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# Early whole-genome transcriptional response induced by benzo[a]pyrene diol epoxide in a normal human cell line

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# ABSTRACT

(±)-*anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) is a carcinogen causing bulky-adduct DNA damage. In this study, we investigated early transcriptional signatures induced by various concentrations (0.005, 0.05, and 0.5  $\mu$ M) of this carcinogen in a normal human cell line (FL human amnion epithelial cells) using the whole-genome Affymetrix HG-U133 Set microarray. The numerous identified genes were involved in multiple functions and higher doses of BPDE elicited more robust expression changes. The disturbance of genes involved in cell cycle regulation, growth and apoptosis was correlated with the S and G<sub>2</sub>/M phase cell cycle arrest and cytotoxic phenotypes induced by different levels of BPDE. Bioinformatic analysis showed that several transcription factors and their related stress signaling pathways might partly account for the transcriptional signature induced by BPDE. Additionally, gene ontology analysis of the microarray data showed down-regulation of transport, cytoskeleton and DNA repair by 0.5  $\mu$ M BPDE exposure. In conclusion, this genomic analysis helps to understand the mechanism of cellular response to BPDE.

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## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants that have been found in cigarette smoke and charred food, as well as the exhaust from internal combustion engines and coal-burning factories [1]. Benzo[a]pyrene (BaP) is one of the most widely studied PAHs. It is metabolized by cytochrome P450 s to form the ultimate carcinogen, (±)-anti-benzo[a]pyrene-7,8-diol-9,10epoxide (BPDE). BPDE can bind covalently to deoxyribonucleic acids (DNAs) or non-covalently intercalate into double-stranded DNA, which result in bulky-adduct DNA damage and conformational abnormalities, respectively [2]. BPDE preferentially reacts with the N<sup>2</sup> position of deoxyguanosine residues to form the dG-N<sup>2</sup>-BPDE major adduct, and principally induces a G:C to T:A transversion mutation [3]. This point mutation is consistently found at the hotspot codons on the p53 gene in lung cancers from smokers but not from nonsmokers, thus implicating BPDE as the direct carcinogen accounting for cigarette smoking-induced lung cancers [4].

In recent years, genomic tools represented by microarrays have been implemented into traditional toxicology to form toxicogenomics, which involves many applications based on gene profiles, and in the

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end helps to understand the complex gene–toxicant interactions. For instance, gene signatures in exposure to ionizing radiation (IR) [5] and ultraviolet radiation (UV) [6] have provided insight into mutagenesis and stress response induced by these classical genotoxic agents, respectively. Gene profiling studies on BPDE have also been performed in different cell types and treatment models [7–12]. The alteration of genes involved in p53 pathway, cell cycle, cell growth, apoptosis, DNA repair, glutathione detoxification pathway and inflammation etc. have been identified, and helps to explain the cell cycle-arrest, mutagenic, cytotoxic and pro-inflammatory effect of BPDE in the related models. However, the previous *mRNA* transcriptomic studies on BPDE did not reach the whole-genome level in their used microarray or rapid analysis of gene expression (RAGE) technologies. This drawback impeded obtaining full-scale transcriptional responses induced by BPDE.

We thus used the whole-genome Affymetrix HG-U133 Set microarray that covers ~33,000 human genes and ESTs to explore responsive genes after exposure to different doses of BPDE in human amnion epithelial FL cells. We have used FL cell line as an *in vitro* model to study low doses of environmental chemical pollutantsinduced responses in normal human cells for the exposure dosage to them is usually low in human daily life [1,7,13–15]. We performed genomic analysis at an early 4-h time point after BPDE exposure for early cellular changes could be useful indicators of the harmful exposure and help to understand the underlying mechanisms of chemical-caused damages, also be convenient to compare the BPDE-induced transcriptional responses with several previously reported studies being in a similar time-point context [7,8,12]. Through Affymetrix HG-U133 Set microarray analysis, we have characterized



Abbreviations: BPDE, (±)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide; AP-1, activator protein-1; NF-KB, nuclear factor kappa B; ATF, activating transcription factor; CREB, cAMP responsive element binding.

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the gene signatures in response to different doses (0.005, 0.05, and 0.5  $\mu$ M) of BPDE. The numerous genes are involved in multiple functions including cell cycle regulation, proliferation, apoptosis, transcription, metabolism, transport, cytoskeleton and DNA repair etc. Gene ontology analysis of the microarray data using GSEA software has revealed the down-regulation of cell cycle, proliferation, transport, cytoskeleton and DNA repair by 0.5  $\mu$ M BPDE exposure. A medium-throughput quantitative real-time RT-PCR validation based on Taq-Man<sup>®</sup> Low-Density Array has confirmed more than one hundred of gene expression changes. The validated gene sets showed the correlation between gene expression change and the cell cycle and cytotoxicity phenotypes induced by different doses of BPDE, also provided insight into the transcriptional regulation and stress signaling pathways triggered by BPDE damage.

