RESEARCH ARTICLE

# Development of a Cellular System to Identify Modulators of B-Raf Induced Senescence in Human Fibroblasts

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Keywords:
***Tumorigenesis:*** *The transformation of normal cells-which grow only under favorable conditions- to cancer cells, which grow uncontrollably.*
***Oncogene:*** *A gene that has the potential to cause cancer when mutated.*
***Oncogene Induced Senescence:*** *A state of growth arrest in cells driven by the expression of an oncogene.*
***Genetic Screen:*** *An experimental technique used to identify potential genes involved in the expression of a phenotype of interest.*

## Abstract

**Background:** B-Raf is one of the earliest and most common genetic mutations observed in many different types of cancers. A single mutation in B-Raf cannot cause full-blown cancer, but may cause an observed phenotype called oncogene induced senescence (OIS). This suggests the need for cooperation between B-Raf and other genes for successful tumorigenesis.

**Objective:** We look to characterize Human Fibroblast cells that are able to senesce in response to elevated oncogenic expression of B-Raf.

**Methods:** We introduced ectopic expression of inducible B-Raf into human fibroblast cells. We characterized the successfully infected cells based on their ability to induce senescence.

**Results:** We isolated cells of clonal origin and we identified the clone most responsive to B-Raf expression.

**Conclusions and Future Research:** Our methodology proved to be effective in creating a model of B-Raf expression that can be used to study OIS. The next step is to screen the cells to identify genes that enable the cells to evade senescence. These genes could prove to be valuable chemotherapeutic targets.

## Introduction

The MAPK/ERK pathway plays an important role in the regulation of cell proliferation, differentiation, survival and apoptosis in many organisms (1). This signal cascade starts with the binding of a mitogen to a cell surface receptor, activating members of the Ras GTPase family (1). Activation of Ras involves switching from a GDP-bound state to GTP-bound state and activating the downstream element Raf (1). There are 3 homologs of Raf proteins, *A-RAF*, *B-RAF*, and *C-RAF*, but research has been focused on B-Raf as its mutations have been observed in melanomas, thyroid and, colorectal cancers (2). Raf is a serine/threonine protein kinase that activates the downstream element MEK by phosphorylation. Activation of MEK, another serine/threonine kinase, allows it to phosphorylate p42 and p44 Erk kinases, converting them to the active states p42 phospho-Erk and p44 phospho-Erk (1). Finally, the Erk kinases can phosphorylate other transcription factors, ultimately leading to cell growth and proliferation. The loss of function or gain of function of any component of this pathway can have detrimental effects to the overall function of the organism.

Normal expression of Ras and Raf causes cell growth, however, constitutive expression of Raf and Ras in normal cells induces the phenotype of oncogene-induced senescence (OIS) (3, 4). This state of irreversible growth arrest is thought to be a protective mechanism used by the cell as a defense against tumorigenesis and to allow for the repair of cell cycle machinery (5). Senescent cells display special cell properties such as an elongated cell morphology and beta-galactosidase activity (6). OIS greatly resembles replicative senescence

where a cell's telomeres are shortened to the point where cells can no longer proliferate (7).

The biochemical mechanisms governing how senescence is induced are unknown, however candidates include p53, cyclin-dependent kinase inhibitors (CDK) inhibitors, $p21^{Cip1}$, $p16^{Ink4a}$, and other factors involved in telomere maintenance, such as telomerase, TRF1 and TRF2 (3). Aberrant expression of p53, p16, and p21 genes often leads to tumorigenesis, likely due to the ability of the cancerous cells to bypass the senescent mechanism (8). The loss of p53 may promote transformation through evasion of apoptosis as well(9). On the other hand, overexpression of telomerase can bypass the Hayflick limit - the number of times a normal cell will divide before cell division stops, and lead to the immortalization of cells(10).

Upregulation of the oncoproteins Ras or Raf is insufficient to induce uncontrolled growth in normal cells- rather cells need the cooperation of other genes in order to successfully transform (11). A likely mechanism of transformation is the loss of tumour suppressor genes, as their expression usually inhibits cell growth. The candidate approach and looking at specific genes greatly limited previous studies scope, and these experiments likely missed many relevant genes involved in the senescence mechanism. The question is also raised of whether or not changes in gene expression are of causality or of correlation.

