



Microarray Analysis of Bmi-1 Downstream Genes in Normal Human Oral Keratinocytes

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ABSTRACT Bmi-1 is a polycomb group oncogene highly overexpressed in premalignant and malignant oral lesions. Bmi-1 is believed to promote oral carcinogenesis in part by allowing normal cells to evade the senescence checkpoint and extending their replicative life span. To determine the mechanisms underlying the role of Bmi-1 in oral carcinogenesis, the authors performed microarray analysis in normal human oral keratinocytes, NHOK, overexpressing Bmi-1. The authors report here several potential target genes of Bmi-1.

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The senescence process, or “cellular aging,” in normal human oral keratinocytes has been studied extensively in the context of its tumor suppressive function. It has been found that the senescence block in NHOK can be overcome by expression of Bmi-1.¹ Bmi-1 is a polycomb group transcription repressor originally found by provirus (Moloney murine leukemia virus; MuLV) tagging to identify the cellular gene cooperating with *c-myc* in pre-B cell lymphomagenesis.² Transgenic mice overexpressing *c-myc* in their lymphoid tissues develop pre-B cell lymphomas with greater efficiency when infected with MuLV, which was frequently (49 percent) found integrated near the cellular Bmi-1 locus, resulting in increased level of Bmi-1 expression.² These findings indicate that Bmi-1 overexpression contributes to tumorigenesis. Recent studies also showed that proliferative capacity of leukemic and nor-

mal hemopoietic stem cells derived from Bmi-1^{-/-} mice was compromised, suggesting the possible role of Bmi-1 in the maintenance of tumor stem cell phenotype.³

Bmi-1 is believed to promote cellular proliferation by inhibiting the expression of p16^{INK4A} tumor suppressor protein.⁴ During normal replication of human diploid fibroblasts (HDF), Bmi-1 expression level is decreased notably during senescence.⁵ When overexpressed, Bmi-1 is able to extend the replicative life span of HDF, HMEC, and NHOK.^{5,6,1} Thus, in normal human cells, diminution of Bmi-1 expression may be necessary for the onset of senescence, which can be overcome during carcinogenesis by sustained overexpression of Bmi-1. The association between Bmi-1 and oral carcinogenesis has been studied by Kang et al. who showed that overexpression of Bmi-1 occurs very early in oral carcinogenesis upon dysplastic epithelial transformation and is required

for sustained cancer cell replication and survival.⁷ It is possible that Bmi-1 evades the senescence block and results in cellular transformation during oral carcinogenesis.

Identification of the downstream genes of Bmi-1 in oral carcinogenesis is important for understanding the pathophysiology of the disease progression and to identify novel early markers of oral squamous cell carcinoma. With this purpose, the authors expressed exogenous Bmi-1 in rapidly proliferating NHOK and determined its effect on altered gene expression profiles by DNA microarray analysis. The authors found several candidate target gene groups of Bmi-1 in NHOK, including those involved in apoptosis, cell cycle regulation, and DNA replication. Further study is necessary to elucidate their biological roles in oral carcinogenesis.

MATERIALS AND METHODS

Cells and Cell Culture

Primary NHOK cultures were prepared from separated epithelial tissue discarded from routine oral surgery procedures and subsequently serially subcultured in keratinocyte growth medium (KGM) containing a low level (0.15 mM) of Ca⁺⁺ and supplementary growth factor bullet kit (Cambrex, East Rutherford, N.J.) as described previously.⁸ Briefly, epithelial cells were isolated from the basal surface of the epithelial tissue of approximately 25 mm² × 0.5 mm in size by trypsin digestion. The duration of the enzymatic digestion was limited to three min. to avoid harvesting cells from the suprabasal layers. These cells were seeded onto two collagen-treated T25 culture flasks and allowed to proliferate until 60 percent to 70 percent confluency. Primary NHOK were serially subcultured in KGM, with passage at every 60 percent to 70 percent confluency level.

