and continued to grow until 20 weeks or so. However, after that, most papillomas stop growing. So we classified these growing papillomas as the early stage papilloma and non-growing papillomas as the late stage papillomas. The p16-luc mice were subjected to noninvasive BLI, and the significantly elevated bioluminescent signals were detected in the late stage papilloma. The color bar indicates photons with minimum and maximum threshold values. (Yamakoshi et al., 2009 originally published in the Journal of Cell Biology, Vol.186, No.3 393-407.)

cellular senescence through up-regulation of p16^{INK4a} expression in cultured normal human fibroblasts, this is not the case in freshly isolated normal human fibroblasts[4]. It remains, therefore, unclear whether the induction of *p16^{INK4a}* gene expression by oncogenic Ras expression in cultured cells truly reflects an anti-cancer process or an artifact of tissue culture-imposed stress. To explore this notion in a more physiological setting rather than using ectopic expression of oncogenic Ras in cultured cells, the *p16-luc* mice were subjected to a conventional chemically induced skin papilloma protocol with a single dose of DMBA followed by multiple treatments with TPA. Because this protocol induces benign skin papillomas, more than 90% of which harbors oncogenic-mutation in *H-ras* gene, it appears to be ideal for studying physiological response against oncogenic mutation in endogenous *H-ras* gene *in vivo*.

When p16-luc mice were treated with DMBA/TPA protocol, benign skin papillomas began to appear after 7 weeks of treatment and continued to grow to a larger size for a further 18 weeks (early-stage papilloma). Although bioluminescent signals were hardly detectable during this time, a significant level of bioluminescent signal was induced as papillomas stopped growing (late-stage papilloma). The levels of bioluminescent signals were well correlated with those of endogenous p16^{INK4a} expression as well as other senescence markers such as senescence-associated (SA) β-galactosidase (β-gal) activity and de-phosphorylation of pRb, indicating that oncogenic Ras signaling derived from the endogenous *H-ras* gene indeed provokes p16^{INK4a} expression accompanied by senescence cell cycle arrest *in vivo*[6]. This is also suggesting that p16^{INK4a} may play important role(s) in late papillomas, presumably preventing malignant conversion of benign tumors. In agreement with this notion, by 30 weeks after DMBA/TPA treatment, approximately 33 % of *p16^{INK4a}* knock-out mice (C57BL/6 back ground) had at least one carcinoma compared with 5 % of the wild type mice[9]. These results are also consistent with a previous study showing that the tumor-free survival of DMBA-treated mice was substantially reduced in *p16^{INK4a}* knockout mice[10].

Epigenetic regulatory mechanism underlies the *p16^{INK4a}* gene induction

Given that oncogenic mutation in the *H-ras* gene is known to occur immediately after DMBA treatment[6], it was puzzling that *p16^{INK4a}* gene expression was fully induced in late- but not early-stage papillomas. Interestingly, the levels of DNMT1, which is known to repress *p16^{INK4a}* gene expression, were significantly increased in early-stage papilloma and were subsequently reduced in late-stage papillomas. Intriguingly moreover, the status of the histone 3 Lys 9 methylation (H3K9me) but not CpG methylation around the *p16^{INK4a}* gene promoter was well correlated with the levels

of DNMT1 expression during the course of papilloma development[6]. These results, together with a recent observation that DNMT1 possesses an activity to enhance H3K9 methylation through interacting with G9a, a major H3K9 mono- and di-methyltransferase[11], suggest that DNMT1 serve to counterbalance the activation of the *p16^{INK4a}* gene promoter mediated by oncogenic Ras during skin papilloma development. Of note, the levels of DNMT1 were initially increased by oncogenic Ras expression and were subsequently reduced as cells reached to the senescence stage in cultured human primary fibroblasts. Together, these results indicate that a similar mechanism is likely to be involved in the regulation of *p16^{INK4a}* gene expression by oncogenic Ras signaling both *in vitro* and *in vivo*.

DNA damage response regulates *p16^{INK4a}* gene expression through DNMT1

It has previously been shown that oncogenic Ras signaling activates *DNMT1* gene promoter through AP1[12]. Thus, the induction of DNMT1 expression appears to be caused by a direct effect of oncogenic Ras expression. However, it was unclear how DNMT1 is reduced in late stage of papilloma development. Our results strongly suggest that DNA damage response (DDR) triggered by hyper-cell proliferation plays critical role(s) in blocking the level of *DNMT1* gene expression, at least partly, through the elevation of reactive oxygen species (ROS) level in late-stage papillomas[6]. Because *DNMT1* gene expression is known to be regulated by E2F[13] and that E2F activity is reduced by H_2O_2 treatment (Yamakoshi & Hara unpublished data), it is most likely that ROS regulate *DNMT1* expression, at least in part, through E2F. These results, together with the observation that depletion of DNMT1 causes up-regulation of *p16^{INK4a}* gene expression in cultured human cells[6, 14], indicate that DDR plays key role(s) in induction of *p16^{INK4a}* gene expression through blocking the levels of *DNMT1* expression in the setting of Ras-induced senescence *in vivo*.

