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Microarray Gene Expression Profiling of Bisphosphonate-Treated Normal Human Oral Keratinocytes

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Microarray Gene Expression Profiling of Bisphosphonate-Treated Normal Human Oral Keratinocytes

A thesis submitted in partial satisfaction

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in Oral Biology

by

Rachel Suhhee Lee

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ABSTRACT OF THE THESIS

Microarray Gene Expression Profiling of Bisphosphonate-Treated
Normal Human Oral Keratinocytes

by

Rachel Suhhee Lee

Master of Science in Oral Biology
University of California, Los Angeles, 2014
Professor Reuben Han-Kyu Kim, Chair

Bisphosphonates (BPs) are the most widely used anti-resorptive agents for osteoporotic and cancer patients. With frequent use of BPs in multiple clinical settings, a growing body of evidence supports a notion that some BPs cause side effects that are specific to soft tissues. Previously, we reported that BPs inhibit proliferation and induce senescence in normal human oral keratinocytes (NHOKs). Cell cycle analysis in BP-treated NHOKs revealed an increase in the S phase cell cycle arrest (Kim et al, 2011). In this study, we further examined the underlying mechanisms by which BPs inhibit the proliferation of NHOKs.

Primary NHOKs were established from normal human oral epithelium. These cells were treated with pamidronate (PAM) and zoledronate (ZOL) and subjected to microarray gene profiling. Microarray analysis showed that genes associated with cell cycle control of chromosomal

replication were significantly dysregulated. Among the validated genes, expression of CCNA2, a cyclin that is associated with S-phase in the cell cycle, was drastically inhibited by both PAM and ZOL as determined by qRT-PCR and Western blotting. Expression of CCND1, Cdk6, and Cdk2 were not affected by PAM and ZOL. The luciferase reporter assay using CCNA2 promoter showed suppression of luciferase activity in ZOL-treated NHOK, indicating that the loss of cyclin A2 expression is transcriptionally regulated. Immunohistochemistry also showed the inhibition of cyclin A2 expression in NHOK in ZOL-treated oral mucosa tissue constructs. Our study, through the microarray gene expression profiling, demonstrates that inhibition of NHOK proliferation by BPs is, in part, mediated by suppressing CCNA2 expression at the transcriptional level, which may explain the underlying mechanisms of soft tissue toxicity by BPs.

The thesis of Rachel Suhhee Lee is approved.

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Mo Kwan Kang

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Ki-Hyuk Shin

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Reuben Han-Kyu Kim, Committee Chair

University of California, Los Angeles

2014

DEDICATION

I dedicate this thesis to my parents, Jin Ok Lee and Esther Sangja Lee, for their limitless support and prayer throughout my academic career, and also to my siblings, Kevin Lee and Joyce Lee, for their encouragement. Last but not least, I would like to dedicate this work and give thanks to God who guided me throughout my hardships and helped me to become who I am now. Without God, I would not have met a wonderful mentor, Dr. Reuben Han-Kyu Kim, who gave me the opportunity to pursue DDS/MS degree.

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INTRODUCTION

The development of bisphosphonates (BPs) changed the prognosis for bone associated diseases. The idea of developing BPs first came up by the discovery of inorganic pyrophosphate in the urine and plasma. Pyrophosphate has high affinity for calcium, chelates circulating calcium, and binds to the mineral at bone surface. These calcium-targeting characteristics made the pyrophosphate considered to be a potential chemical to treat calcium-related symptoms. However, because of its high metabolic rate, pyrophosphates get rapidly inactivated, which is clinically undesirable.

In 1960, Fleisch and David Francis worked together to develop pyrophosphate-like compounds with desired properties that would withstand the hydrolysis, which are BPs (Fleisch et al., 1982). The oxygen molecule in pyrophosphate backbone is substituted by a carbon in BPs, and this makes the BPs more resistant to the degradation and suitable for long term use in clinical setting. BPs have hydroxyl groups, which are responsible for the moiety to the bone, and R-groups, which give the variance of anti-resorptive activities (van Beek et al., 1999a; Leu et al., 2006). Different types of BPs are designated based on the R-groups.

Currently, there is a variety of commercially available BPs. The two prominent BPs that are used to treat and prevent malignant hypercalcemia and bone-related diseases are pamidronate and zoledronate. Both pamidronate and zoledronate are nitrogen-containing BPs, and zoledronate is considered the most potent inhibitor of the growth of the hydroxyapatite crystals because of its high binding affinity to the bone mineral (Nancollas et al., 2006).

Many studies have shown that BPs reduce the bone resorption via inhibition of farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway in the osteoclasts. Nitrogen-

containing BPs are shown to inhibit mevalonate pathway, which is involved in the synthesis of cholesterol. Specifically, BPs inhibit farnesyl diphosphate synthase (FPP synthase) (van Beek et al., 1999b). FPP synthase produces FPP, which can then partake in production of either squalene for cholesterol production or geranylgeranyl disphophate (GGPP) for osteoclast activities. FPP synthase is also responsible for isoprenylation of small GTPases that promote an array of activities in the osteoclasts that control bone resorption. Small GTPases, such as Rab, Rac, Ras, and Rho, are important for the regulation of osteoclast function and survival (Luckman et al., 1998). Bone resorption is initiated by osteoclast activity, which is a part of bone remodeling activities. The inhibition of FPP synthase, which is closely involved in osteoclast functions by isoprenylating small GTPases, reduces bone resorption. The inhibition disrupts the attachment of osteoclasts to the bone surface, which promotes early cell death (Favus et al., 2010).

The studies have also shown that during the direct interaction between the BPs and the bone remodeling process, the administered BPs bind to the bone surfaces. BPs have high affinity to the bone where the osteoclast is activated. This is due to the presence of high concentration of free calcium released from the bone by osteoclast, which allows more interaction between the BPs and the calcium. As a result, BPs that are highly concentrated in the resorbing surfaces later get liberated by the osteoclast, and the amount is sufficient to initiate the inhibition of FPP synthase (Sato et al., 1991).

Because of their affinity to bone and their ability to reduce bone resorption, BPs are recognized as a mode of treatments for bone diseases that require sustained bone level. Malignant hypercalcemia, which can be due to the failure of renal functions or post-menopausal effect, and other skeletal-related symptoms are some of the therapeutic indications for the BPs administration.

Osteoporosis is the most commonly treated by BPs. Osteoporosis often post-menopausal and is characterized by loss of bone density with a high risk for bone fractures (Poole and Compston et al., 2006). Studies indicated that intake of estrogen during the menopause decreases the risk of osteoporotic fracture and act as an anti-resorptive agent (Weiss et al., 1980; Steiniche et al., 1989). However, estrogen placement therapy increases the risk for other side-effects, such as breast cancer. This turned the public attention to BPs as potential drugs to use to treat osteoporosis as non-hormonal anti-resorptive agents to reduce the risk of having side effects from the hormone therapy.

Furthermore, BPs, because of its cytotoxic effect on inducing apoptosis of the cells, have been tested and used as a mode of cancer therapy. Several clinical surveys show improved conditions of the cancer patients who use BPs as anti-tumor agents (Rennert et al, 2010; Kirk et al, 2010; Green et al, 2004). BPs are shown to reduce bone and visceral metastasis (Hiraga et al, 2004) by reducing the recurrence of tumor cells (Chang SS et al, 2004), and preventing the proliferation of cancer cells that have potential to metastasize (Senaratne and Colston et al, 2002).

The possible molecular mechanisms of the BPs on tumor cells have been widely studied to support the anti-tumor activity of BPs via in vitro studies targeting the molecules involved in the mevalonate pathway (Goffinet et al, 2006; Sonnemann et al, 2001). BP treatments induce the production of cytotoxic ATP analogs in tumor cells, leading to growth arrests (Monkkonen et al, 2008) and suppress angiogenesis via hypoxia-induction factor (HIF)-1α/ vascular endothelial growth factor (VEGF) signaling pathways in human breast cancer cells (Tang et al, 2010). Furthermore, BPs induce cell-cycle prolongation in murine lung cancer cells by alteration of cyclin and Ras expression (Li et al, 2011).

BPs have been widely used to treat osteoporosis and cancer. Many of the BP-cancer related studies have been geared toward the effect of BPs on breast, prostate, and lung cancers, which are mostly involve with bone metastasis. However, recent findings suggest the involvement of BPs on increased risk of having mucosal cancers, such as esophageal cancers (Green et al, 2010). This brings in the possibility of BPs effecting, not only at the bone level, but also, at the oral epithelial level.

A growing body of evidence suggests that BPs may have direct effects on soft tissues. For instance, one of the major side effects of orally administered BPs is inflammation and ulceration of gastrointestinal (GI) tracts and, to a lesser extent, oral mucosa. GI toxicity is one of the major dropout reasons during BP clinical trials (Van Holten-Verzantvoort et al., 1993). Esophageal inflammation and ulceration are frequently reported in BP-users (De Groen et al., 1996), and oral ulceration is also observed in patients who suck BP tablets (Rubegni et al., 2006).