Retrovirus Construction

For this study, the authors constructed a two retroviral vectors: RV-Bo and RV-Bmi-1. These viral vectors were prepared, respectively, from pBabe and pBabe-Bmi-1 retroviral expression plasmids, which were a kind gift of Dr. Goberdhan Dimri (Northwestern University, Evanston, Ill.). pBabe-Bo or pBabe-Bmi-1 was introduced into GP2-293 packaging cells (BD Biosciences, Bedford, Mass.) by the calcium phosphate precipitation method. Briefly, the

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day before transfection, GP2-293 cells were plated into T-175 flasks (Corning, Corning, N.Y.) at 1.5 × 10⁷ cells, with 30 ml of DMEM (Invitrogen) supplemented with 10 percent SCS (Gemini). Upon 60 percent to 70 percent confluency the next day, media was replaced with 25 ml of fresh media and cells were additionally incubated with 2 ml of 1x Hepes Buffered Saline solution containing 15 µg of pBabe-Bo or pBabe-Bmi-1, 15 µg of pCMV-VSV-G envelope plasmid, and 0.1 mM CaCl. Viral supernatant was collected 24 and 48 hours after transfections, filtered through 0.45 µm-pore-size filter, and harvested at 5 × 10⁵ g for 1.5 hrs at 4 degrees Celsius. The resulting viral pellet was then resuspended in 2 ml of KGM overnight at 4 degrees Celsius, aliquoted, and stored at -80 degrees Celsius.

Retrovirus Infection and Selection

NHOK were cultured to ~70 percent confluency in BD Falcon 6-well dishes (BD Biosciences), and subsequently incubated for four hours at 37 degrees Celsius with the retroviral vectors and polybrene (Sigma) at a concentration of 8 µg/ml. The supernatant was then changed with fresh media and the infected cells were selected with puromycin (Sigma) two days after infection at a concentration of 1 µg/ml. The infected cells were then serially subcultured in the presence of puromycin (1 µg/ml) and collected at various time points for further analysis.

RNA Isolation and Analysis of Gene Expression by Microarray

Gene expression profiling was performed between senescing NHOK infected with the empty vector control (RV-Bo) and NHOK with the virus expressing Bmi-1 (RV-Bmi-1), using the GeneChip Human Genome U133 Plus 2.0 Array from Affymetrix (Santa Clara, Calif.).

Total RNA was extracted from cultured NHOK (RV-Bo at population doubling (PD) 12 and RV-Bmi-1 at PD 19) using Trizol reagent (Invitrogen) and further purified through RNeasy columns (Qiagen, Chatsworth, Calif.) using the standard protocol.⁹ The probe synthesis, labeling, hybridization, and data acquisition were performed by the DNA Microarray Core facility at University of California, Los Angeles.

The expression profiling of more than 47,000 annotated genes was compared between senescing NHOK 05-12 infected with RV-Bo, and NHOK 05-12 with extended life span infected with RV-Bmi-1. Data analysis was followed after calibrating the Cy3 signals from the two NHOK cultures based on the mean value of the overall signal intensity and the distribution of the individual spot intensities.

RESULTS

Cellular Proliferation and Kinetics of Replication

Primary NHOK undergo a finite number of replications during serial subcultures before entering a senescing phase, characterized by the loss of replicating potential and lack of DNA synthesis, immediately followed by senescence and differentiation. The maximum replication potential of NHOK in the authors' previous report was determined to be 22 ± 3 population doublings (PD).⁸ In this study, the NHOK strain used (05-12) reached a maximum accumulated 19 PDs at senescence.