We used a lentiviral vector containing the conditionally active ΔB-Raf-ER* construct to successfully transfect the cells. ΔB-Raf-ER* is stabilized by 4-hydroxy-tamoxifen (4-HT) binding, causing dimerization of the chimeric protein, translocation into the nucleus and increased B-Raf expression. We screened multiple clones obtained from infection based on responsiveness to 4-HT and B-Raf signaling, and the induction and maintenance of senescence. Having clones being able to grow in the presence of 4-HT and B-Raf signaling would result in false positives. After careful selection, we identified clone 2 as the best candidate.

The next step of the experiment would be to screen the genome through the systematic knockdown of genes with clone 2. The identification of these genes as requisites for B-Raf driven oncogenic transformation would provide new targets for drug development and a better understanding of oncogenic mechanisms.

# Materials and Methods

## Construction of Lentivirus Expression Vectors

We conducted the experiment using immortalized HF-E1T cells (human fibroblasts) transduced with the appropriate pLEGblast lentiviral stocks.

Lentiviral vectors encoded either ΔB-Raf-ER* or mCherry. ΔB-Raf-ER* is a fusion protein consisting of the protein kinase domain of mouse B-Raf linked to mouse estrogen receptor (ERα). The ER receptor is engineered to be non-responsive to β-estradiol, but retains responsiveness to 4-hydroxy-tamoxifen (4-HT) and the ICI series of estrogen receptor antagonists (12). We gateway cloned the B-Raf-ER* cassette from the pBabe vector into the pLEG vector (13). mCherry is a monomeric fluorescent protein. Cells with the mCherry vector lack an estrogen receptor and are not responsive to 4-HT.

## Cell Culture, Lentiviral Production, and Infection

We cultured all cells in Dulbecco's modified Eagle's Medium (DMEM) supplemented by 10% Fetal Bovine Serum, penicillin and streptomycin. We cultured cells in a humidified environment containing 5% (v/v) CO2 at 37 °C. We prepared 4-HT (Sigma) as 1 mM stock solution in ethanol, stored at -20 °C and diluted to appropriate concentrations.

We obtained lentiviral stocks by polyethylenimine transfection of the appropriate vectors along with lentiviral packaging plasmids into 293T cells. We cultured targeted cells in blasticidin to select for successfully infected cells. We isolated individual cell clones into different plates. We obtained 9 independent clones, labeled as clones 1-3, 6,7, 9-12. Following selection we isolated cells into individual plates and allowed to grow on their own. We tested their responsiveness to 4-HT in the Characterization of B-Raf clones experiment, dose-response curves and time courses.

## Cell Characterization

Characterization of B-Raf Clones

We seeded the clones and cells expressing mCherry at 40,000 cells per well in a 6-well plate which was left for a day in normal media. We then treated wells with either 1 uM of Ethanol or 10 nM of 4-HT. Each condition was done in triplicate. We allowed the cells to grow for 4 days before counting them with a hemocytometer.

Dose-Response Curve and Time Course

For dose-response curves, we seeded the cells at 15,000 cells per well in a 12-well plate and left for a day. We then treated wells with increasing concentrations of 4-HT. Each concentration was done in triplicate. We allowed the cells to grow for 7 days before counting. We replaced media containing 4-HT after 3 days with media containing the same concentration of 4-HT. We constructed the characterization of B-Raf clones and time course experiments in a similar manner to that of dose-response curves, except that the wells were all treated with 100 nM 4-HT. The cells were counted using a hemocytometer on days 2,4, and 6.

Screen for Escape Frequency

We seeded mCherry cells and HF-E1T:ΔB-Raf-ER* , clones 2,6, and 12

at 106 cells in 150 mm dishes. We either seeded clones 2,6, and 12 alone or with 100 or 1000 mCherry cells. We the treated all cells with 100 nM 4-HT for 4 days. We replaced the media with normal media without 4-HT and changed every 3-4 days over the span of 2 weeks. We seeded the mCherry expressing cells with clones to insure that the 4-HT media allows cell growth. We then stained the plates with Giemsa and visualized for colonies that grew out. Giemsa stains for DNA, therefore the darker the shade of purple from Giemsa stain, the more colonies that are present. The experiment was conducted twice.