Interestingly, the possible association of BPs with oral mucosa also correlates with the defective wound healing of bisphosphonate-related osteonecrosis of the jaw (BRONJ). The administration of high-dose BPs, such as pamidronate and zoledronate, gives BRONJ as a devastating side effect (Marx et al., 2003). The primary risk factor of BRONJ has been attributed to invasive dental procedures (Van den Wyngaert *et al*., 2006). Clinically, it is characterized by chronically exposed necrotic bone with unhealed overlaying oral mucosa due to the compromised wound healing of the lesion. BPs may have direct effect on the wound healing of oral mucosal (Landesberg et al., 2008). This furthermore implies that oral epithelium may be one of the major targeting tissues of BPs. However, the role of BPs on soft tissue toxicity is poorly understood.

The primary characteristic of wound healing is wound closure. In order for the complete healing, adequate closure of the wound must be achieved. In oral epithelium, this mainly occurs during the reepithelialization by keratinocytes (Gosain et al., 2004; Nickoloff et al., 2006). Reepithelialization can be divided into three stages, which are migration, proliferation, and differentiatation (Raja et al., 2007). Keratinocytes migrate into the wound, which then proliferate and differentiate. Impairment in any of these stages of reepithelialization by BPs may lead to defective wound healing.

To embark on elucidating the role of oral mucosal soft tissue, we investigated the phenotypic effects of BPs on normal oral mucosal cells of human origin. Our previous study showed that BPs elicit differential cellular responses in normal human oral keratinocytes (NHOK) (Kim et al., 2011). We performed microarray and selected several dysregulated genes. The previous data suggested a cell-cycle arrest and cell-accumulation at S phase in NHOK (Kim et al, 2011). We hypothesize that BPs target cyclin-Cdk complex molecules, involved in S phase. To further investigate the possible targets of BPs during S phase, Cyclin A2 was selected, which was one of the genes that are dysregulated in the microarray data. Cyclin A2 is important for initiation of DNA replication during S phase of cell cycle, and expression of cyclin A is found to be elevated in a variety of tumors (Yam et al, 2002).

In this study, we report that Cyclin A2, which activates S phase when it forms as a Cyclin A2-Cdk2 complex, is inhibited by BPs, both pamidronate (PAM) and zoledronate (ZOL), at the transcriptional level, specifically at its promoter region. This may serve as the leading cause of the cell-cycle arrest and the inhibition of proliferation of NHOK. Our 3-dimensional (3D) oral mucosal tissue constructs also demonstrated that ZOL inhibits the expression of cyclin A2, suggesting that the development of soft tissue toxicity in patients receiving ZOL or other

BPs may be associated with BP-induced growth arrest of oral mucosal cells and subsequent defective soft tissue wound healing. Moreover, the cell cycle arrest due to the down-regulation of Cyclin A2 can serve as an explanation to the molecular mechanism behind the anti-cancer activity of BPs.

MATERIALS AND METHODS

Cells and cell culture. Primary normal human oral keratinocytes (NHOK) were prepared from oral mucosal tissues according to methods described in elsewhere (Kang *et al.,* 1998). NHOK were cultured in in EpiLife supplemented with HKGS (Cascade Biologics, Portland, OR. USA). Zoledronate (ZOL) and Pamidronate (PAM) was purchased from LKT Laboratories, Inc. (Saint Paul, MN, USA).

Reverse Transcription (RT)–PCR. Total RNA was isolated from the cultured cells using TrizolTM reagent (Invitrogen) and was subjected to RNases-free DNase I digestion at 37°C for 2 h to eliminate any genomic DNA contamination. DNA-free total RNA (5 µg) was dissolved in 15 µl diethylpyrocarbonate-treated water, and the reverse transcription reaction was performed in first-strand buffer (Invitrogen) containing 300 U Superscript II (Invitrogen); 10 mM DTT and 0.5 µg random hexamer (Promega Corporation, Madison, WI) and 125 µM deoxynucleoside triphosphates. The annealing reaction was carried out for 5 min at 65°C, and complementary DNA synthesis was performed for 2 h at 37°C, followed by incubation for 15 min at 70°C to stop the enzyme reaction. The reverse transcription product was diluted with 70 µl H2O. The following primers were used for PCR amplification: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers, 5'-GAC CCC ATT GAC CTC AAC-3' (forward) and 5'-CTT CTC CAT GGT GGT GAA GA-3' (reverse); Cyclin A2 primers, 5’-GAG GAC CAG GAG AAT ATC AAC CCG G-3’ (forward) and 5’-AGC CAG GGC ATC TTC ACG CTC TAT T-3’ (reverse); and β-Actin primers, 5’-GCT CGT CGT CGA CAA CGG CTC-3’ (forward) and 5’-GTA CAT GGC TGG GGT GTT GAA GG-3’ (reverse).

Western Blotting. Cells were lysed and subjected to Western blotting as described previously (Kim *et al.*, 2010). The following antibodies were used: Cyclin A2 (C-19), Cyclin D1 (C-20), Cdk6 (C-21), Cdk2 (M2), and β-actin (I-19) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and PCNA (Ab-1) from Oncogene Resesarch Product (Boston, MA, USA).

Immunohistochemical (IHC) staining. IHC staining was performed as described previously (Shin *et al.*, 2006). Cyclin A2 expression was determined in bisphosphonate-treated or -untreated oral mucosal tissue samples from raft cultures, which were formalin-fixed, paraffin-embedded, and sectioned. The specimens were subjected to immunohistochemical analysis for the expression of Cyclin A2 (C-19, Santa Cruz) at 1:200 concentration. Stained tissues were developed using the 3,3'-diaminobenzidine (DAB) chromogen substrate (Vector Laboratories Inc., Burlingame, CA. USA). The samples were counterstained with hematoxylin.

Analysis of BP-responsive reporter activity. The pALUC-CCNA2 reporter plasmid, kindly provided by Dr. Berthold Henglein (Institut National de la Santé et de la Recherche Médicale, Paris, France) was used to construct pGL3-CCNA2 plasmid. The pGL3-Luc plasmid (Promega), in which expression of the firefly luciferase gene is controlled by the SV40 promoter, was used for Cyclin A2 promoter assay. The pRL-SV40 plasmid, which contains the *Renilla* luciferase gene is driven by the SV40 promoter, was used as an internal control for transfection efficiency. The pGL3-Cyclin A2 promoter plasmid was constructed by performing double-digestion on PALUC-Cyclin A2 promoter vector and pGL3-Luc plasmid with SacI (New England Biolabs) and XhoI (Invitrogen). The Cyclin A2promoter insert and the pGL3-Luc plasmid were ligated to form pGL3-Cyclin A2 promoter plasmid.

Approximately 5×10^4 cells/well were plated in a 24-well culture dish and cultured for 24 hours. Normal and bisphosphonate-treated NHOKs were then transiently transfected with 1

μg of either pGL3-Prom, the control luciferase reporter vector that has SV40 promoter, or pGL3-Cyclin A2 promoter, which has Cyclin A2 promoter sequence (608bp) at the 3' end of luciferase gene. For a better comparison among cells with different transfection efficiencies, the pRL-SV40 plasmid (0.005 μg/well) using the Lipofectin Reagent (Invitrogen) was also transfected into each cell and used for normalization of the pGL3-Luc reporter activities. After 4 hours of transfection, the transfection medium was replaced with regular culture medium. After 48 hours, cells were harvested. Cell lysates were prepared according to the Promega's instruction manual. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) and a luminometer (Promega). The *Renilla* luciferase activity was used to normalize for transfection efficiency. The experiment was performed in triplicates.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the cultured cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and was subjected to the optional column DNA digestion with the RNase-Free DNase (Qiagen) to eliminate any contaminating genomic DNA. DNAfree total RNA (5 μg) was dissolved in 15 μl of H2O, and the RT reaction was performed in the first-strand buffer containing 300 U of Superscript II (Invitrogen, Carlsbad, CA, USA),10 mM dithiothreitol, 0.5 μg of random hexamer (Promega, Madison, WI, USA), and 125 μM of dNTP mixture (Promega). Real-time PCR was performed in triplicates for each1 μl cDNA sample with LC480 SYBR Green I master (Roche, Indianapolis, IN, USA) using universal cycling conditions on LightCycler 480 from Roche.A total of 45 cycles were executed, and the second derivative Cq value determination method was used to compare fold differences. The primer sequences were obtained from the Universal Probe Library (Roche). Primer sequences are available upon request.