#### **Results and discussion**

#### Early global changes in gene expression after BPDE treatment

Three concentrations of BPDE (0.005, 0.05, and 0.5 µM) were used to treat human amnion epithelial FL cells for the microarray profiling study. 0.005 and 0.05 µM BPDE did not affect cell viability evidently and 0.5 µM BPDE produced a moderate cytotoxicity (88% cell viability) compared with DMSO vehicle exposure as determined by MTT reduction assay (Fig. 1). With Affymetrix HG-U133 Set microarray analysis, we found that the expression of 74, 103, and 2176 probe sets representing 70 (31 up- and 39 down-regulated), 88 (38 up- and 50 down-regulated), and 1707 (307 up- and 1400 downregulated) genes were significantly altered at 4 h after exposure to 0.005, 0.05, and 0.5 µM BPDE, respectively, compared with the vehicle exposure (Supplementary Table 1). These profiles constituted a total of 1764 unique genes that were differentially expressed in at least one of the three concentrations of BPDE treatment. Hierarchical clustering of the 1764 genes based on their fold changes relative to the control was performed to visualize the change patterns with the two biological replicates separated (Fig. 2a). It could be cognized that the microarray profiling replication was acceptable with the replicate samples clustered most close, and the 0.5 µM BPDE provoked much more extensive transcriptional changes than the two lower doses did.

Affymetrix gene ontology annotation showed that the differentially expressed genes were involved in multiple biological functions such as cell cycle regulation, signal transduction, transcription, translation, metabolism, transport, cytoskeleton, DNA repair, and etc. (Supplementary Table 1). We further used Gene Set Enrichment Analysis (GSEA) software to analyze whether some functional categories of genes were significantly enriched in the differentially



**Fig. 1.** Cell viability measured by MTT assay. Human amnion epithelial FL cells were exposed to different concentrations of BPDE for 2 h. At 10 and 22 h after exposure, cell viability for each treatment was determined based on spectrometry of formazan formation, and relative viability represented the viability percentage relative to vehicle exposure. Triplicate experiments were carried out, and  $\bar{x}\pm SD$  was calculated for diagram.

expressed dataset, and coordinately regulated to enhance or impair some cellular activities after BPDE treatment. By searching the mSigDB gene ontology gene set collection, GSEA analysis filtered out 233 gene sets in the 0.5  $\mu$ M BPDE dataset, 1 gene set in the 0.05  $\mu$ M BPDE dataset, and none in the lowest dose dataset. The enriched gene sets in the 0.5  $\mu$ M BPDE group were also mainly involved in cell cycle, proliferation, apoptosis, signal transduction, transcription, RNA processing, protein metabolism, transport, cytoskeleton, and DNA repair etc. (Supplementary Table 2). These identified gene expression changes prominently indicated down-regulation of cell cycle, proliferation, transport, cytoskeleton, and DNA repair (Supplementary Fig. 1).

# Quantitative real-time RT-PCR validation

A medium-scale quantitative real-time RT-PCR validation of the microarray results based on TaqMan<sup>®</sup> Low-Density Array was performed using independently prepared cell samples. Of 35, 47, and 234 responsive genes selected from 0.005, 0.05 and 0.5  $\mu$ M BPDE-treated samples, 7, 16, and 111 genes showed same change trends in their expression as that identified by microarray analysis. Among which, 2, 6, and 57 genes were confirmed with statistical significance (p < 0.05). The validated gene list covers many functional categories, and includes many genes related with cell cycle regulation, proliferation, apoptosis, transcription, metabolism, transport, cytoskeleton, and DNA repair etc. (Table 1 and Fig. 2b).

#### Dose effect of BDPE on cell cycle regulation

Microarray analysis revealed an early alteration in expression of numerous cell cycle-regulating genes after BPDE treatment. A portion of these genes was selected and validated by quantitative RT-PCR (Table 1). Following 0.05 and 0.5 µM BPDE treatment, the expression of H1F0 (H1 histone family, member 0), AURKA (aurora kinase A), CCNB1 (cyclin B1), and CENPA (centromere protein A) was decreased. The down-regulation of H1F0 implied slow down of DNA synthesis [16], and the decreased expression of the other three genes that function in promoting G<sub>2</sub>/M progression indicated G<sub>2</sub>/M blockade [17-19]. Upon 0.5 µM BPDE exposure, the expression of four more genes was affected, among which three genes that act in promoting  $G_2/M$ progression including CDC20 (cell division cycle 20 homolog) [20], KIF14 (kinesin family member 14) [21], and KIF2C (kinesin family member 2C) [22] were down-regulated, and one gene that functions in blocking the onset of mitosis, i.e., PKMYT1 (protein kinase, membrane-associated tyrosine/threonine 1) [23], was up-regulated. These gene expression changes indicate that 0.5 µM BPDE induced a more potently inhibition of  $G_2/M$  progression compared with 0.05  $\mu$ M BPDE treatment. No gene expression changes related to cell cycle regulation was validated by quantitative RT-PCR in 0.005 µM BPDEtreated samples.