## Preparation of Cell Extracts and analysis by Western Blotting

We serum starved cells through treatment with DMEM with penicillin and streptomycin for 12 h. For the first Western blot, we treated ΔB-Raf-ER* human fibroblast clones with increasing amounts of 4-HT (0-100 nM) for 24 hours. We treated mCherry cells with 0 and 100 nM of 4-HT and used these cells as a control. For the second Western blot, we treated ΔB-Raf-ER* human fibroblast clones with 100 nM 4-HT for increasing periods of time. We counted 510,000 cells from each condition and lysed in 100 uL of 1X Laemmli Lysis Buffer. We directly loaded and electrophoresed aliquots of cell lysates through 12% polyacrylamide gels. We transfered the gels to PVDF membranes (Bio-RAD) and incubated in primary antibodies overnight at 4 °C. Primary antibodies used are as follows: Polyclonal rabbit anti-ERα (Santa Cruz Biotechnology, sc-543) used at a dilution of 1:200, monoclonal mouse anti-p-ERK (Cell Signalling Technology, 9106) used at a dilution of 1:2000, and monoclonal rabbit anti-ERK (Cell Signalling Technology, 9102) used at a dilution of 1:1000. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins were from Amersham and used at a dilution of 1:5000. We developed Western blots with the ECL Western Blotting detection kit (Amersham).

# Results

We obtained 9 independent clones after infection with pLEG-B-Raf-ER* vector and blasticidin selection. In order to test responsiveness to 4-HT, the characterization of B-Raf clones experiment was conducted. The results are shown in Fig. 1. 4-HT treatment caused all clones to adopt pronounced morphological changes. Cells displayed an extended shape with a spherical cell body. Relative cell numbers were obtained by dividing the cell count from 4-HT treatment by the cell count obtained from ethanol treatment. The relative cell counts are shown in Fig. 2. Varying levels of growth arrest were observed in the clones. Clone 1 showed a very low level of growth arrest whereas clones 2,6 and 12 showed the highest levels. Clones 2, 6 and 12 were selected for further characterization with dose-response curve and time course experiments.

## Dose-Response Curves

We wanted to determine the minimal concentration of 4-HT required for the activation of B-Raf and senescence induction in the maximal number of cells. A concentration that was too low would result in insufficient levels of 4-HT and B-Raf signaling, whereas too much could result in cell apoptosis(14). We incubated cells in full media containing increasing concentrations of 4-HT (0-100 nM) for 7 days before their numbers were quantified. The values obtained were normalized to that of cells treated with 0 nM of 4-HT. The results are shown in Fig. 3.

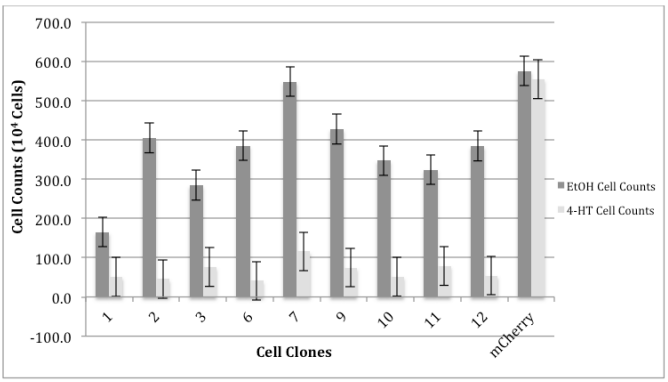


**Fig. 1**

Characterization of B-Raf Clones: B-Raf induced senescence of HF-E1T cells. Average cell count comparison between ethanol and 4-HT treatments for clones and mCherry cells. Growth curve I was conducted by first seeding 40,000 cells into 6 well plates and leaving them for a day. The cells were then treated with either Ethanol or 10 nM 4-HT and allowed to grow for 4 days before counting. Each condition was done in triplicate.

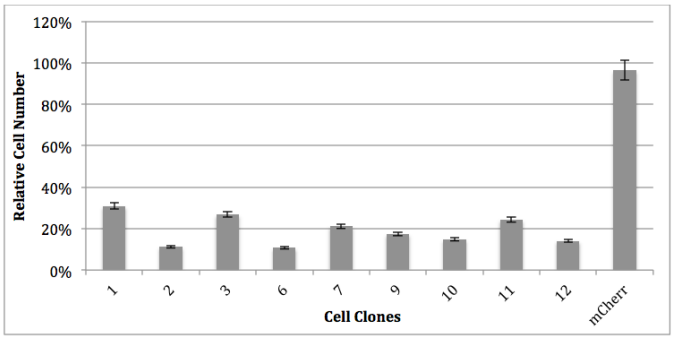


**Fig. 2**

Characterization of B-Raf Clones: B-Raf induced senescence of HF-E1T cells. Relative cell counts were obtained by dividing the cell count obtained from 4-HT treatment by the cell count obtained from ethanol treatment. Growth curve I was conducted by first seeding 40,000 cells into 6 well plates and leaving them for a day. The cells were then treated with either Ethanol or 10 nM 4-HT and allowed to grow for 4 days before counting. Each condition was done in triplicate.