Organotypic raft cultures. Cells were grown as organotypic raft cultures using techniques established previously (Dongari-Baqtzoglou *et* al, 2006) with slight modification. Briefly, 10^6 cells were seeded on the submucosal equivalents consisting of type I collagen and normal human oral fibroblasts. The cells were grown to confluence, submerged in the culture medium, and then exposed to the liquid-air interface by lowering the medium level. The cultures were maintained in this "rafting" fashion for 14 days and were harvested by fixing in 10% buffered formalin. Subsequently, hematoxylin-eosin (H&E) staining was performed on thick (6 μm) sagittal sections of each reconstructs to reveal the histological features. Sample processing, paraffin-embedding, sectioning, and H&E staining were performed at the Translational Pathology Core Laboratory at UCLA.

cDNA microarray. Gene expression profiling was performed using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix). Total RNA was extracted from the cells using the RNeasy Mini kit (Qiagen) according to the manufacture's instruction. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and purity/concentration was determined using a NanoDrop 8000 (NanoDrop Products, Wilmington, DE). Microarray experiments were performed at UCLA CMC. Microarray targets were prepared using NuGEN WT-Ovation Formalin-Fixed Paraffin-Embedded RNA Amplification System and FL-Ovation cDNA Biotin Module V2 (NuGEN Technologies, San Carlos, CA) and then hybridized to the array, all according to manufacturers' instructions. The arrays were washed and stained with streptavidin phycoerythrin in Affymetrix Fluidics Station 450 using the Affymetrix GeneChip protocol, and then scanned using an Affymetrix GeneChip Scanner 3000. The acquisition and initial quantification of array images were conducted using the AGCC software

(Affymetrix). The subsequent data analyses were performed using Partek Genomics Suite Version 6.4 (Partek). Differentially expressed genes were selected at ≥2-fold and $P<0.005$.

RESULTS

BPs inhibit proliferation of NHOKs

To evaluate the effect of BPs on NHOK, an experimental design was formulated (Figure 1A). NHOK were cultured with PAM and ZOL. NHOK was treated with PAM and ZOL using the protocol that was performed in the previous study (Kim *et* al., 2011). Each sample was tripled. NHOK, treated with 10 μM PAM and 4 μM ZOL, had a significant loss of proliferation in with rounded and flattened phenotypes, compared to the control (Figure 1B). The cells in 3 samples of each were counted and averaged. The cell number of BP-treated NHOK significantly decreased compared to the control (Figure 1C). This indicates that BPs may be involved in the proliferation of NHOKs. In order to confirm the normalization of the genes in each sample, RT-PCR was performed to probe against rRNA (Figure 1D). Relatively similar amount of the rRNA was depicted in all samples, which means the endogenous contents of the cells were not altered. Collectively, these data confirms the previous study (Kim et al., 2011) and that PAM and ZOL inhibit proliferation of NHOKs.

Microarray gene profiling for differentiated genes in BP-treated NHOKs

In order to identify the genes that are altered when NHOKs are treated with BPs, cDNA microarray analysis was performed (Figure 2A). In ZOL-treated NHOKs, 480 genes were dysregulated compared to the control NHOKs. In PAM-treated NHOKs, 383 cells showed differentiated expression compared to the control. 280 genes were differentiated in both samples (Figure 2B). Out of those 280 genes, several genes, such as CDC20, CDC6, CCNA2, CCNB1, E2F8, and CDC2, which are related to cell cycle regulation showed decreased expression, whereas genes that are more involved in structural formation, such as MMP10, LCE3E, MAFB,

CLITRK6, LCE3D, and IFIT1, showed increased expression (Table 1). The values indicate the fold induction of the expression. Negative values indicate down-regulated gene expressions.

BPs decrease the expression of genes associated with cell cycle

In order to further validate the values of the microarray analysis in Table 1, qRT-PCR was conducted, using 10 μM PAM and 4 μM ZOL treated NHOKs. CDC20, CDC6, CCNA2, CCNB1, E2F8, CDC2, HELLS, FOXM1, BRCA2, and RBL1 mRNA expressions were decreased (Figure 3A and 3B), whereas MMP10, LCE3E, MAFB, CLITRK6, LCE3D, and IFIT1 mRNA expressions were increased (Figure 3C). These results confirm the validity of the microarray data. Moreover, the decreased expression of the cell-cycle regulation genes correlates with the findings of our previous study (Kim et al., 2011) that BPs may be involved in cell-cycle alterations in NHOKs.

BPs alter G1/S phase in cell cycle of NHOKs

Functional analysis of the differentiated genes in PAM and ZOL-treated NHOKs was performed using the microarray data (Figure 4). The black bar indicates the p-value of the each category and the gray points indicate the ratio of the p-values among the genes in each category. Low p-value indicates high statistical significance. The data indicate that among the differentiated genes in PAM and ZOL-treated NHOKs, a significant number of the genes are functionally involved in G1/S phase of cell cycle regulation.

BPs inhibit the expression of Cyclin A2 in NHOK in dose- and time-dependent manner

G1 and S phases are involved in the DNA duplication, promoting the cellular proliferation. Therefore, the expression of proteins that are involved in the G1 and S phases of

the cell cycle were tested, using the BP-treated NHOKs. The expression levels of cell-cycle proteins, such as Cyclin-Cdk complex molecules for G_1/S transition, were analyzed along with PCNA, which can serve as a proliferation marker, by Western blot (Figure 5A and 5B). First, NHOKs were treated with 10 μM PAM for 2 and 4 days or treated 10 μM and 50 μM PAM for 4 days. This was to monitor if the expression of the proteins are time- and/or dose- dependent. The levels of PCNA seemed to decrease in time and dose-dependent manners, which supports the theory that the cells, when treated with BPs, may undergo cell-cycle arrest. Interestingly, there seemed to be no change in the expression levels of Cyclin D1 and Cdk6, which regulates the transition from G1 to S phase, and also in Cdk2, which regulates S phase with Cyclin A2 as a part of Cyclin A2-Cdk2 complex.

Cyclin A2 is one of the essential cell-cycle proteins that control the S phase. Western blot result showed a significant decrease in the expression of Cyclin A2 in the cells treated for Day 2, and even more so for Day 4 (Figure 5A). However, there is no significant difference between the expression of Day 2 and Day 4, which indicates that the effect of the BPs can be immediate. Also, there was a significant decrease in Cyclin A2 expression in NHOKs that were treated with PAM for 4 days in different doses of 10 μM and 50 μM PAM (Figure 5B). Again, even though less expression of Cyclin A2 in higher dosage of PAM was shown, there was no significant difference in expression between 10 and 50 μM PAM- treated cells, which suggests that as small as the amount of 10 μM PAM can bring to the optimal effect.

When NHOKs were treated with ZOL in 1μM and 2μM, there was a decrease in the expression of Cyclin A2 as the dosage increased, which suggests that ZOL also effect Cyclin A2 expression in NHOK cells as PAM does in a dose-dependent way (Figure 5C). This data suggests that Cyclin A2 maybe the primary target of BPs to reduce the proliferation of oral

mucosa cells during the S-phase of the cell cycle. The overall data suggest that BPs inhibit the expression of Cyclin A2 in NHOK in time- and dose-dependent ways.

BPs inhibit the expression of Cyclin A2 in NHOK at the transcriptional level

In order to check the possibility of BPs affecting the Cyclin A2 at the post-transcriptional level, RT-PCR was performed with the NHOKs that were treated with PAM at different time and dosage. Similarly to the previous Western blot analysis, the mRNA level of Cyclin A2 decreased in time- and dose-dependent ways (Figure 6A and B) as NHOKs were treated with PAM. This leads to the possibility of BPs' involvement in the transcription of CCNA2.

BPs interact at the CCNA2 promotor region, transcriptionally regulating the gene expression

In order to validate whether BPs down regulates the CCNA2 expression at the promoter, luciferase assay was performed. Using the constructed plasmid with CCNA2 promoter, luciferase assay was performed with NHOK that were treated with different dosages of PAM (10μM, 50μM, and 100μM) and ZOL (2μM and 4μM). The luciferase assay showed relatively 4- to 5-fold reductions in luciferease activity in PAM- and ZOL-treated NHOK (Figure 7), supporting the notion that Cyclin A2 expression is indeed transcriptionally regulated by BPs.

BPs inhibit the expression of CCNA2 in NHOKs, but not NHOFs

In the previous study, BPs were shown to target specifically to NHOKs (Kim et al., 2011), and not normal human oral fibroblast (NHOFs). In order to check if the effect of BPs on CCNA2 expression is also NHOK-specific, the levels of Cyclin A2 in BP-treated NHOF were tested using RT-PCR (Figure 8A) and Western blot analysis (Figure 8B). When NHOFs were treated with 10 μM PAM, there was no change in Cyclin A2 expression in NHOF at both post-

transcriptional and post-translational levels. Also, when NHOKS and NHOFs were treated with1μM and 2μM ZOL, CCNA2 levels in NHOKs decreased in dose-dependent manner, whereas no change was noted in ZOL-treated NHOFs (Figure 8C). When the luciferase promoter assay was performed on 2μM ZOL-treated NHOFs, no significant difference in the promoter activity was shown, compared to the control (Figure 8D). Therefore, the inhibition of CCNA2 expression by BPs is specific to NHOKs at the transcriptional level, which correlates with the earlier findings (Kim et al., 2011).