In order to determine the effects of ectopically expressed Bmi-1 on the replicative capacity of NHOK, the authors infected secondary NHOK with RV-Bo or RV-Bmi-1, which represent empty vector and the retroviral vector expressing full-length Bmi-1, respectively. These viral vectors were prepared by transfection of pBabe and pBabe-Bmi-1 plasmids into GP2-293 packaging cells along with pCMV-VSV-G. Transfecting with VSV-G allows for the produced viral particles to be concentrated by ultracentrifugation at 50,000g; this is not otherwise possible because other envelope proteins are linked by weak disulfide bonds that may not withstand centrifugation and filtration.¹⁰

NHOK were infected with concentrated RV-Bo or RV-Bmi-1 for four hours in media containing 8 $\mu\text{g}/\mu\text{l}$ of polybrene. Polybrene is a small, charged molecule that enhances the efficiency of infection by neutralizing surface charges, facilitating the interaction between the retrovirus and cellular membrane surface.¹¹ At 24 hours postinfection, 1 $\mu\text{g}/\mu\text{l}$ of puromycin was introduced to the culture medium as a selection marker. The drug-resistant cells were maintained at 70 percent confluence to avoid contact-regulated

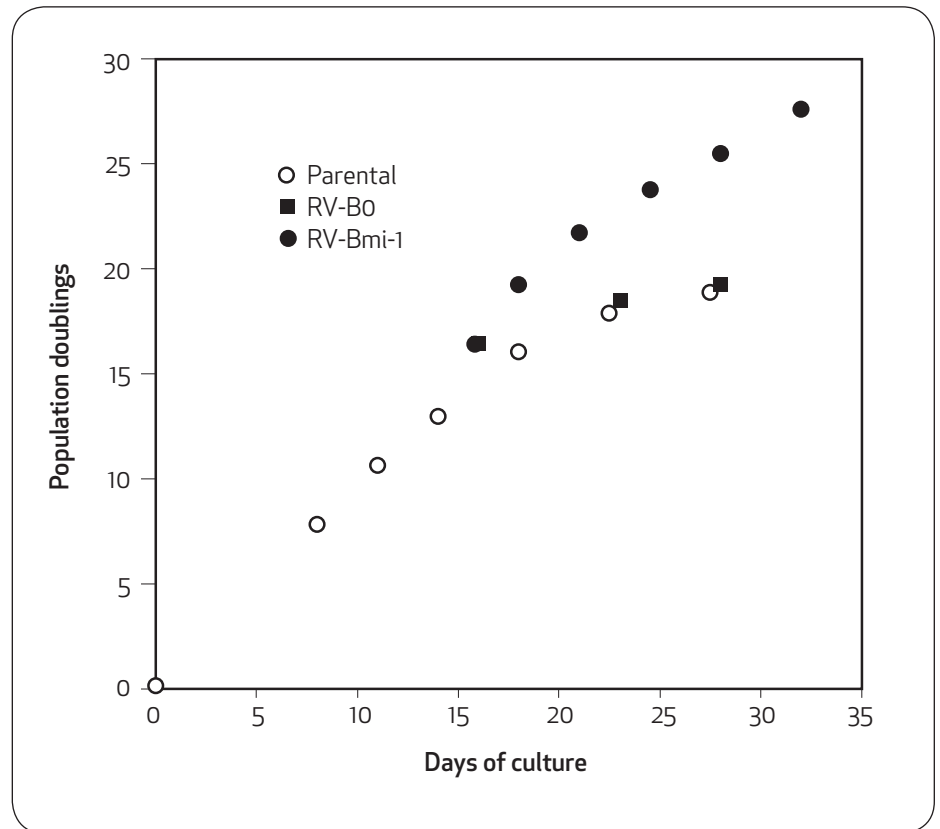


FIGURE 1. Replication kinetics of parental NHOK and NHOK ectopically expressing Bmi-1. Replication kinetics of parental NHOK (Parental); NHOK infected with RV-Bo (RV-Bo); and NHOK infected with RV-Bmi-1 (RV-Bmi-1). NHOK was infected with RV-Bo and RV-Bmi-1 14 days after the initial primary culture of NHOK. Parental NHOK reached a maximum 19 PDs, NHOK/RV-Bo reached a maximum 19 PDs, and NHOK/RV-Bmi-1 reached a maximum 28 PDs.

growth inhibition and differentiation. NHOK stably expressing Bmi-1 were able to extend their replicative potential from a maximum cumulated 19 PDs in RV-Bo infected cells to over 27 PDs in RV-Bmi-1 transduced cells (FIGURE 1), but underwent a delayed senescence and were unable to reach immortalization.