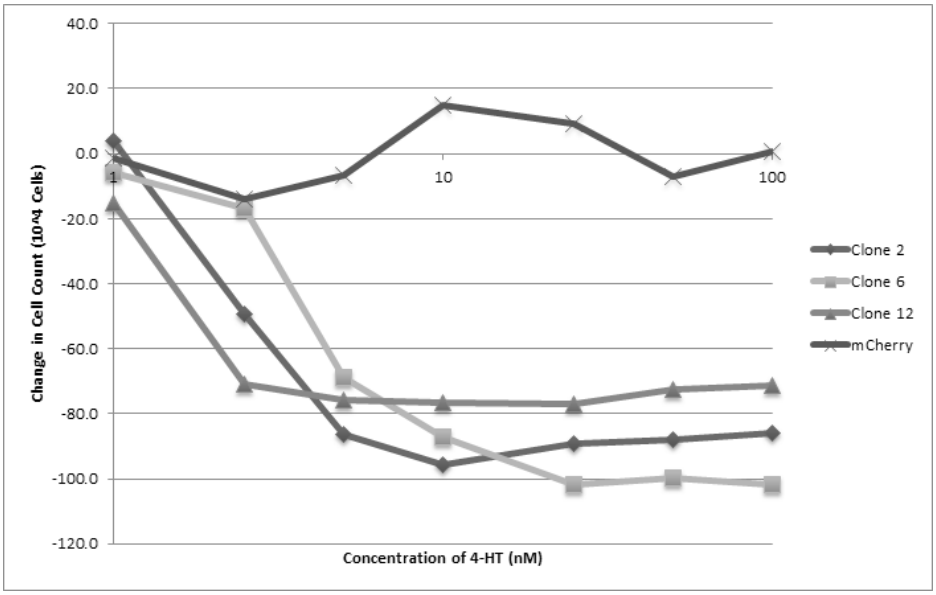


**Fig. 3**
**Dose Response Curve**: B-Raf induced senescence of HF-E1T cells with increasing concentrations of 4-HT. The cell counts were normalized to the cell count obtained at a concentration of 0 nM. Dose response curves were conducted by first seeding 15,000 cells into 12 well plates and leaving them for a day. The cells were then treated with increasing concentrations of 4-HT and allowed to grow for 7 days before counting. Each concentration of 4-HT treatment was done in triplicate.

mCherry cells were unresponsive to 4-HT treatment and show relatively consistent cell counts across the concentrations of 4-HT. Clones 2, 6 and 12 show growth arrest at increasing concentrations of 4-HT. Clones 2 and 6 have more of a gradual decrease in cell count as concentration increases, whereas clone 12 has a more abrupt drop in cell count. 25 nM appears to be the minimal concentration required to induce cellular senescence. However, concentrations 25-100 nM may also show the ratio of apoptosis and proliferation near one.

## Time Course

The amount of time required for the induction of senescence is directly dependent on the activation of ΔB-Raf-ER*, its downfield cascade and growth arrest machinery. We wanted to determine how quickly cells would respond to ΔB-Raf-ER* activation and how its activation would affect cell proliferation. We chose a concentration of 100 nM of 4-HT to treat the cells, well above the minimal concentration required for senescence induction determined from the dose-response curves. We chose a concentration of 100 nM to make sure that the least number of cells would escape senescence and to keep it consistent with the Characterization of B-Raf Clones experiment. We normalized the results obtained to the cell counts obtained on day 0. The results are shown in Fig. 4.

In all cases, treatment with ethanol did not affect growth of cells. mCherry cells treated with 4-HT were unaffected and grew as quickly as in ethanol. Clones 2, 6 and 12 all exhibit senescence after 2 days of 4-HT treatment and stay at a similar cell count until day 6.