Inhibition of Cyclin A2 expression in NHOK in BP-treated tissues

Immunohistochemical staining was performed to see the Cyclin A2 expression in the 4 μM ZOL-treated and –untreated oral mucosa tissue construct (Figure 9). 3-dimensional (3D) oral mucosal wound healing model was established using the organotypic raft culture system. Five days after airlifting the transwell, the oral mucosal tissue constructs were fed with medium only (CTL) or medium containing 4μM ZOL. The oral tissue constructs were harvested two weeks after air-lifting, and subjected to immunohistochemistry staining against IgG (1:200) and Cyclin A2 (1:200) (bottom panel). Stained tissues were developed using the 3,3'-diaminobenzidine (DAB) chromogen substrate and counterstained with hematoxylin. There is a reduction in the epithelial layer in the ZOL-treated tissues, compared to the untreated sample which supports the figure. In correlation to the previous data, the inhibition of Cyclin A2 by ZOL was demonstrated using 3D oral mucosal constructs as determined by immunohistochemistry.

DISCUSSION

BP's mechanisms have been widely studied, primarily focused on its affect on bone cells, due to its use in treating osteoporosis and cancer therapy. Osteoclasts are unique cells that have the ability to resorb bone and therefore have become a major target in bone disease treatment modalities. BP is known to interact with osteoclasts. BPs suppress bone turnover via interference with the internal enzymatic cell system of osteoclasts leading to cytoskeletal disruption. This mechanism found its clinical relevance in reducing bone resorption, stabilizing bone mass and reducing fracture risk in osteoporosis patients (Carano et al., 1990). Furthermore, changes in osteoclast morphology and function, similar to osteoclast apoptosis, were detected when BP was given to osteoporosis patients (Jobke et al., 2014). The recent appearance of BRONJ as a side effect of using BP in high dosage heightened the focus of the BP study on hard tissues.

Fractured jaw is the secondary result of the impaired wound healing around the BRONJ. In despite of the risk of having BRONJ as a side-effect, BPs are clinically determined to be administered on patients because of the ability to suppress bone resorption and anti-tumor effect (Migliorati et al, 2010). However, the clinical management of BRONJ is still uncertain, and complete treatment is not currently available. Because osteoclast activity is essential for bone remodeling and healing, it has been hypothesized that inhibition of osteoclasts' functions may slow down the wound healing process of the jaw, leading to BRONJ. However, there is no difference in healing of fractured bone other than jaw with or without BPs (Lyles et al., 2007), which brings the possibility that BP's inhibitory effect on osteoclasts alone may be not be sufficient to explain the source of the side effect.

BRONJ involves impaired wound healing of soft tissues. Wound healing process in oral environment involves several cell types and is complex. There are several supporting evidence that defected oral mucosa may play a role in ONJ pathogenesis. In vitro, BP has been shown to inhibit the proliferation, migration, and adhesion of mature endothelial cells, fibroblasts, epithelial cells, vascular smooth muscle cells and oral keratinocytes (Santini et al., 2003). Our recent study also showed that BP may localize to the outer-layer of the epithelium and cause soft-tissue toxicity (Bae et al., In Press).

In this study, we propose that BPs alter the regulation of the genes that are involved in the cell cycle, primarily CCNA2 during the S phase. Our study demonstrates that BPs negate the normal properties of NHOK by showing the following: 1) The inhibition of proliferation of NHOK by PAM and ZOL treatment, 2) Decreased gene expression of the cell cycle regulatory genes, including CCNA2, 3) Decreased expression of CCNA2 by BPs in NHOK at the transcriptional level in dose- and time-dependent manner, 4) decreased CCNA2 promoter activity by BPs in NHOK, 5) BP-CCNA2 transcript interaction in NHOK, not NHOF, and 6) decreased CCNA2 expression of oral mucosa cells ex-vitro.

Microarray assay showed highly down regulated genes that are involved in cell cycle in BP-treated cells. Specifically, the genes regulating S phase seemed to be differentiated by BPs more so than other genes in the other stages of the cell cycle. Interestingly, our previous study showed S phase-arrest in BP-treated NHOK (Kim et al., 2011). The microarray data support and confirm our previous finding that BPs have effect on S phase during the cell cycle of NHOK and be the possible cause of delayed wound healing process.

NHOK is highly involved in wound healing process of the oral mucosal tissues. Proliferation of NHOK during the reepithelialization is one of the critical stages, which needs to be maintained during the wound healing process (Raja et al., 2007). During the S phase, cells undergo DNA replication, which ultimately leads to the proliferation of the cells. Cyclin A2 is one of the main proteins that regulates and facilitates the S phase of the cell cycle. The BP-treated NHOK consistently showed the decreased expression of Cyclin A2 throughout the study, which supports the previous discovery (Li et al., 2011) that BPs elicit cell-cycle alteration that seems to be associated with changes in the levels of certain cyclins and cyclin-related regulatory proteins.

Interestingly, BP's interaction with Cyclin A2 is seemed to be NHOK-specific, which suggests that BPs not only localize in the outer epithelial layer in the soft tissues (Bae et al., In Press), but also selectively effect those cells. The decreased Cyclin A2 expression in the BP-treated oral mucosa tissue construct (Figure 9) further shows the localized effect of BP on NHOK. The steady data of the decreased expression of the Cyclin A2 in NHOK by BPs, not in NHOF, suggest that BPs may prevent the wound healing of the oral mucosal tissue by affecting the proliferation stage of the NHOK through the alteration of the S phase. A schematic representation of BP's role in NHOK proliferation is portrayed in Figure 10.

In conclusion, this study provides novel information on the interaction between BP and Cyclin A2 in NHOK through microarray gene expression profiling. Our findings are the evidence for plausible mechanism underlying defected wound healing and soft tissue toxicity caused by BP treatments.

FIGURE LEGENDS

Figure 1. BPs inhibit proliferation of NHOKs

(A) A schematic design of the experiments. **(B)** NHOKs were seeded with 2 X 10^4 in 6-well plates and treated with 10 μM PAM and 4 μM ZOL for 4 days. Each sample size is tripled. One dish from each sample is selected and the cells were photographed at 100X. **(C)** After 4 days from seeding, control and BP-treated NHOKs were counted and averaged. **(D)** RT-PCR was performed to quantify and confirm the internal control of each sample by probing against rRNA.

Figure 2. Microarray gene profiling for differentiated genes in BP-treated NHOKs

(A) cDNA microarray was performed on 10 μM PAM and 4 μM ZOL treated NHOKs. The top right indicates the genes with decreased expressions, whereas the bottom right refers to the genes with increased expression, in both PAM and ZOL treated cells, compared to the control. **(B)** A Van-diagram showing the number of differentiated genes from each sample. Out of 480 differentiated genes in ZOL treated cells, 280 genes were also appeared to be differentiated in 383 differentiated genes in PAM treated cells.

Figure 3. BPs decrease the expression of genes associated with cell cycle

In order to validate the values of the microarray analysis in Table 1, qRT-PCR was conducted, using 10 μM PAM and 4 μM ZOL treated NHOKs. CDC20, CDC6, CCNA2, CCNB1, E2F8, CDC2, HELLS, FOXM1, BRCA2, and RBL1 mRNA expressions were decreased **(A and B)**, whereas MMP10, LCE3E, MAFB, CLITRK6, LCE3D, and IFIT1 mRNA expressions were increased **(C)**.

Figure 4. BPs alter G1/S phase in cell cycle of NHOKs

Functional analysis of the differentiated genes in PAM and ZOL-treated NHOKs was performed using the microarray data from Figure 2A. The black bar indicates the p-value of the each category and the gray points indicate the ratio of the p-values among the genes in each category. Low p-value indicates high statistical significance.

Figure 5. BPs inhibit the expression of Cyclin A2 in NHOK in dose- and time-dependent manner

Western blot was performed against PCNA, Cyclin D1, Cdk6, Cyclin A, Cdk2, and β-Actin, using both NHOKs that were treated with 10μM PAM for 2 and 4 days **(A)**, and the cells that were treated with 10μM and 50μM for 4 days **(B)**. B-Actin served as a loading control. **(C)** NHOK treated with 1μM and 2μM ZOL were harvested for Western blot analysis against Cyclin A2, using β-Actin as a loading control.

Figure 6. BPs inhibit the expression of Cyclin A2 in NHOK at the transcriptional level

(A)NHOKs were treated with 10 μM PAM for 2 and 4 days and harvested. Total RNAs were isolated, cDNAs were synthesized, and RT-PCR was performed for the expression of Cyclin A2, using GAPDH as loading control. **(B)**NHOKs were treated with 10 and 50 μM PAM for 4 days, and cells were harvested for RT-PCR. β-Actin was probed for a loading control.