Differential Gene Expression Profiling in NHOK With or Without Bmi-1 Overexpression

In order to elucidate the potential downstream targets of Bmi-1, RNA was extracted from NHOK infected with RV-Bo and undergoing senescence and NHOK overexpressing Bmi-1 exhibiting an extension of life span (FIGURE 2). Fluorescent-labeled cDNA was prepared from the RNA of senescent NHOK,

infected with RV-Bo (NHOK/RV-Bo) at PD 19, and from NHOK overexpressing Bmi-1 (NHOK/RV-Bmi-1) with an extended life span at PD 22. Senescent NHOK/RV-Bo (PD 19) showed characteristics of senescence as previously described, including flattened morphology, perinuclear vacuolization, increased cytoplasmic to nuclear ratio, and SA- β -Gal expression, in contrast to NHOK/RV-Bmi-1 (PD 22), which demonstrated normal morphology and remained in the exponentially replicating phase.⁸

Hybridizing the labeled cDNA to the Affymetrix Human Genome U133 Plus 2.0 Array, the authors were able to analyze the expression of over 47,000 different transcripts corresponding to 38,500 genes. Spot intensities from the microarray were analyzed using Affymetric Gene

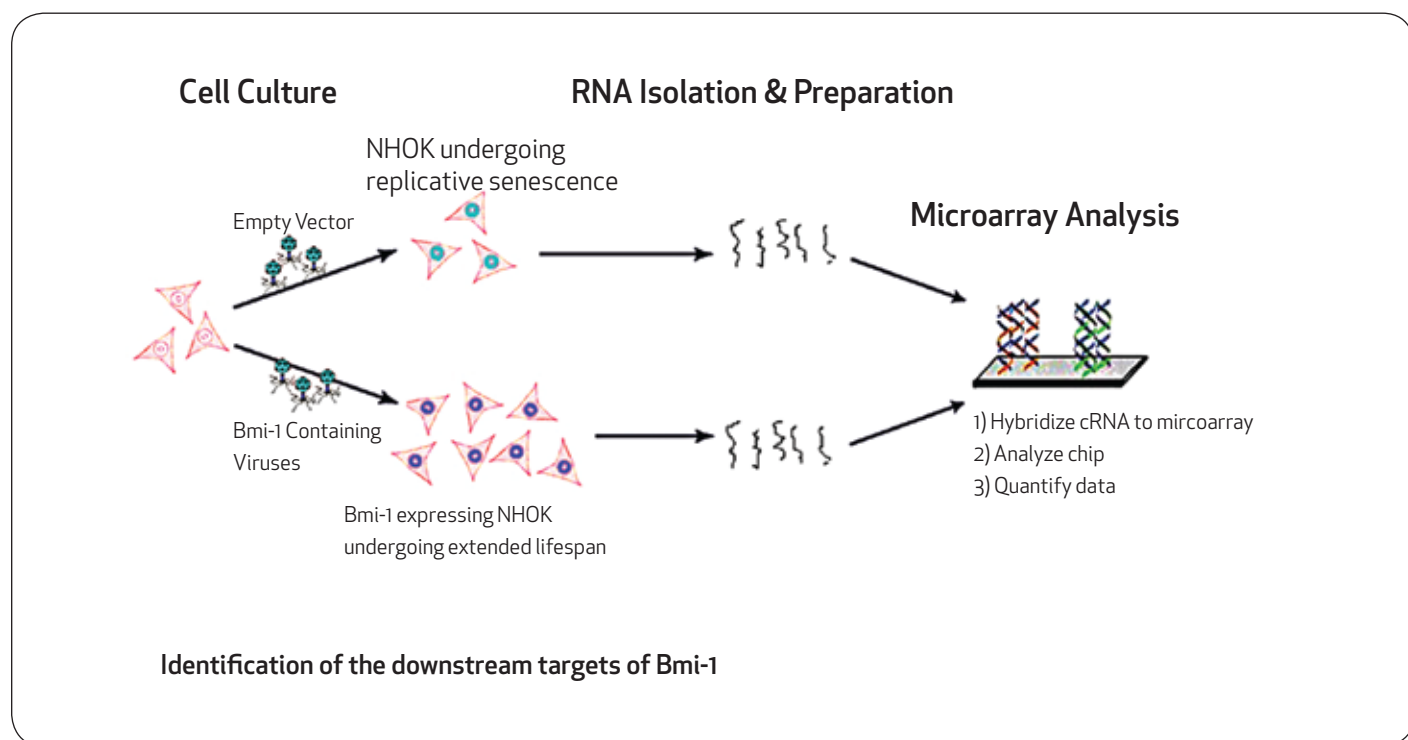


FIGURE 2. NHOK cultured from discarded epithelial tissue was infected with vectors containing Bmi-1. RNA was isolated from NHOK undergoing replicative senescence and Bmi-1 overexpressing NHOK with an extended life span. RNA was hybridized to an affymetrix microarray chip containing probes representing more than 38,000 genes. Chips were analyzed and quantified for intensity of hybridization, and genes with a greater than two-fold difference of expression between the two groups were selected for further study.

Chip Operating Software. Statistically significant ($P < 0.05$) differences were further reduced using the selection criteria cutoff (> 2 -fold difference). As a result, the authors observed a total of 3,024 transcripts that were differentially expressed between NHOK/RV-Bo (PD 19) and NHOK-Bmi-1 (PD 27). Of these, 1,321 genes were upregulated in Bmi-1 overexpressing NHOK, and 1,703 genes were downregulated compared to NHOK/RV-Bo (PD 19). A detailed analysis of their functions was conducted and the genes were grouped into major biochemical pathways, i.e., cell cycle control, cellular differentiation, and apoptosis.