Flow cytometry assay was performed to explore the correlation between these gene expression changes and cell cycle phenotype. Cell cycle distributions were recorded at 4, 13, and 22 h post BPDE treatment (Fig. 3). 0.05 µM BPDE elicited S phase delay as early as at 4 h post treatment and caused G<sub>2</sub>/M arrest later as identified at 13 h and 22 h post treatment. 0.5  $\mu$ M BPDE evoked much more severe S phase delay than 0.05 µM BPDE at all three time points. The S phasedelayed cells induced by 0.5 µM BPDE were partially released but still arrested at G<sub>2</sub>/M transition at 22 h post treatment. The G<sub>1</sub> peak diminished at 22 h after 0.5 µM BPDE treatment, indicating a more profound S phase and G<sub>2</sub>/M blockade was triggered compared with 0.05 µM BPDE treatment. The 0.005 µM BPDE seemed to only elicit much milder G<sub>2</sub>/M arrest as late as at 13 and 22 h post treatment. Thus, these data showed the consistency of gene expression change with cell cycle phenotype and the dose effect of BDPE on the downregulation of cell cycle progression.



**Fig. 2.** (a) Hierarchical clustering analysis. Hierarchical clustering of the 1764 genes that differentially expressed in at least one of the three doses of BPDE treatment was performed with the two biological replicates separated. Experimental conditions are on the horizontal axis and affected genes are grouped along the vertical axis. Gene expression changes are colored red for up-regulation or green for down-regulation. The scale of colorbar was ranged from –2 to 2, representing 4-fold change of down and up-regulation, respectively. (b) Enlargement of two clusters (arrow pointed) in the dendrogram. Among which, a dozen genes involved in cell cycle, proliferation and apoptosis, etc. have been validated by quantitative RT-PCR.

In respond to 0.5  $\mu$ M BPDE treatment *CDKN1A* (cyclin-dependent kinase inhibitor 1A (p21, Cip1)) and *CDKN1C* (cyclin-dependent kinase inhibitor 1C (p57, Kip2)) were induced, and *CDK6* (cyclin-dependent kinase 6) was repressed, which would lead to the impairment of G<sub>1</sub>/S transition [24]. However, the simultaneous up-regulation of *CCNE1* (cyclin E1) and *CCNE2* (cyclin E2) could enhance G<sub>1</sub>/S transition [24]. Cell cycle analysis showed that a G<sub>1</sub>/S arrest was not established upon all three doses of BPDE treatment (Fig. 3).

# Responsive genes and related pathways involved in cell growth and apoptosis

Important genes including membrane receptors, signaling molecules, and transcription factors that determine cell fate were affected by  $0.5 \mu$ M BPDE at 4 h post treatment. These included repression of *EGFR* (epidermal growth factor receptor) and *IGF1R* (insulin-like growth factor 1 receptor), both of which belong to single-transmembrane receptor tyrosine kinases (RTK). Physiological binding of ligands to these receptors leads to activation of phosphatidylinositol-3-kinase (PI3K)/Akt, Ras/MAPK (mitogen-activated protein kinase), and phospholipase C (PLC<sub>y</sub>)-mediated pathways, which regulate a wide variety of downstream targets related with cell metabolism, migration, proliferation, differentiation, and survival [25]. Downstream of the PLC $\gamma$ -mediated pathways were also affected. PLC<sub>y</sub> catalyzes PtdIns(4,5)P<sub>2</sub> to generate second messengers including diacylglycerol (DAG) and Ins(1,4,5)P<sub>3</sub> (IP<sub>3</sub>). DAG activates protein kinase C (PKC). IP<sub>3</sub> induces Ca<sup>2+</sup> release from the calcium pool by binding to the ion channel receptor ITPR1 (inositol 1,4,5-triphosphate receptor type 1)/IP3R. The released Ca<sup>2+</sup> binds with calmodulin, thereby activating Ca<sup>2+</sup>/calmodulin-dependent protein kinases (CaMKs) [25]. Upon 0.5 µM BPDE treatment, the expression of PRKCA (protein kinase C, alpha) and ITPR1 were reduced. Thus, the down-