Figure 7. CCNA2 promotor luciferase reporter assay in NHOK treated with BPs

PALUC, which is a construct carrying the full promoter fragment of cyclin A (Henglein et al., 1994), and pGL3-Basic were double digested with XhoI and SacI. The cyclin A promoter insert (608bp from -516 to +92) from the PALUC was then ligated and cloned into the pGL3 vector, forming the cyclin A promoter/luciferase construct, pGL3-CCNA2p. NHOK cells were transfected with pGL3-CCNA2p to perform luciferase reporter assay. After 24 hours, the cells

were treated with PAM (10μM, 50μM, and 100μM) and ZOL (2μM and 4μM) and incubated for additional 24 hours. Cells were harvested to measure luciferase activity. The pRL-SV40 plasmid was also transfected to normalize the transfection efficiency.

Figure 8. BPs inhibit the expression of CCNA2 in NHOK, but not NHOF

NHOFs, treated with 10 μM PAM, were harvested for RT-PCR **(A)** and Western blot analysis **(B)** against Cyclin A2, using β-Actin as a loading control. **(C)** NHOKs and NHOFs treated with 1μM and 2μM ZOL were harvested for Western blot analysis against Cyclin A2, using β-Actin as a loading control. **(D)** NHOFs were transfected with pGL3-CCNA2p to perform luciferase reporter assay. After 24 hours, the cells were treated with 2μM ZOL and incubated for additional 24 hours. Cells were harvested to measure luciferase activity. The pRL-SV40 plasmid was also transfected to normalize the transfection efficiency.

Figure 9. Inhibition of Cyclin A2 expression in NHOK in BP-treated tissues

Immunohistochemical staining was performed to see the Cyclin A2 expression in the 4 μM ZOL-treated and –untreated oral mucosa tissue construct. The antibody for Cyclin A2 was used in 1:200. IgG was used as a negative control. Stained tissues were developed using the 3,3'-diaminobenzidine (DAB) chromogen substrate and counterstained with hematoxylin.

Figure 10. Summary of the role of BP in CCNA2 regulation

A schematic summary of interaction of BP with CCNA2 transcript.

Table 1. Microarray analysis: list of fold induction of differentiated genes in BP treated NHOKs

FIGURE 1

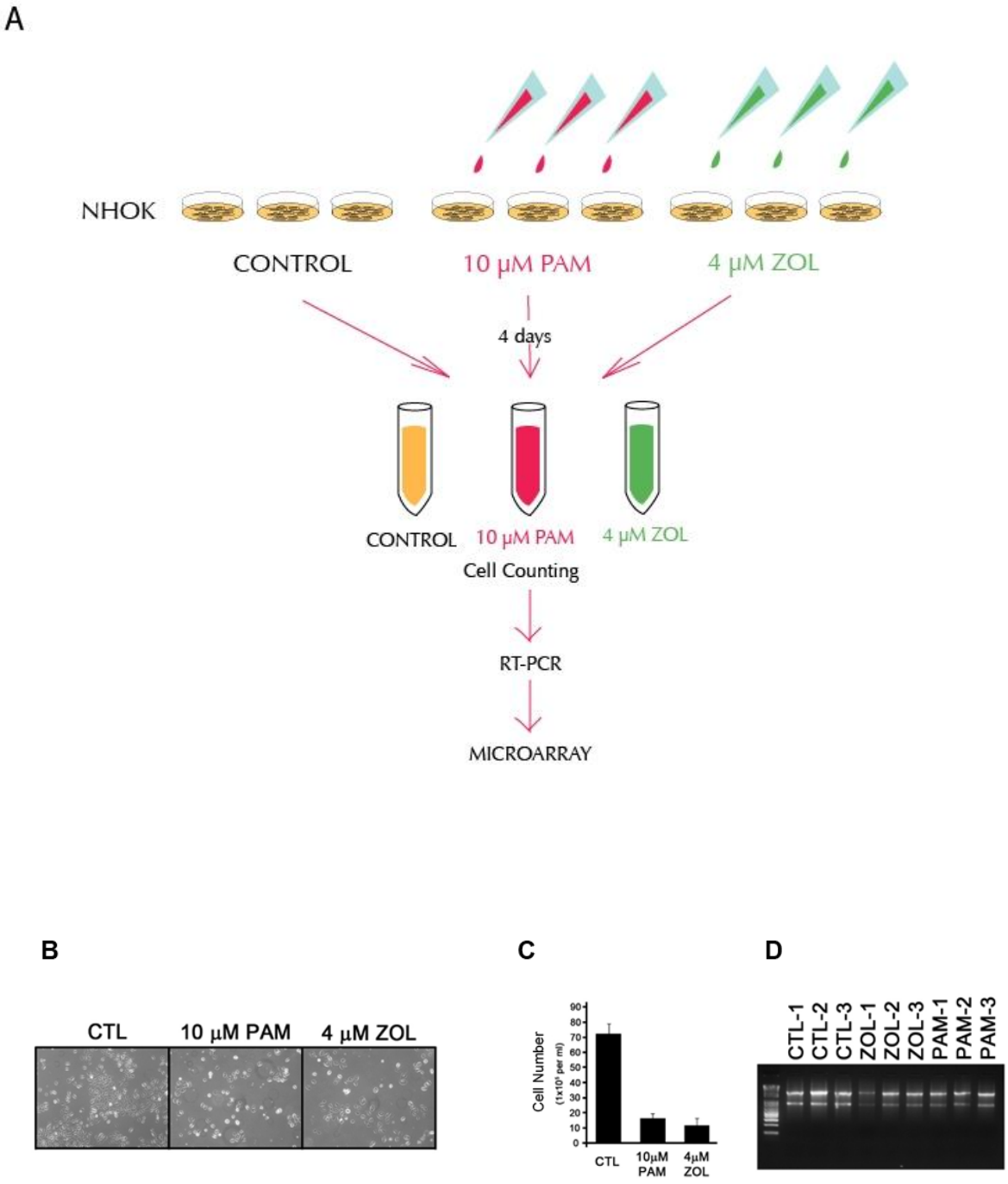

A
NHOK
CONTROL
10 µM PAM
4 µM ZOL
4 days
CONTROL
10 µM PAM
4 µM ZOL
Cell Counting
RT-PCR
MICROARRAY
B
CTL
10 µM PAM
4 µM ZOL
C
Cell Number
(1x10⁵ per ml)
CTL
10µM PAM
4µM ZOL
D
CTL-1
CTL-2
CTL-3
ZOL-1
ZOL-2
ZOL-3
PAM-1
PAM-2
PAM-3

FIGURE 2

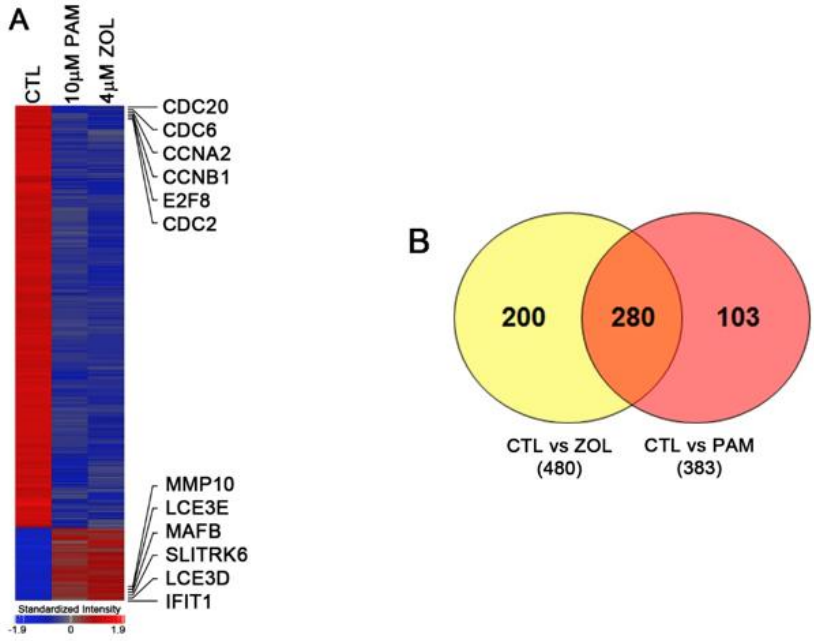

A
CTL
10μM PAM
4μM ZOL
CDC20
CDC6
CCNA2
CCNB1
E2F8
CDC2
MMP10
LCE3E
MAFB
SLITRK6
LCE3D
IFIT1
Standardized Intensity
-1.9
0
1.9
B
200
280
103
CTL vs ZOL
(480)
CTL vs PAM
(383)