In order to compile a broader understanding of the patterns associated with Bmi-1 expression in NHOK, previous gene expression data were normalized using the Robust Multichip Average. The authors used the RMA Express software developed by the Ben

Bolstad group at University of California, Berkeley, in 2003 in order to attain a global perspective on differential gene expression. The protocol consists of three steps: a background adjustment, quantile normalization, and summarization, which ultimately allows for the comparison of data across several experiments. RMA normalization was utilized to allow the comparison of differential gene expression data between genes differentially expressed 1) when Bmi-1 is overexpressed in late passage NHOK (denoted by NHOK/RV-Bmi-1 at PD 19); 2) when Bmi-1 is overexpressed in early passage NHOK (NHOK/RV-Bo at PD 11) and NHOK-RV-Bmi-1 at PD 11); and 3) in parental exponentially replicating and senescing NHOK (NHOK at PD 11) and NHOK at PD 19).

The normalized data were compared using NHOK-RV/RV-Bo (PD 19) as the baseline, in order to demonstrate whether

NHOK/RV-Bmi-1 (PD 22) exhibited a pattern of expression more similar to exponentially replicating parental NHOK (PD 11), exponentially replicating NHOK/RV-Bo (PD 11), and exponentially replicating NHOK/RV-Bmi-1 (PD 11), or senescing parental NHOK (PD 19).

Genes were then categorized according to the Affymetrix Netaffx online module Gene Ontology Biological Process and Gene Ontology Molecular Function criteria for further analysis. The TIGR Multiexperiment Viewer software was then used to visualize global gene expression patterns and cluster genes using K-means hierarchical clustering statistical algorithms.