#### Table 1

Validation of gene expression changes at 4 h post BPDE treatment with quantitative real-time RT-PCR

synthetic     Apring     PRE       Regulation of conservations     Apring     Apring     PRE       Regulation of conservations     Apring     Apring     -1.14     -1.15       Regulation of conservations     Apring     -1.14     -1.13     -1.23       Signal restrictures     Constraine risk family protein 2     Constraine risk family protein 2     -1.23     -1.23       Signal restrictures     Constraine risk family protein 2     Constraine risk family protein 2     -1.23     -1.23       Mill Dield     2005 protein 2     Constraine risk family 23, member 3     Mill Dield     -1.25     -1.22       Mill Dield     2010 p. 1, at     Adaptar-velated protein complex 1, signal 1 suburit     AP151     -1.52     -1.23       Mill Dield     2010 p. 1, at     Adaptar-velated protein complex 1, signal 1 suburit     AP151     -1.52     -1.24       Mill Dield     2010 p. 1, at     Adaptar-velated protein complex 1, signal 1 suburit     AP151     -1.25     -1.27       Mill Dield     2010 p. 1, at     Const family for member 3     AP1524     -1.52     -1.52       Mill Diston     2010 p	RefSeq ID	Probe ID	Gene title	Gene		Fold change	
DBS JM PDC     Addition of recurrention     Addition of recurrention       NNL 13207     226900_AH     Addition protein 2     Addition of recurrention       NNL 13207     226900_AH     Chromodomana helicase DNA binding protein 2     GR02     -1.34     -1.35       Signal manufaction     NRL 13207     22690_AL     Cyrchic rich trassmenthates BMP regulator 1 (chordin-like)     CBMP     -1.46     -1.33       MRL 13807     220595_LL     Oblighooth like EGF report commang     DWRP     -1.46     -1.33       MRL 13807     220596_LL     Adapta-related potein complex 1, signa 1 autouit     AP151     -1.52     -1.23       Sidar remoter     MRL 101812     21093_AL     Adapta-related potein complex 1, signa 1 autouit     AP151     -1.52     -1.23       MRL 101812     21093_AL     Adapta-related potein complex 1, signa 1 autouit     AP151     -1.52     -1.23       MRL 101812     21093_AL     Adapta-related potein complex 1, signa 1 autouit     AP151     -1.52     -1.23       MRL 101810     21093_AL     Adapta-related potein complex 1, signa 1 autouit     AW1221     -1.52     -1.23       MRL 101810				symbol	Array	PCR	
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Signed mathematication     228408_3_24     Cynterine rich transmenbrane BMP regulator 1 (chordin-like)     CMIN1     -1.23     -1.20       NNL.19072     22028_1_2.1     Delay inchrine like FCF regret containing     DMPR2     -1.35     -1.20       MNL.19072     22028_1_2.1     Adaptor-related protein complex 1, signa 1 subunit     AP151     -1.52     -1.20       MNL.19072     22158_3_2.4     Adaptor-related protein complex 1, signa 1 subunit     AP151     -1.52     -1.21       MNL.19072     22159_1_2.4     Adaptor-related protein complex 1, signa 1 subunit     AP152     -1.22     -1.71       MNL.19072     20164_1_2.4     Addic (inscine + ich) nuclear phosphoprotein 32 family, member A     AP122A     -1.23     -1.71       MNL.091500     20078_3_2.4T     Arona kines A     AM22A     -1.23     -1.70       MNL.091500     2008_3_1.4     Cyrclin Bit     Cyrclin Bit     -1.70     MXL1999     -1.48     -1.50       MNL.09150     20164_1_1.4     Cyrclin Bit     Cyrclin Bit     -1.70     MXL199     -1.70     MXL199     -1.70     MXL199     -1.70     MXL199     -1.71	NM_001271	228999_at	Chromodomain helicase DNA binding protein 2	CHD2	-1.78	-1.28	
NULDEAL     224885_Lat     Cystein rich transmeniume fMP regulator (1 (mulin-like)     CMU + 1.23     -1.20       NULDEAL     20208_Lat     Delayaotch-like EGF repact contailing     DMER     -1.36     -1.30       NULDEAL     203106_Lat     Adaptor-related protein complex 1. signs 1 subunit     AP157     -1.52     -1.29       Solar François     203106_Lat     Adaptor-related protein complex 1. signs 1 subunit     AP157     -1.52     -1.20       Solar François     203106_Lat     Adaptor-related protein complex 1. signs 1 subunit     AP157     -1.52     -1.21       Solar François     203106_Lat     Adaptor-related protein complex 1. signs 1 subunit     AP157     -1.22     -1.71       NULDOSIG     20404_S.s.#     Cultic flexion-rich) moder phosphoprotein 32 family, member A     AP1521     -1.23     -1.71       NULDOSIG     208078_S.s.#     Culti B1     CUN 41     -1.84     -1.81       NULDOSIG     208078_S.s.#     Certain remember O     IIII     -1.82     -1.82       NULDOSIG     20808_S.#     Certain remember O     IIII     -1.42     -1.53       NULDOSIG     2	Signal transduction						