Because the p53 tumor suppressor is known to be activated immediately after detection of DNA damage, preventing accumulation of DNA damage, it is possible that p53 might block the DDR pathway activating *p16^{INK4a}* gene expression. To explore this idea, we again took advantage of using bioluminescence *in vivo* imaging technique, in conjunction with *p16-luc* mice lacking the *p53* gene. Indeed, although bioluminescent signals were only slightly induced after treatment with doxorubicin (DXR), a DNA damaging agent, in *p16-luc* mice, this effect was dramatically enhanced by *p53* deletion, especially in highly proliferating tissues such as thymus or small intestine[6]. Furthermore, the DDR-pathway activating *p16^{INK4a}* gene expression and consequent cellular senescence was provoked naturally in the thymus of nearly all mice lacking *p53* gene around 10 to 20 weeks after birth[6]. It is therefore possible that *p16^{INK4a}* may play a back-up tumor suppressor role in case p53 is accidentally inactivated, especially in highly proliferative tissue such as thymus.

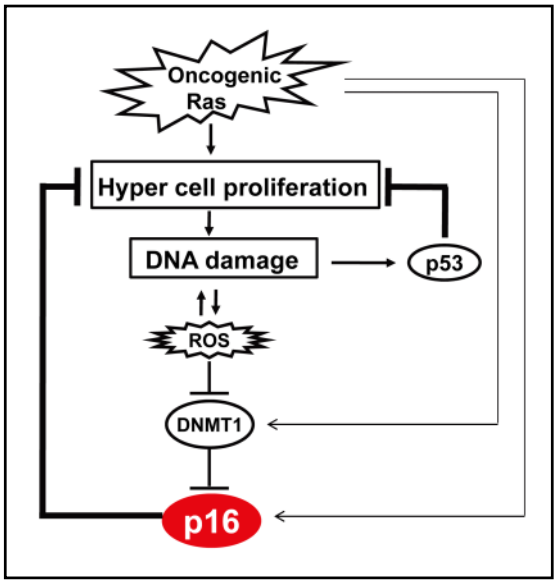

Fig.4 Cross talk between the p53 and p16 pathways through DDR

Although oncogenic Ras signaling has a potential to activate *p16^{Ink4a}* gene expression, this effect is initially counteracted by an elevation of DNMT1 level and thereby causes a strong proliferative burst, resulting in the accumulation of DNA damage. The accumulation of DNA damage activates ROS production, which in turn blocks *DNMT1* gene expression, thereby causing epigenetic derepression of *p16^{Ink4a}* gene expression and thus senescence cell cycle arrest. This pathway is counterbalanced by the p53 pathway because p53 is immediately activated by DNA damage and blocks proliferation of damaged cells that cause further accumulation of DNA damage. Thus, the DDR pathway-induced *p16^{Ink4a}* expression is accelerated in the event of p53 inactivation.

(Yamakoshi et al., 2009 originally published in the Journal of Cell Biology, Vol.186, No.3 393-407.)

A regulatory circuit between p53 and *p16^{INK4a}* tumor suppressors

Our results draw a following model in which although oncogenic Ras signaling has a potential to activate *p16^{INK4a}* gene expression immediately[15, 16], this effect is initially counteracted by elevation of DNMT1 levels and thereby causes hyper-cell proliferation. However, since hyper-cell proliferation tends to cause DNA damage and elevation of ROS, *DNMT1* gene expression is eventually reduced by this ROS leading to epigenetic de-repression of *p16^{INK4a}* gene expression and hence senescence cell cycle arrest (see model in Fig.4). Interestingly moreover, this pathway is more potentiated in the setting of p53 deletion because p53 tends to prevent proliferation of damaged cells that cause further accumulation of DNA damage (Fig.4)[17, 18]. It is therefore most likely that *p16^{INK4a}* may play a back-up tumor suppressor role in case p53 is accidentally inactivated. In agreement with this notion, it has recently been shown that the levels of *p16^{INK4a}* gene expression are substantially increased in the mice lacking *p53* gene[19]. Moreover, over-expression of Aurora A resulted in a significant induction of *p16^{INK4a}* expression in the mammary glands of p53 knock-out mice[20]. It is also worth emphasizing that p53 inactivation alone is not sufficient to fully abrogate telomere-directed cellular senescence, but combined inactivation of p53 and p16^{Ink4a} does so[21]. These results, together with our recent findings, help to explain why mice doubly deficient for p53 and p16^{INK4a} showed an increased rate of tumor formation[22, 23] and why the combination of p53 and p16^{INK4a} loss is frequently occurred in human cancer cells.

Concluding remarks

It is clear that all aspects of *p16^{INK4a}* regulation cannot be explained by factors described here and that the *p16^{NKk4a}* gene is subject to multiple levels of control[16, 24-35]. Nonetheless, we uncovered an unexpected link between p53 and *p16^{INK4a}* gene expression[6], expanding our understanding of how *p16^{INK4a}* gene expression is induced by oncogenic stimuli *in vivo* and open up new possibilities for its control. Visualizing the dynamics of *p16^{INK4a}* gene expression in living animals, therefore, provides a powerful tool for not only help to resolve and clarify issues connecting *in vitro* studies but also reveals unrecognized functions of this key senescence regulator in various physiological processes *in vivo*.

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Disclosure

We have no conflicts of interest.

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