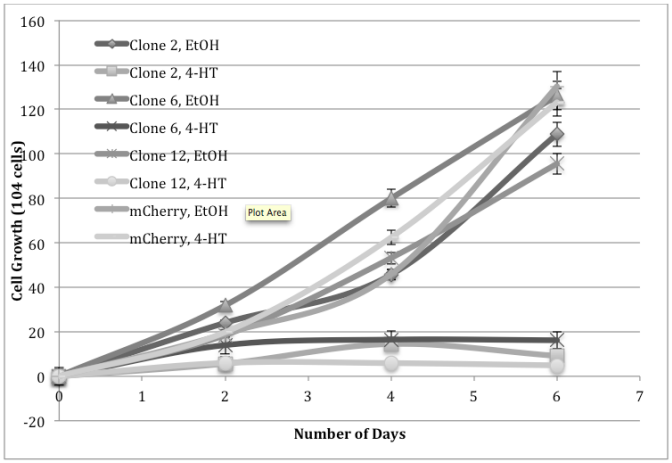


**Fig. 4**
**Time Course:** B-Raf induced senescence of HF-E1T cells with increasing time of exposure to 4-HT. The cell counts were normalized to the cell count obtained at day 0. Growth curve II was conducted by first seeding 15,000 cells into 12 well plates and leaving them for a day. The cells were then treated with 100 nM of 4-HT and counted on days 0, 2, 4 and 6. Each condition was done in triplicate.

## Screen for Escape Frequency

The ultimate goal of the genetic screen is the determination of genes that are involved in the evasion of senescence. It's therefore imperative that cells are responsive to 4-HT and B-Raf signaling to become senescent. Any cells able to evade the senescence mechanism without the knock down of a gene would lead to a false positive. We conducted the screen for reversion frequency as described in methods.

Clones 2 and 12 without any mCherry cells had uniformly lightly coloured plates without any dark spots illustrating that no colonies had escaped senescence. Clone 6 without any mCherry cells had many colonies grow out and had a much darker stain compared to that of clone 2 and 12. The plate with only mCherry cells had a uniformly darker shade compared to that of clones 2 and 12 without mCherry cells, and was similar in colour to that of clone 6 without mCherry cells. This observation illustrates that mCherry cells and clone 6 cells had a similar number of cells in the wells. Clones 2 and 12 with mCherry cells showed darker regions on the plates due to mCherry cells being unaffected by 4-HT and growing out. There was very little difference in colour between clone 6 plates with and without mCherry cells, demonstrating that clone 6 cells were able to evade senescence. The ability of clone 6 to grow out in the presence of 4-HT removed it from any further experiments. The results are shown in Fig. 5.

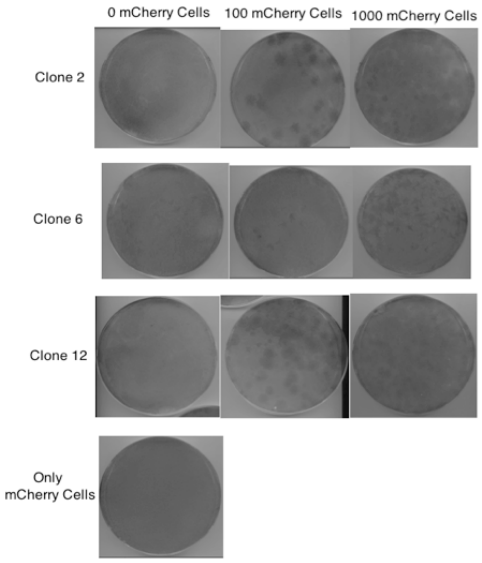


**Fig. 5**

**Screen for Escape Frequency:** B-Raf induced senescence of HF-E1T cells in the presence of 4-HT with or without mCherry cells. The screen for escape frequency was conducted by first seeding $10^6$ mCherry cells, clones 2,6 and 12 into 150 mm plates. The plates of clones were either seeded alone or with 100 or 1000 mCherry cells. After a day, all the plates were treated with 100 nM of 4-HT for 4 days before replacement with normal media for two additional weeks.

## ΔB-Raf-ER* Signaling in 4-HT treated Cells

We wanted to demonstrate that 4-HT treatment caused an increase in ΔB-Raf-ER* signaling in cells by conducting Western blots. We conducted the first blot by treating cells with increasing concentrations of 4-HT; we conducted the second one by treating cells with increasing times of exposure. We blotted both Western blots for estrogen receptor ERα, protein kinase Erk and its phosphorylated form phospho-Erk (p-Erk). Estrogen receptor is usually degraded by the proteasome (15), however 4-HT binding stabilizes it (16). Also, human fibroblasts should not have estrogen receptor present, therefore any expression of estrogen receptor would be from 4-HT binding and stabilizing the estrogen receptor of ΔB-Raf-ER*. Stabilization of receptors causes dimerization and translocation into the nucleus, leading to increased B-Raf expression. To ensure that stabilization of the receptor does indeed lead to the activation of the MAPK/ERK pathway, we also blotted for downstream element of B-Raf, p-Erk. We used Erk as a loading control.