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Membran trafficting     Adaptor related protein complex 1, signa 1 subunit     P151     -1.52     -1.29*       Solar comport     NNL01023     22020, s., st     Solar carrier family 25, member 37     SL225A37     1.95     1.21       0.05 JM HPD Regulation of romacription     20043, s. st     Addie (location-sich) nuclear phosphoprotein 32 family, member A     AM92A     -1.23     -1.71       0.05 JM HPD Regulation of romacription     Constitus benneebox 1     COD4     -1.24     -1.72       NL.000305     20043, s. st     Addie (location-sich) nuclear phosphoprotein 32 family, member A     AM92A     -1.23     -1.71       NL0030500     20079, s. st     Aurora kinase A     410000     -1.80     -1.80       NL0104611     200456, s. st     Centromere protein A     CD14     -1.32     -1.35       NL010461     200456, s. st     Cystein erich transmembrane BMP regulator 1 (chordin-like)     CRMH     -1.32     -1.41       NL010461     200450, s. st     Cystein erich transmembrane BMP regulator 1 (chordin-like)     CRMH     -1.32     -1.42       NL010461     200450, s. st     Periodio famosphomit regolat and Fidomain 1     A62P1							
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Nu.0.166112   221920_x.xt   Solute carrier family 25, member 37   SL225A37   1.95   1.21     0.05 jub MPDE Regulation of monocription NUL000015   201043_x.xt   Acidic (leacine-rich) nuclear phosphoprotein 32 family, member A   AMP2A   -1.23   -1.71     NUL000015   201043_x.xt   Acidic (leacine-rich) nuclear phosphoprotein 32 family, member A   AMP2A   -1.23   -1.71     NUL000015   206079_x.st   Curvitie homeobox 1   CUV   -1.50   -1.70     NUL000015   206079_x.st   Curvitie homeobox 1   CUV   -1.83   -1.83     NUL000015   206079_x.st   Curvities family, member 0   HII   -1.83   -1.83     NUL001601   206017_x.st   Curvities family, member 0   CUV   -1.32   -1.35     NUL001601   200101_x.st   Connectic taisse growth factor   CUF   -1.26   -1.39     NUL001601   201901_x.st   Curvities regulation of force in complex 1. sigma 1 subunit   PUEN   -1.28   -1.59     NUL10072   235581_x.st   Affabre related protein complex 1. sigma 1 subunit   PUEN   -1.59   -1.59     NUL10070   235581_x.st   Affabre related protein complex 1. sigma	Solute transport						
Acids jul BPDE Begulation (Junscription NML009105     201043_s.at 201473_s.at     Acids (feucine-rich) nuclear phosphoprotein 32 family, member A Cut-file homenbox 1     ANY22A - 1.23     - 1.71 - 1.52       Cief orde regulation NML009100     208779_s.at     Aurora kinase A Aurora kinase A NML009100     Aurora kinase A Aurora kinase A NML009100     AURORA     - 1.50     - 1.70       NML091060     208779_s.at     Cyclin B1     CCNB1     - 1.36     - 1.37       NML091080     204962_s.at     Centromere protein A NML009101     - 1.38     - 1.31     - 1.32     - 1.31       Signal monubarritom NML009101     204901, s.at     Connective tissue growth factor     CUT     - 1.42     - 1.43       NML019502     225281, at     Deta/notch-like EGF repeat containing     DNER     - 1.40     - 1.93       NML019207     225281, at     Deta/notch-like EGF repeat containing     DNER     - 1.52     - 1.52       NML019208     235368, s.at     Adptor-related protein complex 1, signa 1 submit     AP151     - 1.53     - 1.52       NML019208     235368, s.at     Adptor-related protein complex 1, signa 1 submit     AP151     - 1.30     - 1.39       MML019208	NM_016612	221920_s_at	Solute carrier family 25, member 37	SLC25A37	1.95	1.21	
