

Extra Views

# The Normal Response to RAS Senescence or Transformation?

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## ABSTRACT

Normal cells are thought to protect against transformation by undergoing a permanent cell cycle arrest, cellular senescence, in response to the expression of activated oncogenes such as RAS. We recently found that freshly established neonatal human fibroblasts are resistant to RAS-induced senescence. Moreover, extended passaging of normal fibroblasts leads to increased levels of the cyclin dependent kinase inhibitor p16 and sensitizes cells to senescence induced by RAS. These findings implicate exogenous stress as a necessary cofactor in RAS-induced senescence and demonstrate that RAS expression can promote some characteristics of transformation in the absence of other genetic changes.

## ALL FIBROBLASTS ARE NOT EQUAL

Senescence was first described by Hayflick in the 1960's when he reported that human diploid fibroblast strains have a limited in vitro lifespan.<sup>1,2</sup> Since this first description, senescence has been studied extensively in human fibroblasts and a great deal has been learned about the changes that occur upon senescence. However, one area that has been overlooked until recently is how various isolates of fibroblasts differ from one another. Commonly used fibroblast strains differ in their tissue of origin, donor age, genetic background and in vitro culture conditions. Despite this, most laboratories define a normal fibroblast as any diploid strain with a limited lifespan in vitro. Perhaps not surprisingly, recent studies suggest that all fibroblasts are not necessarily equal and that there may be significant variation among fibroblast strains. For example, gene expression array analysis has demonstrated that fibroblasts from different anatomic sites have distinct expression profiles.<sup>3</sup> Furthermore, fibroblast strains differ with respect to the pathways used to initiate cellular senescence.

Telomere shortening is known to be a primary trigger of replicative (passage-induced) senescence in human cells. The lifespan of some human fibroblasts such as BJ and other primary foreskin fibroblasts is limited exclusively by telomere length and expression of the catalytic subunit of telomerase, hTert, is sufficient to immortalize these strains.<sup>4-6</sup> In contrast, other human fibroblasts such as Hayflick's WI-38 strain and IMR90 lung fibroblasts are not immortalized by expression of hTert alone. Instead, these fibroblast strains senesce in response to high levels of the cyclin dependent kinase inhibitor p16, and reducing p16 levels extends their lifespan.<sup>7,8</sup> Although little is known about why p16 levels vary among cell types, one likely explanation for this difference is that fibroblast strains that are limited by p16 levels may express more p16 because they were exposed to a greater amount of culture-imposed stress during their establishment. This idea is supported by the observations that p16 levels increase as cells are passaged in culture,<sup>9-11</sup> and that culturing fibroblasts and epithelial cells in suboptimal conditions further increases p16 expression.<sup>12</sup>

Fibroblast strains also appear to differ in the pathways that are required for senescence induced by oncogenic RAS. Both the p53/p21 and p16/Rb pathways have been implicated in RAS-induced senescence of human fibroblasts, however results differ between cell strains and experimental protocols. Several groups have shown that IMR90, LF1, and BJ fibroblasts senesce in response to RAS and that this arrest is only bypassed following disruption of both the p53/p21 and p16/Rb pathways.<sup>13-16</sup> In contrast, a recent report found that knock-down of p53 alone could confer resistance of BJ fibroblasts to RAS-induced senescence.<sup>17</sup> To our knowledge, the only primary human fibroblasts that have been reported to be resistant to senescence induced by RAS are two strains established from individuals carrying inactivating mutations in the p16 gene.<sup>18,19</sup> These findings argue that a functional p16/Rb pathway is all that is required for RAS-induced senescence.

While these reports appear to contradict one another, some of these discrepancies can be explained by the use of different fibroblast strains among studies. As mentioned above, some fibroblast strains have higher p16 levels that may block proliferation when p16 is further induced following RAS expression. In these strains it is likely that the p16/Rb pathway needs to be disrupted in order to prevent RAS-induced arrest. It is less clear why the results differ with respect to inactivation of the p53 pathway. The main difference seems to be that some groups find that the p53 pathway is activated in response to RAS,<sup>13-15,17</sup> while others do not.<sup>18,20</sup> Whatever the reason for this difference, if p53 is not activated by RAS in a particular system, it should not need to be inactivated to prevent senescence.