FIGURE 3A shows the global pattern of expression of all 3,024 transcripts that were differentially expressed between NHOK/RV-Bo (PD 19) and NHOK/RV-Bmi-1 (PD 22) in the original microarray data, as compared across all experimental

conditions in a dendrogram heatmap. These genes showed a clear, consistent pattern of expression in NHOK/RV-Bmi-1 (PD 22) with NHOK/RV-Bmi-1 (PD 11), NHOK/RV-Bo (PD 11), and NHOK (PD 11). This is in contrast with NHOK (PD 19), which showed a generally opposite pattern of expression. **FIGURE 3B** also shows the pattern of expression of various apoptosis-related genes, again detailing the opposing pattern of expression in senescing NHOK, but the recovery of exponentially replicating expression via the ectopic overexpression of Bmi-1 in NHOK with an extended life span.

As expected, several genes associated with cell cycle arrest also exhibited a consistent pattern between Bmi-1 overexpressing strains and parental NHOK (PD 11), and an opposing pattern in senescent parental NHOK (PD 19) (**FIGURE 3C**). Lastly, Bmi-1 appears to recover the exponentially replicating pattern of expression of several genes associated with differentiation. Again, the pattern is clear that Bmi-1 overexpressed in extended-life span NHOK allows genes that are otherwise oppositely expressed in senescing NHOK (NHOK (PD 19)) to follow the patterning of exponentially replicating parental (NHOK at PD 11) and Bmi-1 overexpressing NHOK (NHOK/RV-Bo at PD 11, NHOK/RV-Bmi-1 at PD 11) (**FIGURE 3D**).

DISCUSSION

The authors reported changes in the gene expression profile of NHOK with an extended life span after ectopic expression of the Bmi-1 oncogene. Of the 47,000 transcripts screened utilizing DNA-microarray in this study, 3,024 transcripts were found to be differentially expressed with greater than two-fold differences. These transcripts reflect the phenotypic differences exhibited between Bmi-1 overexpressing NHOK with an extended life