FIGURE 3

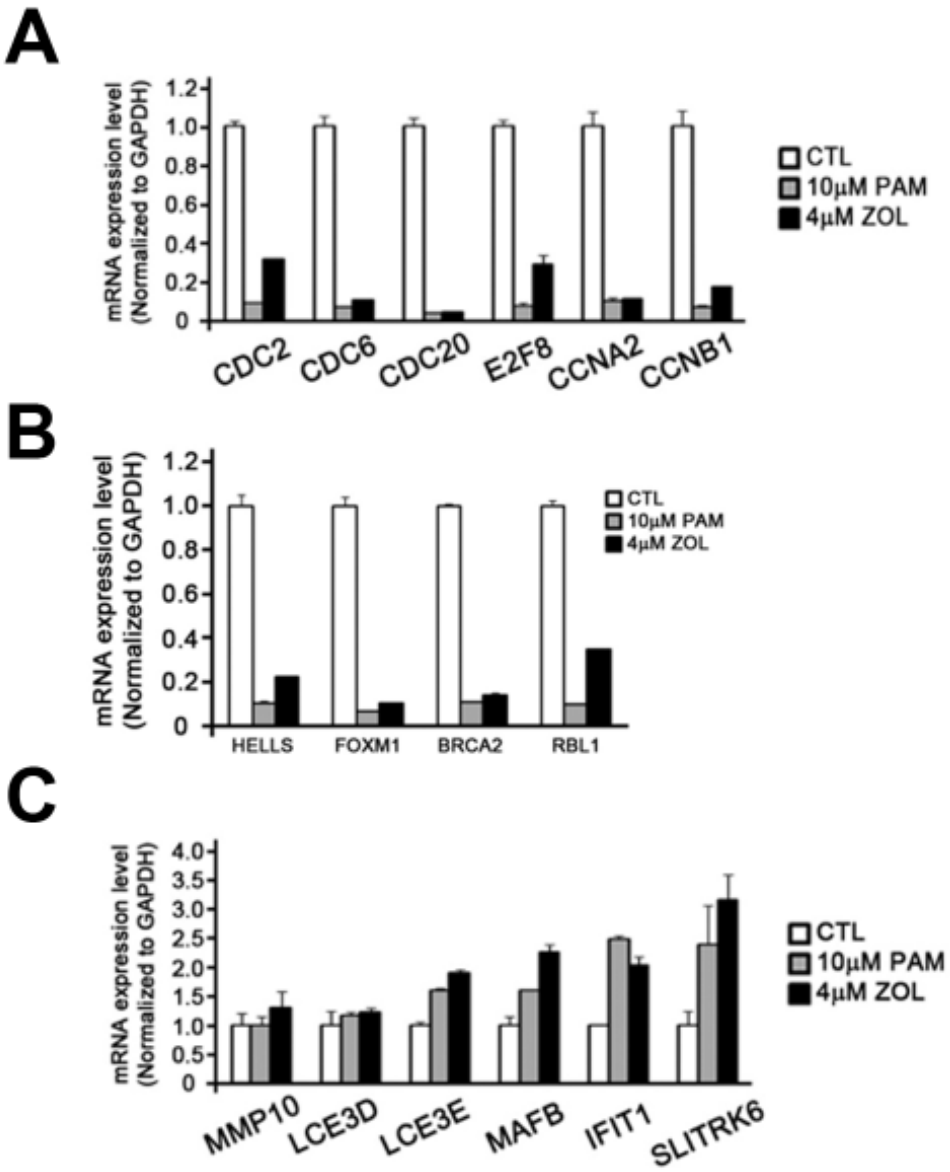

A
mRNA expression level (Normalized to GAPDH)
1.2
1.0
0.8
0.6
0.4
0.2
0
CDC2
CDC6
CDC20
E2F8
CCNA2
CCNB1
CTL
10μM PAM
4μM ZOL
B
mRNA expression level (Normalized to GAPDH)
1.2
1.0
0.8
0.6
0.4
0.2
0
HELLS
FOXM1
BRCA2
RBL1
CTL
10μM PAM
4μM ZOL
C
mRNA expression level (Normalized to GAPDH)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
MMP10
LCE3D
LCE3E
MAFB
IFIT1
SLITRK6
CTL
10μM PAM
4μM ZOL

FIGURE 4

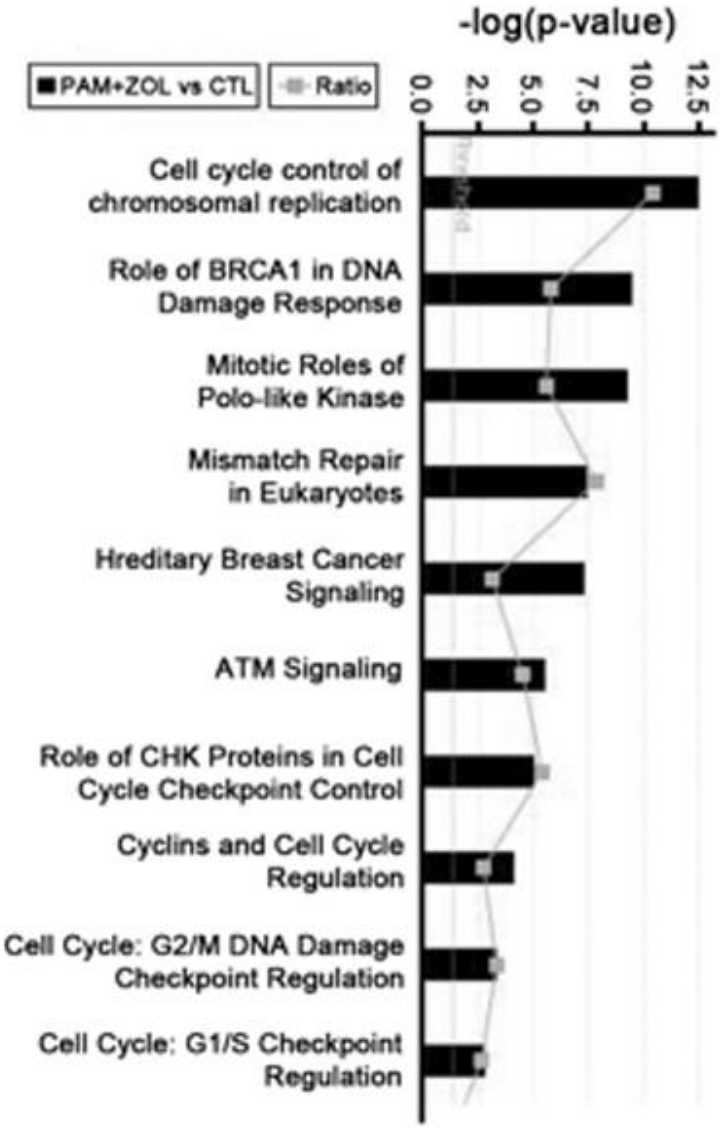

-log(p-value)
PAM+ZOL vs CTL
Ratio
0.0
2.5
5.0
7.5
10.0
12.5
Cell cycle control of chromosomal replication
Role of BRCA1 in DNA Damage Response
Mitotic Roles of Polo-like Kinase
Mismatch Repair in Eukaryotes
Hreditary Breast Cancer Signaling
ATM Signaling
Role of CHK Proteins in Cell Cycle Checkpoint Control
Cyclins and Cell Cycle Regulation
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
Cell Cycle: G1/S Checkpoint Regulation