We rendered plates of almost confluent cells quiescent by serum starvation. For the first part of the experiment, we treated clones with increasing concentrations (0-100 nM) of 4-HT for 24 hours. We treated cells expressing mCherry with either 0 or 100 nM of 4-HT and used them as a control. For the second part of the experiment, we treated clones with 100 nM of 4-HT for increasing periods of time (0-24 hours). After exposure to 4-HT, we lysed cells with 1X Laemmli buffer. We ran the lysates on a Western blot and blotted with ERα, Erk and p-Erk antibodies. The Western blots are shown in Fig. 6, Fig. 7 whereas the levels of expression of ERα, Erk and p-Erk are shown in Table 1 and Table 2.

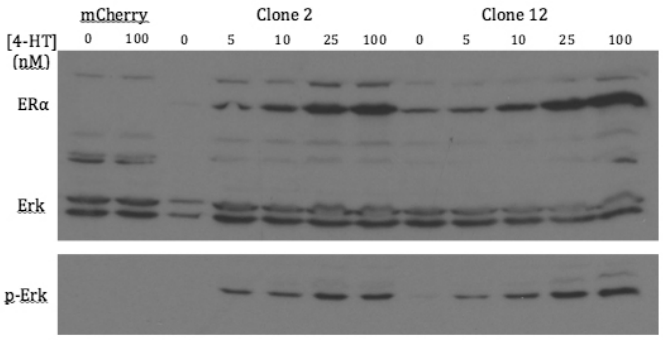


**Fig. 6**

ΔB-Raf-ER* signaling in HF-E1T cells. Effect of increasing concentration of 4-HT on protein expression. Almost confluent plates of mCherry cells and clones 2 and 12 were serum starved overnight. They were then treated with increasing concentrations of 4-HT for 24 hours. Cell lysates were prepared, and Western blotting assessed the levels of expression of ERα, Erk and p-Erk.

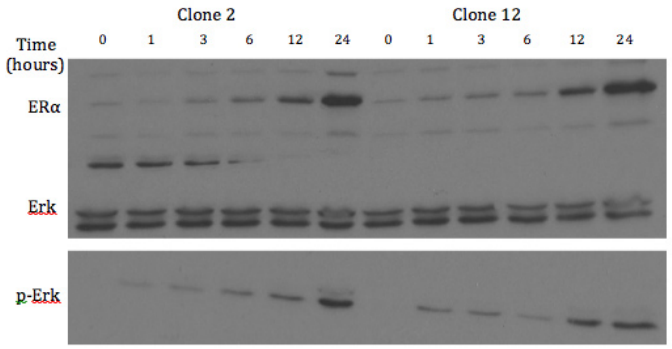


**Fig. 7**
ΔB-Raf-ER* signaling in HF-E1T cells. Effect of time of treatment to 4-HT on protein expression. Almost confluent plates of mCherry cells and clones 2 and 12 were serum starved overnight. They were then treated with increasing times of exposure to 100 nM of 4-HT. Cell lysates were prepared, and Western blotting assessed the levels of expression of ERα, Erk and p-Erk.

| **Cell Type** | **mCherry** | | **Clone 2** | | | | |
|---|---|---|---|---|---|---|---|
| *[4-HT]* | *0 nM* | *100 nM* | *0 nM* | *5 nM* | *10 nM* | *25 nM* | *100 nM* |
| *p-Erk Level* | 2367.39 | 2002.64 | 2703.39 | 14558.15 | 15365.56 | 24312.86 | 21386.27 |
| | | | **Clone 12** | | | | |
| *p-Erk Level* | | | 2479.86 | 9367.56 | 15703.10 | 26386.98 | 29542.22 |
| **Cell Type** | **mCherry** | | **Clone 2** | | | | |
| *[4-HT]* | *0 nM* | *100 nM* | *0 nM* | *5 nM* | *10 nM* | *25 nM* | *100 nM* |
| *ERα Level* | 1109.08 | 890.64 | 1277.69 | 8893.81 | 14866.15 | 26636.51 | 30717.64 |
| | | | **Clone 12** | | | | |
| *ERα Level* | | | 8647.74 | 10285.98 | 17220.81 | 30188.46 | 44964.61 |
| **Cell Type** | **mCherry** | | **Clone 2** | | | | |
| *[4-HT]* | *0 nM* | *100 nM* | *0 nM* | *5 nM* | *10 nM* | *25 nM* | *100 nM* |
| *Erk Level* | 29068.81 | 28656.10 | 11706.29 | 27977.05 | 28844.76 | 30007.51 | 27867.69 |
| | | | **Clone 12** | | | | |
| *Erk Level* | | | 25207.34 | 26512.66 | 24335.22 | 24685.83 | 23928.51 |