## NORMAL FIBROBLASTS ARE RESISTANT TO RAS-INDUCED SENESCENCE

Because of the discrepancies among studies carried out in different fibroblast strains, we have chosen to study senescence in neonatal human foreskin fibroblasts (HFFs) directly after isolation from the primary tissue. Unlike many of the fibroblast strains used by others, these cells have not been subjected to freeze-thaw cycles or prolonged passaging in culture. Previous work in our laboratory has shown that early passage HFFs have very low levels of p16 (when compared to primary epithelial cells and other fibroblast strains) and that they can be immortalized by the expression of hTert alone.<sup>5</sup> For these reasons, we believe that early passage HFFs may more closely resemble fibroblasts *in vivo*.

Since the pathways involved in RAS-induced senescence remain controversial, we decided to investigate the response of early passage HFFs to RAS. Surprisingly, we found that unlike other fibroblast strains, HFFs do not undergo senescence in response to RAS.<sup>20</sup> When experiments are repeated alongside the well-studied IMR90 fibroblasts, RAS efficiently arrests the IMR90 cells, but not HFFs. This is not because HFFs simply have a delayed response to RAS, as RAS-expressing HFFs have an *in vitro* lifespan similar to vector transduced controls. Moreover, this response is not specific to cells from a particular donor, since we have repeated the experiment in several freshly established HFF strains. To our knowledge, this was the first description of a genetically normal, cultured cell type that is resistant to RAS-induced arrest.

In order to understand the molecular differences that account for the resistance of HFFs to RAS, we have compared the activation of the p53/p21 and p16/Rb pathways in RAS-transduced IMR90 and HFF cells. While we do not observe a strong induction of p53 or p21 in either cell type following RAS expression, p16 levels do increase in both strains. However, there is a significant difference in overall levels of p16. IMR90 fibroblasts have very high levels of p16 and these levels increase further upon RAS expression. In contrast, early passage HFFs do not express any detectable p16 and although the levels are increased following RAS expression, they are still lower than the amount of p16 seen in normally cycling IMR90 cells. Furthermore, while increased p16 correlates with both cell cycle arrest and hypophosphorylated Rb protein in IMR90 cells, Rb protein remains hyperphosphorylated in RAS-expressing HFFs. These results strongly suggest that p16 levels determine how a cell will respond to RAS.

Since RAS-induced senescence is observed in all other cultured fibroblast strains analyzed, we reasoned that the unique feature of the cells we tested was that they were freshly established from the primary tissue. Because of this, cells had been exposed to considerably

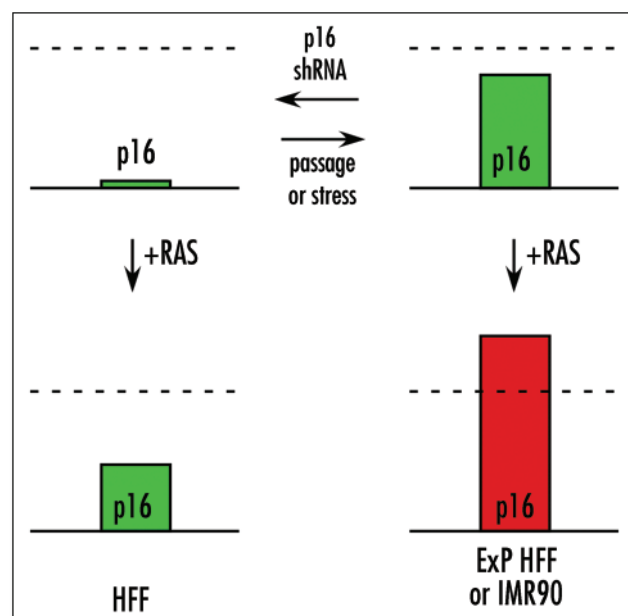


Figure 1. Threshold Model of RAS-induced Senescence. Early passage HFFs express very low levels of p16 and although RAS leads to increased p16 expression, levels are still below a threshold amount (dashed line) so cells continue to cycle (green bars). In contrast, extended passage HFFs (ExP HFF) and/or other types of fibroblasts such as IMR90 express higher initial levels of p16. RAS expression in these cell types increases p16 past the threshold and cells enter senescence (red bar). In the case of ExP HFFs, expression of an shRNA that reduces p16 levels renders cells resistant to RAS-induced senescence.

less culture-imposed stress and did not express any detectable p16. We hypothesized that if culture-imposed stress sensitizes cells to RAS-induced arrest, extended passaging of HFFs may render them sensitive to RAS. In fact, hTert-immortalized HFFs that have undergone extensive passaging in culture have elevated levels of p16 and are sensitive to RAS-induced arrest. Moreover, this effect is reversed in extended passage HFFs that express a short hairpin RNA (shRNA) that knocks-down levels of p16 protein. These data, along with the findings from IMR90 cells, suggest a model in which cells can tolerate a certain level of p16 and continue cycling, however when p16 levels become high enough cells undergo senescence. In freshly established cells, p16 levels are extremely low and although these levels increase following RAS expression, levels are still below the threshold amount required to initiate a senescent arrest (Fig. 1). Cells that have been exposed to extensive passaging or a great deal of culture-imposed stress (extended passage HFFs or IMR90) have higher initial levels of p16, and expression of RAS pushes this level past the threshold and induces a senescent arrest.