FIGURE 5

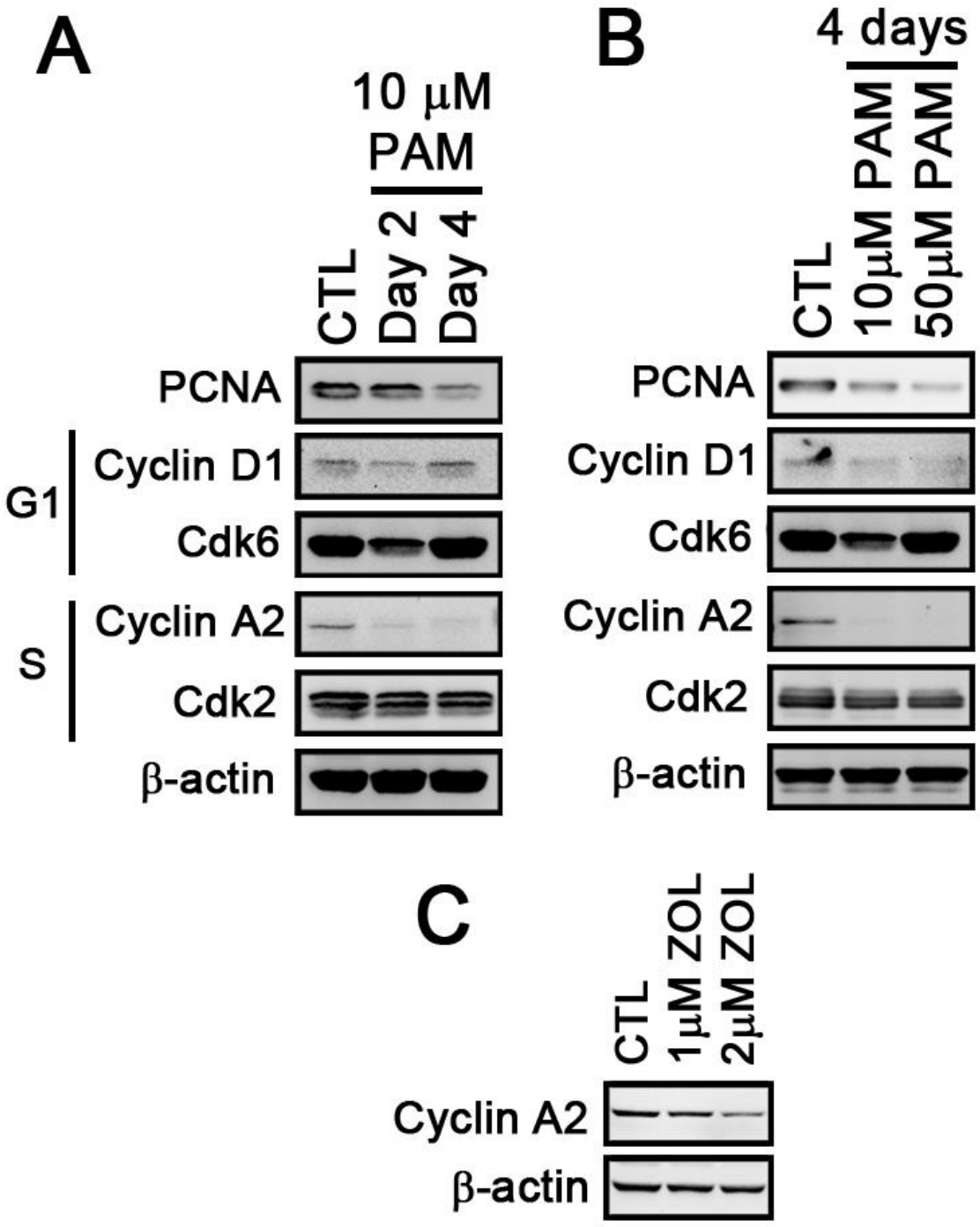

A
10 μM PAM
CTL
Day 2
Day 4
PCNA
G1
Cyclin D1
Cdk6
S
Cyclin A2
Cdk2
β-actin
B
4 days
CTL
10μM PAM
50μM PAM
PCNA
Cyclin D1
Cdk6
Cyclin A2
Cdk2
β-actin
C
CTL
1μM ZOL
2μM ZOL
Cyclin A2
β-actin

FIGURE 6

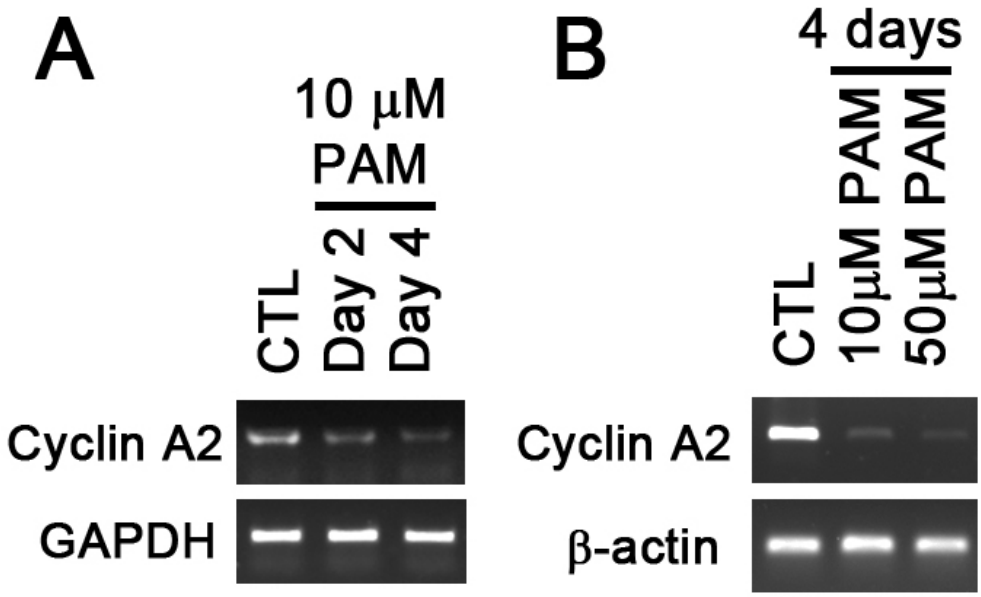
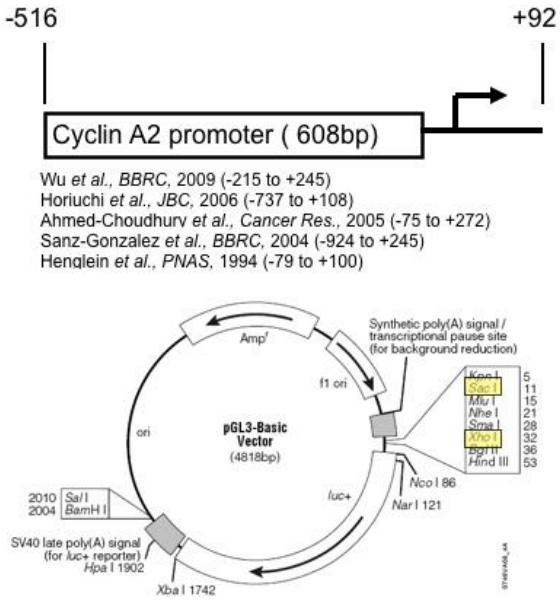

A
10 μM PAM
CTL
Day 2
Day 4
Cyclin A2
GAPDH
B
4 days
CTL
10μM PAM
50μM PAM
Cyclin A2
β-actin

FIGURE 7


-516
+92
Cyclin A2 promoter (608bp)
Wu et al., BBRC, 2009 (-215 to +245)
Horiuchi et al., JBC, 2006 (-737 to +108)
Ahmed-Choudhury et al., Cancer Res., 2005 (-75 to +272)
Sanz-Gonzalez et al., BBRC, 2004 (-924 to +245)
Henglein et al., PNAS, 1994 (-79 to +100)
Amp^r
f1 ori
Synthetic poly(A) signal / transcriptional pause site (for background reduction)
ori
pGL3-Basic Vector (4818bp)
Kpn I 5
Sac I 11
Mlu I 15
Nhe I 21
Sma I 28
Xho I 32
Bgl II 36
Hind III 53
Nco I 86
Nar I 121
luc+
2010 Sal I
2004 BamH I
SV40 late poly(A) signal (for luc+ reporter)
Hpa I 1902
Xba I 1742

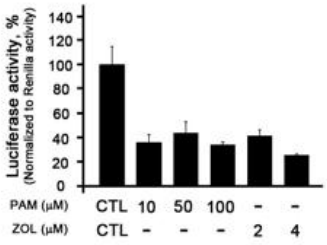

Luciferase activity, %
(Normalized to Renilla activity)
140
120
100
80
60
40
20
0
PAM (μM) CTL 10 50 100 - -
ZOL (μM) CTL - - - 2 4

FIGURE 8

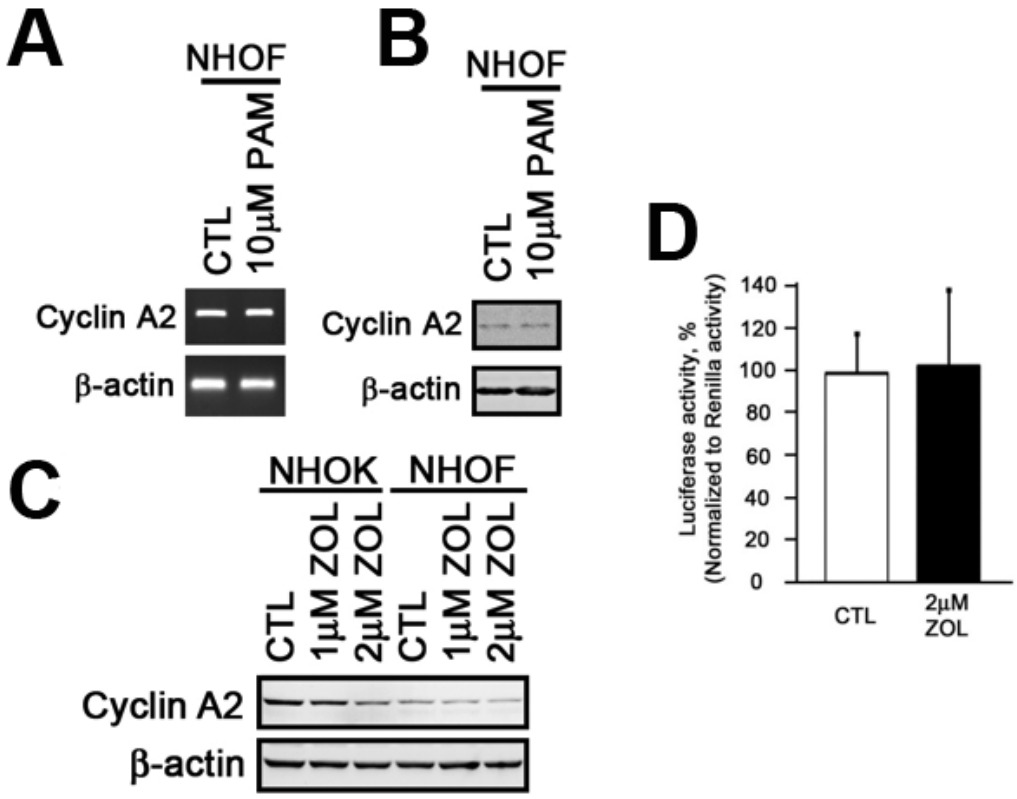

A
NHOF
CTL
10μM PAM
Cyclin A2
β-actin
B
NHOF
CTL
10μM PAM
Cyclin A2
β-actin
C
NHOK
NHOF
CTL
1μM ZOL
2μM ZOL
CTL
1μM ZOL
2μM ZOL
Cyclin A2
β-actin
D
Luciferase activity, %
(Normalized to Renilla activity)
140
120
100
80
60
40
20
0
CTL
2μM
ZOL