**Table 1**
Calculated levels of expression of ERα, Erk and p-Erk for clones 2 and 12 treated with increasing concentrations of 4-HT for 24 hours.

| | **Clone 2** | | | | | |
|---|---|---|---|---|---|---|
| **Time** | *0 hours* | *1 hours* | *3 hours* | *6 hours* | *12 hours* | *24 hours* |
| **p-Erk Level** | 3823.91 | 5354.44 | 7099.05 | 9734.81 | 12945.10 | 20688.46 |
| | **Clone 12** | | | | | |
| **p-Erk Level** | 2432.15 | 6840.98 | 7162.39 | 3870.27 | 14199.56 | 26072.93 |
| | **Clone 2** | | | | | |
| **Time** | *0 hours* | *1 hours* | *3 hours* | *6 hours* | *12 hours* | *24 hours* |
| **ERα Level** | 4545.22 | 2719.74 | 4642.56 | 7067.86 | 12735.86 | 27392.69 |
| | **Clone 12** | | | | | |
| **ERα Level** | 2782.74 | 4048.61 | 4828.98 | 5454.74 | 14586.56 | 34587.29 |
| | **Clone 2** | | | | | |
| **Time** | *0 hours* | *1 hours* | *3 hours* | *6 hours* | *12 hours* | *24 hours* |
| **Erk Level** | 28091.69 | 26433.81 | 26837.44 | 27245.56 | 26734.39 | 24582.74 |
| | **Clone 12** | | | | | |
| **Erk Level** | 24596.98 | 24097.2 | 24936.81 | 23721.56 | 23599.98 | 22947.58 |

**Table 2**
Calculated levels of expression of ERα, Erk and p-Erk for clones 2 and 12 treated with increasing times of exposure to 100 nM of 4-HT.

From the first Western blot, we observe that increasing concentrations of 4-HT caused increased levels of the estrogen receptor and increased levels of p-Erk. We can observe that there are consistently higher levels of p-ERK and ERα for Clone 12 compared to clone 2. The similar levels of Erk present in each sample confirmed equal loading of the Western blots. From the second Western blot, we observe that increasing time of exposure to 4-HT caused increased levels of estrogen receptor and increased levels of p-Erk. We can observe that there are consistently higher levels of p-ERK and ERα for Clone 12 compared to clone 2. Again, the similar levels of Erk present in each sample confirmed equal loading of the Western blots.

## Discussion

Virus infection of human fibroblast cells generated multiple clones, numbered 1-3, 6,7, and 9-12. We screened the clones for their ability to induce senescence through B-Raf signaling and their ability to remain growth arrested after the removal of 4-HT. Clones 2 and 12 were the most efficient clones at senescence induction and its maintenance. We measured the activity of the kinase cascade. ΔB-Raf-ER* showed increased activation with increased concentration or time of exposure to 4-HT for both clones. We eventually abandoned clone 12 because of its low cells counts, likely due to increased expression in the MAPK/ERK pathway leading to apoptosis. We can confirm this observation by looking at the increased levels of ERα and p-Erk of clone 12 compared to clone 2 in the Western Blots in Fig. 6, 7 and Tables 1 and 2.

The results obtained from the current experiment agree with the literature where constitutive expression of oncogenic Ras and Raf in cells elicits premature senescence in fibroblast cells(3, 4). Raf mediated senescent cells exhibited irreversible withdrawal from the cell cycle, elongated morphology, retention of viability, and the induction of senescent enzymatic marker β-galactosidase(4). All these characteristics were also observed in the current experiment, except that the activity of β-galactosidase was not tested, but likely to be present. The question still remains, how do cells achieve senescence, what proteins are involved in the process?