0.05 juli PIPE       Sequelation of musicipation       NNL_063015     201043_s_at     Addic (leucine-tich) nuclear phosphoprotein 32 family, member A     210721     -1.23     -1.71       NNL_063015     201043_s_at     Cuci tike homeobox 1     -1.20     -1.20     -1.20       Cell Cicle regulation     NNL_05106     282729_at     Cuci tike homeobox 1     -1.70     -1.82     -1.36     -1.37       NNL_05106     282729_at     Cyclin B1     CVCN H     -1.46     -1.82     -1.35       NNL_05108     208886_at     H1 histone family, member 0     H1/70     -1.32     -1.35       NNL_05101     200281_at     Contective tissue growth factor     CWFrit     -1.28     -1.55       NNL_05010     200391_at     Contective tissue growth factor     CWFrit     -1.28     -1.52       NNL_04260     233588_x_at     Creative tissue growth factor     CWFrit     -1.58     -1.52       NNL_01261     233588_x_at     ArtCAP with CTPs explaint     APFS     -1.43     -1.53       NNL_01261     233588_x_at     ArtCAP with CTPs explaint     APFS							
Regulation framescription     Addit (leucine-rich) nuclear phosphoprotein 32 family, member A     AMP22A     -1.23     -1.71       NNL00605     201043_s_at     Cut-like homenbox 1     -1.71     -1.82     -1.82       NNL00605     20179_s_s_at     Cut-like homenbox 1     -1.71     -1.72     -1.82       NNL00508     20252_s_at     Cyclin 31     CONEI     -1.86     -1.70       NNL00508     204562_s_at     Centromere protein A     CONEI     -1.88     -1.38       NNL00518     208586_at     H1 bistone family, member 0     H1/F0     -1.88     -1.32       NNL00514     228496_s_at     Cysteline rich transmembrane BMP regulator 1 (chordin-like)     CRF     -1.46       NNL01641     226796_s_at     Delajurich-like EG (repeat contanting     DNE     -1.28     -1.45       NNL01632     205196_s_at     Delajurich-like EG (repeat contanting     DNE     -1.28     -1.59       NNL01623     23588_s_x_at     Artford writh Grave contanting 2     NC     -1.28     -1.52       NNL01623     25196_s_s_at     Adaptor-relate gravith actor     AGW /r     -1.39 <td>0.05 µM BPDE</td> <td></td> <td></td> <td></td> <td></td> <td></td>	0.05 µM BPDE						
NNULBIAD   201042.5.at   Acdate [lexine-rin] nuclear prosphoprotem 2/ tamp, member A   AVE2A   -1.23   -1.21   -1.21     Call cycle regulation   NULDING   Curvit kinase A   AVERA   -1.50   -1.50     NULDING   22897.9.at   Curvit kinase A   CURVI   -1.53   -1.53     NULDING   22897.9.at   Curvit kinase A   CURVI   -1.54   -1.53     NULDING   22898.5.at   Curvit kinase A   CURVI   -1.54   -1.53     NULDING   20888.5.at   H1 histone family, member 0   H1PP   -1.52   -1.55     NULDING   220281.at   Connective tissue growth factor   CON   -1.62   -1.57     NULDING   233588.z.at   Petroin factor   CON   -1.58   -1.52*     NULDING   233588.z.at   Petroin factor   CON   -1.58   -1.52*     NULDING   233588.z.at   Adaptor-related protein complex 1, sigma 1 subunit   APTS1   -1.58   -1.52*     NULDING   233588.z.at   Adaptor-related protein complex 1, sigma 1 subunit   APTS1   -1.58   -1.52*     NULDING   233588.z.at   Adaptor-r	Regulation of transcription	001010		(1) (2000)			
Name     Constrained     Constrained     Constrained     Constrained       NAU_03060     208079_x_att     Colin B1     COWIN     -1.36     -1.37       NAU_030800     204662_x_att     Contromer protein A     COWIN     -1.36     -1.83       NAU_001809     204662_x_att     Contromer protein A     COWIN     -1.38     -1.38       Signal transduction     NAU_00104     Contromer protein A anglogenic ludger, 61     CRMM     -1.22     -1.35       NAU_00101     201010_att     Contromer protein Containing     DWR     -1.42     -1.43       NAU_00101     201010_att     Contromer protein containing     DWR     -1.28     -1.45*       NAU_01023     201289_att     Cysteine rich anglogenic ludger, 61     CYR67     -1.40     -1.93       Membrane traffiching     MUL_014260     DVR     -1.52*     NAU_01233     20196_a_xatt     Adaptor-related protein complex 1, signa 1 subunit     AGP1     -1.58     -1.52*       NAU_01230     232588_xat     AliNAK nucleoprotein     AliNAK     -1.52     NAU_01434     20196_a_xat     Adaptor related protein comp	NM_006305	201043_s_at	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	ANP32A	-1.23	-1.71	
Call gole regulation   constrained and the second an	INIM_001913	214/43_dl	Cut-like homeodox 1	CUXI	- 1.52	- 1.62	