FIGURE 9

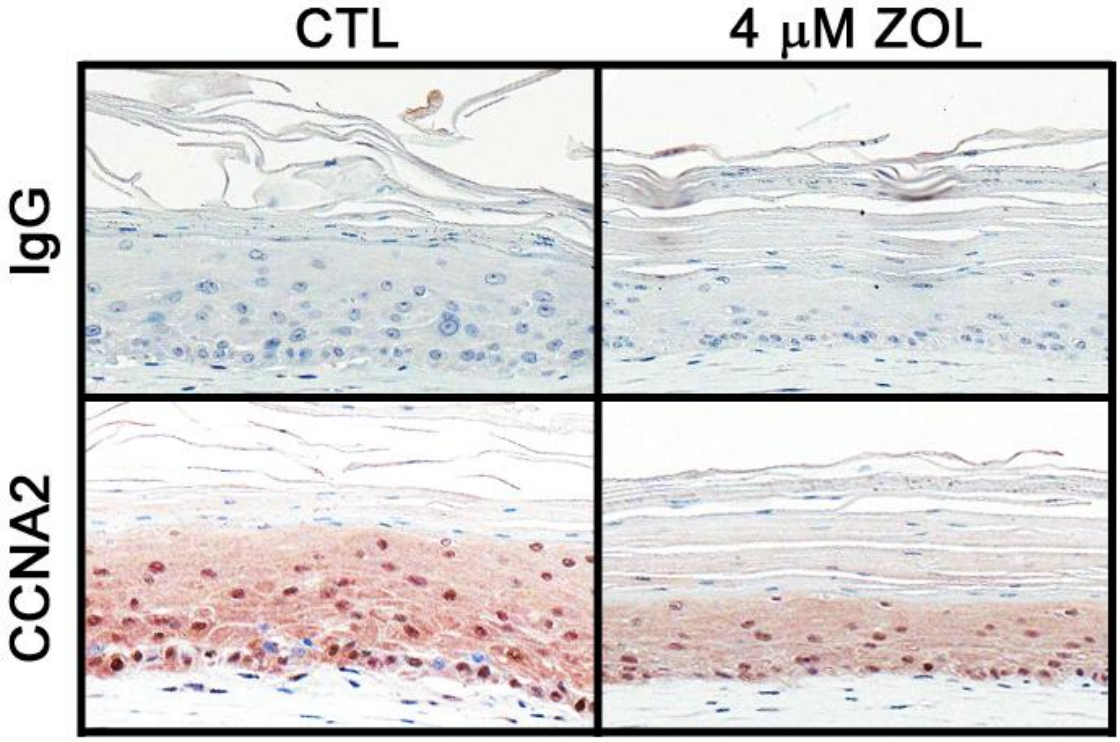


FIGURE 10

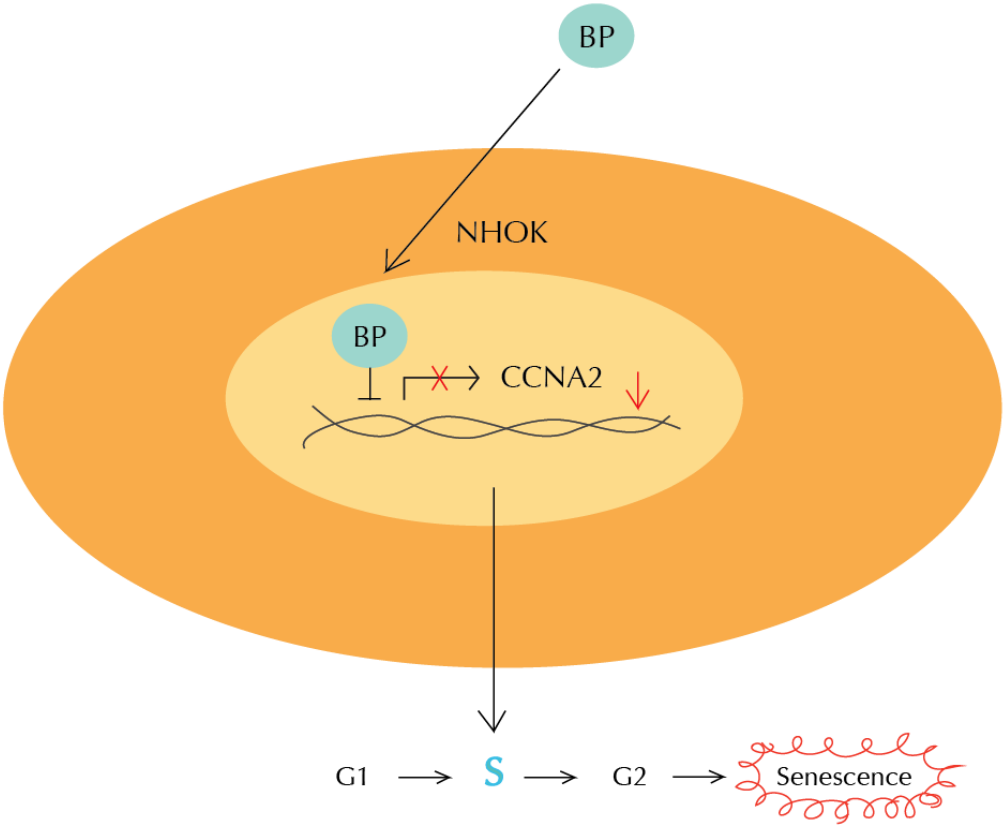


TABLE 1

Gene Symbol	Gene Title	CTL vs. P/Z	CTL vs. P	CTL vs. Z
CLDN11	Claudin 11	-7.48	-7.34	-7.62
CDC20	Cell division cycle 20 homolog	-4.59	-3.77	-5.58
CDC6	Cell division cycle 6 homolog	-4.08	-3.83	-4.36
CDKN3	Cyclin-dep kinase inhibitor 3	-3.89	-3.37	-4.49
LMNB1	Lamin B1	-3.82	-3.41	-4.27
CCNA2	Cyclin A2	-3.78	-3.53	-4.05
CCNB1	Cyclin B1	-3.44	-3.14	-3.77
EPCAM	Epithelial cell adhesion molecule	-3.44	-3.64	-3.24
E2F8	E2F transcription factor 8	-3.37	-3.24	-3.51
HMGB2	High-mobility group box 2	-3.10	-2.95	-3.26
CDC2	Cell division cycle 2	-2.87	-3.03	-2.71
FOXM1	Forkhead box M1	-2.72	-2.60	-2.85
HNRNPD	Hn ribonuclearprotein D	-2.57	-2.30	-2.88
CDKN2C	Cyclin-dep kinase inhibitor 2C	-2.50	-2.32	-2.68
RBL1	Retinoblastoma-like 1 (p107)	-2.28	-2.31	-2.25
FOXA2	Forkhead box A2	-2.26	-2.37	-2.15
BRCA2	Breast cancer 2, early onset	-2.25	-2.31	-2.20
RAD51	RAD51 homolog	-2.21	-2.12	-2.30
MSH2	musS homolog 2	-2.18	-2.17	-2.18
EZH2	Enhancer of zeste homolog 2	-2.18	-2.17	-2.18
RAD54B	RAD54 homolog B	-2.16	-2.15	-2.17
TP53INP2	p53 inducible nuclear protein 2	2.13	2.01	2.24
CD24	CD24 molecule	2.19	2.22	2.16
TNFSF10	TNF superfamily member 10	2.43	2.45	2.40
MMP10	Matrix metallopeptidase 10	2.24	2.25	2.22
TP53INP1	p53 inducible nuclear protein 1	2.37	2.35	2.39
SOX4	SRY-box 4	2.59	2.63	2.54
FN1	Fibronectin 1	2.90	2.70	3.11
IL6	Interleukin 6	3.34	2.95	3.79

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