Through FACS, a tool used to identify cells in different stages of the cell cycle, previous work demonstrated that Raf mediated cell senescence arrested in G1 and G2/M(4). Arresting in these two stages of the cell cycle could suggest different proteins and mechanisms involved at each of these stages. Alternatively, it could also suggest the same protein involved in two different stages of the cell cycle. It would be intriguing to see if the HF-E1T immortalized cell line would exhibit similar properties and arrest in the same cell cycle stages.

Senescent cells are known to express elevated levels of p53, $p21^{Cip21}$, and $p16^{INK4a}$ (17, 18), but evidence suggests that the level and role in senescence these genes may play is still not well understood. In IMR-90 cells, a type of human lung fibroblast, Raf induced senescence did not correlate with induction of p53 or $p21^{Cip21}$, but rather with $p16^{INK4a}$ (4). On the other hand, ectopic expression of $p15^{INK4b}$, $p16^{INK4a}$, $p21^{Cip21}$ and $p27^{Kip1}$ elicited cell cycle arrest in TIG-3 human lung fibroblasts (19). A possible explanation for these results it that, different mechanisms may mediate oncogene-induced senescence in different cell lines.

Finally, much work has also been done on Ras, the upstream regulator of Raf proteins. Ras mediated OIS exhibits similar characteristics to the senescence induced by Raf but has a difference in morphology (20). Ras induced senescence is distinctive in that it has a flattened phenotype like non-senescent cells, as opposed to the elongated phenotype of that of Raf (20). The difference in morphology may be attributed to the additional cell signaling pathways Ras can regulate, such as those involving Rho GTPases. The difference in morphology could also suggest different mechanisms involved in their senescence. Other studies have also been conducted on MEK, the downstream substrate of Raf. MEK mediated OIS exhibits all the same characteristics as Raf mediated OIS including the elongated morphology (21). These observations would be expected as Raf directly activates MEK and has very few substrates (21).

The genes mentioned above in the discussion could all be part of the senescence mechanism; however the increased expression of these genes may at times be observed due to coincidence rather than by causation. In the locus coding for $p16^{INK4a}$, another protein known as $p19^{ARF}$ is also present (22). It has been proven that mice lacking $p19^{ARF}$ but have normal expression $p16^{INK4a}$ develop tumours (22). These results suggest that the elevated expression of $p16^{INK4a}$ observed in senescent human fibroblasts may be indication of correlation rather than the causation of senescence (4). It's possible that $p16^{INK4a}$ may have a role in tumour suppression, however its role in senescence is still not well understood.

Conducting a genetic screen involving the systematic interrogation of the human genome would avoid the problem of correlation and provide results based on causation since we would obtain a visible phenotype. We would also be able to screen thousands of genes at once rather than focus on the expression of specific genes. In a genetic screen conducted by Wajapeyee and al. on human fibroblasts and melanocytes, secreted protein IGFBP7 was identified as a major player in B-Raf oncogene induced senescence (14). However a paper published not long after contradicted this result and concluded that IGFBP7 was actually not required for B-Raf oncogene induced senescence (23). The explanation for this discrepancy was that the tissue culture conditions used in the original experiment allowed activated B-Raf to cause genetic changes. These genetic changes resulted in the selective outgrowth of antibiotic-resistant clones that had escaped oncogene-induced senescence. This scenario is unlikely to occur in our experiment, as our cells are less prone to escape senescence. Our screen for escape frequency of clone 2 did not yield an outgrowth

of a single cell colony. In another genetic screen conducted on epithelial cells, genes previously implicated in proliferative control such as PTEN were confirmed (24). In addition, new genes such as REST that is involved in the transcriptional repression were also identified (24).

For our experiment, we will be using barcoded, retroviral based short hairpin RNA (shRNA) library to enable stable gene knockdown. We will be able to systematically screen the human genome for genes whose loss of function will transform cells into an oncogenic state and bypass senescence. We propose a pooled screen paradigm where cells will be treated with shRNAs to knockdown genes. Cells with successful gene knockdown will be selected for and the ectopic expression of B-Raf will be upregulated to induce senescence. The cells with successful senescence evasion will have the barcode of the shRNA sequenced to determine the gene that was knocked down. There is the possibility that some cells with senescence evasion have more than one gene knocked down. In this case, we will sequence all the barcodes from the shRNAs and identify them. We will then treat senescent cells with the shRNAs individually to determine the gene causing senescence. The genetic screen we would conduct will likely first identify already defined tumour suppressors in the literature, but additionally it may identify novel genes previously undefined in the literature. The newly identified genes would provide novel targets for drug therapies and markers of tumorigenesis.

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