Nul. 0303000000000000000000000000000000000	Cell cycle regulation						
Nul. 031966   228729, at   Cyclin B1   CNPM   -1.36   -1.37     Nul. 001800   204662, ast   Centomere protein A   CNPM   -1.48   -3.21     Nul. 005131   208886, at   H1 histone family, member 0   H1 H10   -1.48   -3.21     Signal runsduction   Signal runsduction   CRM1   -1.32   -1.35     Nul. 001614   2260816, at   Cysteine rich transgenein induce, 61   CRM1   -1.26   -1.41     Nul. 00152   226281, at   Deltajnotch-like ECF repeat containing   DNFR   -1.26   -1.41     Nul. 014260   233588, x, at   Prefoldin subunit 6   PFEN6   -1.40   -1.53     Mul. 014260   233588, x, at   Prefoldin subunit 6   PFEN6   -1.52   -1.52     Nul. 014261   204066, s, at   ArtGAP with GTPase domain, ankyrin repeat and PH domain 1   AGAP1   -1.39   -1.30     Nul. 014914   204066, s, at   FallNA nucleoprotein   Attrans and PH domain 1   AGAP1   -1.30   -1.24     Nul. 016120   235281, x, at   FallNA nucleoprotein   Attrans and PH domain 1   AEM21   -1.40   -2.39     N	NM_003600	208079_s_at	Aurora kinase A	AURKA	-1.50	-1.70	
NNL001809   204862_x.at   Cintromere protein A   CNM   -1.48   -1.38     Signal transduction	NM_031966	228729_at	Cyclin B1	CCNB1	-1.36	-1.87*	
NNL.005318   208886_at   H1 histone family, member 0   H1P0   -1.89   -1.89     Signal transduction	NM_001809	204962_s_at	Centromere protein A	CENPA	-1.48	-3.21	
Signal transduction     State     Cysteine rich transmembrane BMP regulator 1 (chordin-like)     CRM1     -132     -135       NNL,019101     201289_at     Cysteine rich transmembrane BMP regulator 1 (chordin-like)     C/GF     -142     -145       NNL,19072     26281_at     Cysteine-rich, angiogenic inducer, 61     C/R61     -126     -141       NNL,19072     26281_at     Delta/north-like EGF repeat containing     DNER     -128     -145       Protein folding	NM_005318	208886_at	H1 histone family, member 0	H1F0	-1.89	- 1.98	
Signal arroscherion NNL 01641 228406_s_art Cysteine rich transmembrane BMP regulator 1 (chordin-like) CRM 1 - 1.32 - 1.35 NNL 010510 206101_at Connective tissue growth factor CTCF - 1.62 - 1.59 NNL 010520 20258_at Cysteine-rich. anglogenic induce. 61 NNL 198072 22628_at Dela/notci-like ECF repeat containing DNRR - 1.28 - 1.45* NNL 010260 233588_x_at Prefoldin subunit 6 PPDN6 - 1.40 - 1.93 Membrane trafficking NNL 01283 205196_s_at Adoptor-related protein complex 1, sigma 1 subunit API51 - 1.58 - 1.52* NNL 01283 205196_s_at ArGAP with CTBase domain, ankyrin repeat and PH domain 1 API51 - 1.58 - 1.52* NNL 01283 205196_s_at ArGAP with CTBase domain, ankyrin repeat and PH domain 1 API51 - 1.58 - 1.52* NNL 01283 205196_s_at ArGAP with CTBase domain, ankyrin repeat and PH domain 1 AGAP - 1.57 - 1.48 NNL 00120 235281_x_at Family with sequence similarity 83, member D ArM83D - 1.40 - 2.39* NNL 013836 22548_at PCI domain containing 2 PCID - 1.38 NNL 003019 225687_at Family with sequence similarity 83, member D ArM83D - 1.40 - 2.39* NNL 015207 235281_x_at ArJ <sup>1</sup> PCI domain containing 2 PCID - 1.39 NNL 015207 23558_at ArJ <sup>1</sup> API domain 1 ArJ <sup>1</sup> 2.39 - 1.74* NNL 000731 223548_at ArJ <sup>1</sup> ArJ <sup>1</sup> ArJ <sup>1</sup> 4.239 - 1.74* NNL 000731 223548_at ArJ <sup>1</sup> ArJ <sup>1</sup> 4.24* NNL 000731 223548_at ArJ <sup>1</sup> ArJ <sup>1</sup> 4.24* NNL 000734 20067_2_s_at Ar Elinding protein 2 ArJ <sup>1</sup> 4.37 - 2.39* 1.67* NNL 000734 20067_2_s_at Ard <sup>1</sup> ArJ <sup>1</sup> 4.45* 1.74* NNL 000754 20067_2_s_at Ard <sup>1</sup> ArJ <sup>1</sup> 4.45* 1.74* NNL 000754 2007_2_s_at Ard <sup>2</sup> ArJ <sup>1</sup> 4.45* 1.74* NNL 000754 20069_at Mrine receptor repressor ArJ <sup>1</sup> 4.74* 2.75* - 2.06* NNL 000754 2007_2_s_at Ard <sup>2</sup> 4.71* 4.71* 4.71* 4.71* - 2.14* NNL 000754 2007_2_s_at Ard <sup>2</sup> 4.71* 4.71* 4.72* - 2.04* NNL 000757 20059_at Ard <sup>2</sup> 4.71* 4.74* 4.72* 4.72* - 2.04* NNL 000758 20400_at Mrine receptor repressor ArJ <sup>2</sup> 4.75* 2.06* NNL 000758 20400_at Mrine receptor repressor ArJ <sup>2</sup> 4.75* 2.06* NNL 000758 20400_at Mrine receptor repressor ArJ <sup>2</sup> 4.75* 2.75* 2.06* NNL 000758 2047* 4.74* 4.74* 4.74* 4.74* 4.74* 4.74* 4.74* 4.74* 4.74* 4.74*							