It remains possible that other factors such as donor age and tissue type of origin also contribute to the difference between HFFs and other fibroblast strains. However, these factors cannot entirely explain the resistance of HFFs to RAS since BJ fibroblasts were also established from neonatal human foreskin and BJ cells senesce in response to RAS.<sup>16,17</sup> Consistent with the idea that p16 is the critical factor that determines the response to RAS, BJ fibroblasts express more p16 than early passage HFFs.<sup>20</sup> While we have not tested freshly established fibroblasts from other tissue types, the prediction is that they would have low p16 levels and also be resistant to RAS-induced arrest. To be certain of this it will be important to test freshly established fibroblasts from other tissue types and donors of various ages.

## DOES RAS-INDUCED SENEESCENCE OCCUR IN VIVO?

The threshold model we have proposed has significant implications for the idea that oncogene-induced senescence is a tumor suppression mechanism *in vivo*. In fact there is little evidence that RAS-induced senescence actually occurs *in vivo*. In order to model tumorigenesis, several groups have generated transgenic mice that express activated alleles of RAS in various tissue types and the cells in these mice appear to proliferate normally, with no indication of senescence.<sup>21,22</sup> Furthermore, a recent report demonstrates that when mice are generated that express activated K-ras from its endogenous locus *in vivo*, tissues proliferate normally and, similar to our findings, fibroblasts established from these mice do not senesce in culture.<sup>23</sup> Finally, in human cancer RAS mutation is thought to be an early event that promotes cellular proliferation, allowing cells to acquire additional genetic changes.<sup>24</sup> If activated RAS alleles promote senescence in the human body, they would not be predicted to have a tumor promoting effect.

While our model predicts that RAS mutation alone does not cause senescence in normal cells *in vivo*, it does not rule out that possibility that RAS acts together with other factors to induce senescence. Studies in cultured cells demonstrate that p16 levels increase in response to exogenous, culture-imposed stress, as well as oncogenic stress. These same types of stress could induce p16 levels *in vivo*. We propose that p16 levels reflect the cumulative amount of stress placed on a cell and when p16 levels increase past a certain threshold, cells enter a permanent senescent arrest. RAS activation may induce a low level of p16 expression *in vivo*, but only when RAS activation occurs in combination with other types of stress can senescence take hold. Another possibility is that a subset of cells in the body have elevated levels of p16, because of their proliferation history or previous exposure to stress, and RAS activation can induce senescence in these cells. This model is consistent with the fact that p16 is frequently mutated or lost in human tumors.<sup>25</sup> If p16 is essential for RAS-induced senescence *in vivo*, it is likely to be selected against in tumors carrying RAS mutations. Unfortunately, these hypotheses are difficult to test, and there is little data concerning the expression of p16 in normal human tissues *in vivo*. However, the recent development of antibodies that reproducibly detect p16 in tissue sections should make this type of analysis possible in the near future.<sup>26,27</sup>

## THE RESPONSE OF NORMAL CELLS TO ONCOGENES

Our finding that freshly established HFFs behave differently than extensively passaged cells also has implications for the study of oncogenes in cell culture systems. Since HFFs do not senesce in response to RAS, we now have an excellent tool to study how RAS promotes transformation-associated properties in the absence of other genetic changes. In other cell types RAS function can only be studied in the context of disrupted p53 and/or Rb pathways. Preliminary findings suggest that RAS expression results in reduced levels of Rb protein and alters the cell cycle distribution of HFF cultures.<sup>20</sup> In addition, RAS expression alone promotes anchorage-independent growth of HFFs. Since we are studying very early passage cells that are not limited by telomere length, we have also examined the effects of RAS in cells with and without telomerase activity. These studies have uncovered a collaborative effect of hTert and RAS on anchorage-independent growth and the ability to bypass contact inhibition. Dissecting how hTert and RAS cooperate to allow these properties will be of considerable interest. Finally, HFFs can be used as a tool

to study the functions of other oncogenes in normal cells. We have recently used this system to analyze the function of the Myc oncogene in freshly established and late passage HFFs (manuscript in preparation). Comparisons between freshly established primary cells and other established cell strains should contribute greatly to our understanding of differences between cell culture and *in vivo* systems.

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