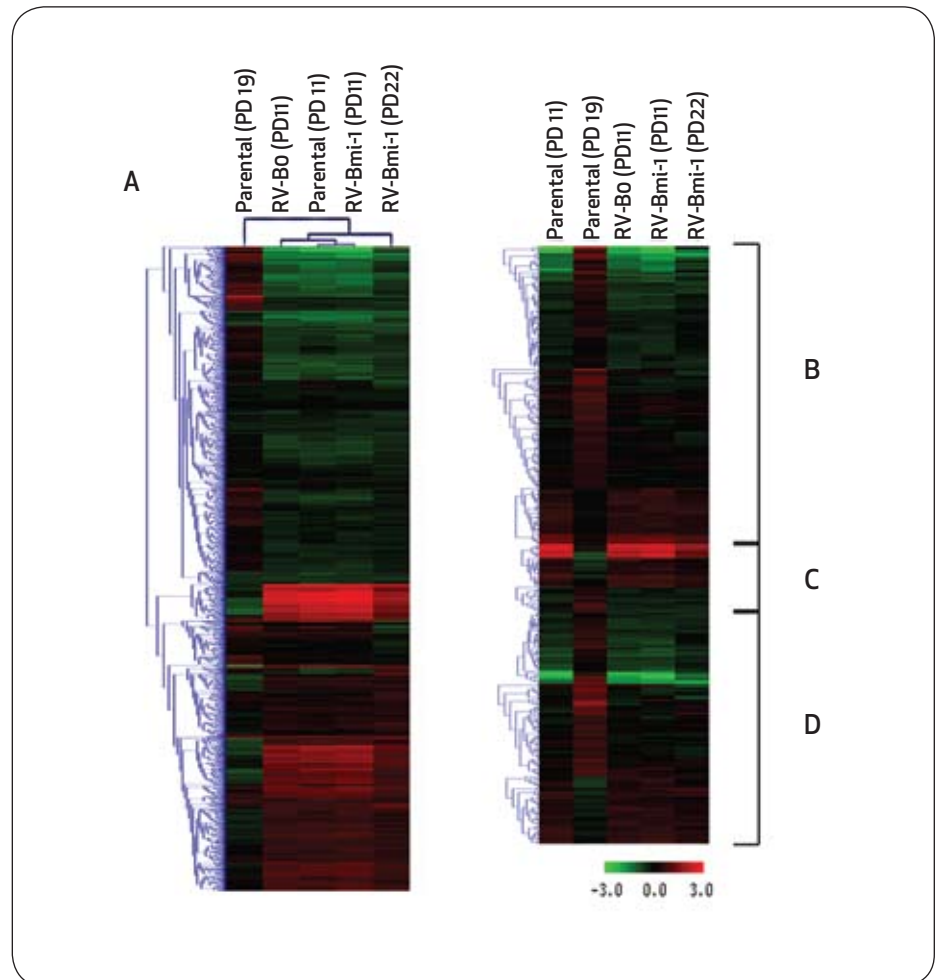


FIGURE 3. Genes are differentially expressed in exponentially replicating NHOK, senescent NHOK, and NHOK with an extended life span. Gene groups and conditions are clustered according to K-means hierarchical clustering statistical algorithms and normalized using RMA normalization with NHOK-RV-Bo (PD 19) as the baseline. Dendrograms display differential expression of 3,024 genes with greater than two-fold change in NHOK-RV-Bo (PD 19) versus NHOK-RV-Bmi-1 (PD 22) (a); genes related to apoptosis (b); genes related to cell cycle arrest (c); and genes related to cellular differentiation (d).

span and senescing late passage NHOK infected with the empty vector control. Expressions of genes involved in cell proliferation, cell differentiation, cell cycle arrest, apoptosis, and senescence were all differentially regulated and profiled.

Of the 3,024 differentially expressed transcripts revealed in this study, there was a clear pattern of recovery when comparing extended-life span, Bmi-1 overexpressing NHOK with senescing NHOK. The global expression pattern of extended-life span, Bmi-1 overexpressing NHOK closely resembled that

of exponentially replicating parental NHOK, exponentially replicating empty vector control NHOK, and exponentially replicating Bmi-1 overexpressing NHOK. There was a generally reduced expression of apoptosis related genes in all exponentially replicating NHOK and Bmi-1 overexpressing NHOK with an extended life span, whereas an opposite pattern was exhibited in senescing parental NHOK.

As expected, those apoptosis-related genes typically overexpressed in exponentially replicating NHOK, were also overexpressed in Bmi-1 overexpress-

ing NHOK with an extended life span. However, they were noted to be under-expressed in senescing parental NHOK. This pattern of opposing expression in senescing NHOK, and recovery of expression to exponentially replicating NHOK levels in Bmi-1 overexpressing extended-life span NHOK, was consistent in genes related to cellular differentiation and cell cycle arrest, and was generally present in all of the 3,024 differentially expressed transcripts. These results may indicate that among these genes, there are critical factors that are maintained by Bmi-1-dependent or other mechanisms in exponentially replicating NHOK and are oppositely expressed in senescent NHOK, but may be recovered to their original status by Bmi-1 overexpression in senescent NHOK, resulting in an extended life span.

In a preliminary examination of the differentially expressed genes, the over-expression of Bmi-1 in NHOK/RV-Bmi-1 (PD 22) upregulated several genes related to cell cycle control and cellular proliferation (TABLE). The cyclin superfamily, for example, is a group of proteins that are unstable and are subject to rapid turnover. They are responsible for activating cyclin-dependent kinases, which in turn effect progression through the cell cycle, such as through the G₁/S-Phase transition.¹² Cyclins A₂, B₁, E₂, and F were all upregulated in Bmi-1 overexpressing NHOK.

Furthermore, another important class of proteins, the cell division cycle (CDC) factors CDC₂, CDC₆, CDC₇, CDC₂₀ and CDC_{45L} were all found to be upregulated in this study. In particular, several of these factors have been suggested to possess oncogenic properties and may be involved in tumorigenesis.^{13,14} CDC₂ for example, is the catalytic subunit of the M-phase promoting factor complex (MPF), which is essential for the

TABLE

Differential Expression of Potential Bmi-1 Target Genes

Gene Symbol	Gene Name	Fold Increase (+)/ Decrease(-)
CDC 2	Cell division cycle 2	+2.6
CDC 6	Cell division cycle 6	+3.0
CDC 7	Cell division cycle 7	+2.5
CDC 20	Cell division cycle 20	+2.0
CDC 25A	Cell division cycle 25A	+1.7
CDC 45L	Cell division cycle 45-like	+2.1
Cdk4	Cyclin-dependent kinase 4	+1.4
CCNA2	Cyclin A2	+2.8
CCNB1	Cyclin B1	+2.0
CCND2	Cyclin D2	+1.7
CCNE2	Cyclin E2	+3.0
CCNF	Cyclin F	-2.4
CCNG2	Cyclin G2	-1.7
CCNI	Cyclin I	-1.5
IL1B	Interleukin 1 beta	-1.6
IL8	Interleukin 8	-2.9
MCM 2	minichromosome maintenance complex component 2	+2.6
MCM 3	minichromosome maintenance complex component 3	+1.9
MCM 4	minichromosome maintenance complex component 4	+2.1
MCM 5	minichromosome maintenance complex component 5	+2.6
MCM 6	minichromosome maintenance complex component 6	+5.9
MCM 7	minichromosome maintenance complex component 7	+1.8
MCM 10	minichromosome maintenance complex component 10	+2.6
TGFB2	Transforming growth factor, beta 2	+2.0

G₁/S and G₂/M-phase transitions.¹⁵

CDC₂ functions as a kinase that is regulated by cyclin accumulation, and complexes with Cyclin B₁ in particular to form MPF, which has also been shown to be upregulated by Bmi-1 in this study. CDC₂ has also been shown to complex with Cyclin A and Cyclin E.^{16,17} There have been numerous targets of the CDC₂/Cyclin complexes documented, and its role in cell-cycle progression and mitosis is widespread. CDC₆ has not been as well documented but has been implicated as an important factor in the cell

cycle, particularly in DNA replication.¹⁸

Reports have also shown that CDC₆ is regulated by p63 in the initiation of DNA replication, and that aberrant CDC₆ expression may be oncogenic in direct repression of the p¹⁶^{INK4A}/ARF locus.^{19,14} Regulators of cell-cycle progression also comprised several of the upregulated genes, totaling 15. Of particular interest is the minichromosome maintenance deficient (MCM) group of proteins, of which six were upregulated by Bmi-1, and have been well documented in DNA-replication. Interestingly, MCM

2, 4, 6, and 7 form a complex that is phosphorylated by the aforementioned CDC2 and CDC7 kinases.²⁰

Overall, several promising avenues of study have been revealed that may further elucidate the physiological mechanisms underlying aberrant Bmi-1 function and its role in oncogenesis. In particular, poorly studied genes and genes with no previously established relationship with Bmi-1 are the most intriguing. Studies are currently being conducted to further validate the data obtained from the microarray analysis, and promising genes are undergoing physiological characterization and functional analysis. Conclusive

validation of the role of Bmi-1 in tumorigenesis is also necessitated. In particular, studies involving the tumorigenic potential of Bmi-1 overexpressing NHOK in nude mice would be the most definitive, and would aid in the characterization of the novel genes related to Bmi-1. Taken together, these studies may provide a better understanding of the roles of Bmi-1 and its key factors in cellular transformation and carcinogenesis. ■■■■■

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