NNL_01641     22494.9	Signal transduction	222.406		CDI (1	1.00	4.05	
NNL_010501     2019 Jat     Connective tissue growth nator     Clor     -1.62     -1.53*       NNL_010524     201289_at     Cytestine-rich, angiogenic inducer, 61     DNER     -1.28     -1.45*       Protein folding     NNL_139072     225281_at     Detatinotch-like EGF repeat containing     DNER     -1.28     -1.45*       Protein folding     NNL_014260     233588_x_at     Prefoldin subunit 6     PTDN     -1.40     -1.93       MNL_014260     235281_x_at     Adaptor-related protein complex 1, sigma 1 subunit     AP151     -1.58     -1.52*       NNL_014914     204066_s_at     ArGAP with GTPase domain, ankyrin repeat and PH domain 1     AGAP1     -1.39     -1.99*       Unknown     NNL_030919     235587_at     Family with sequence similarity 83, member D     FMM320     -1.40     -2.39*       NNL_03386     225149_at     PCI domain containing 2     PCID2     -1.34     -1.35       0.5 µM BPDE     Regulation of transcription     ArV+lydrocarbon receptor repressor     AHRR     -2.01     -3.00       NU_001321     223838_at     Ankyrin repeat domain 11     AVKR011     -3.45	NM_016441	228496_s_at	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	CRIMI	- 1.32	- 1.35	
Null_1007_2     2026.93_At     Cystember 1A, anguegetin funduce, of     CH R0     -1.28     -1.41       NNL_10907_2     22632B_1.at     Detafanotch-line EGF repeat containing     DMSR     -1.40     -1.39       Prinzein folding     NNL_014260     233588_X.at     Prefoldin subunit 6     PFDPN6     -1.40     -1.39       Membrane trafficking     NNL_01283     205196_5_a.at     Adaptor-related protein complex 1, sigma 1 subunit     AP151     -1.58     -1.52*       NNL_010120     235281_X.at     AHNAK nucleoprotein     AHNAK     -1.24     -2.39*       NNL_010120     235281_X.at     AHNAK nucleoprotein     AHNAK     -1.44     -2.39*       NNL_010120     235281_X.at     AHNAK nucleoprotein     AHNAK     -1.24*     -3.35       NNL_010120     235288_A.at     AP1-fytocarbon receptor repressor     AHRR     -2.01     -3.00       NNL_010127_2_3453_B_B_A.at     Aryl-hytocarbon receptor repressor     AHRR     -2.10*     -1.24*       NNL_001674     202672_S_At     Activating transcription factor 3     AF173     2.39     -1.26     -2.75*     -2.06*	NM_001554	209101_at	Connective tissue growth factor	CIGF CVDC1	- 1.62	- 1.59* 1.41	
Number     Labor     Labor     Lab     Lab <thlab< th="">     Lab     Lab     L</thlab<>	NM 130072	201269_dl 226281_st	Delta/notch-like ECE repeat containing	DNER	-1.20	-1.41	
Protein Jolding     233588_x_at     Prefold in subunit 6     Prefold in subunit 6     - 1.39       Membrane trafficking	INN_133072	220201_dt	Dena/noten-nike EGr repeat containing	DIVLK	1.20	1.45	
NML 014260     233588_X_at     Prefoldin subunit 6     PFDN6     -1.40     -1.93       Membraie trafficking	Protein folding						
Membrane trafficking       NM_001283     205196.s., at     Adaptor-related protein complex 1.signa 1 subunit     APIS1     -1.58     -1.52%       NM_0014914     204066.s., at     ArfGAP with CFPase domain, ankyrin repeat and PH domain 1     ACAPI     -1.39     -1.90*       Inknown       ArfGAP with CFPase domain, ankyrin repeat and PH domain 1     ACAPI     -1.58     -1.52%       NM_001620     235281.x., at     AHNAK nucleoprotein     AHNAK     -1.57     -1.48       NM_001836     225149.at     PCI domain containing 2     PCID2     -1.34     -1.34       0.5 µM BPDE      Regulation of transcription     AHRR     -2.01     -3.00       NM_012727     225889.at     AE binding protein 2     AEBP2     -4.13     -1.24*       NM_00174     202672.s., at     Ankyrin repeat domain 11     AHRR     -2.01     -3.00       NM_00174     202692.s.at     Activating transcription factor 3     AHT3     2.39     1.57*       NM_00174     202672.s., at     Activating transcription factor 3     AHR     -2.01     -3.00       NM_000	NM_014260	233588_x_at	Prefoldin subunit 6	PFDN6	-1.40	-1.93	
Membraic trafficking     Adaptor-related protein complex 1, sigma 1 subunit     APISI     -1.58     -1.52*       NML_014914     204066_s_at     ArfGAP with GTPase domain, ankyrin repeat and PH domain 1     AGAP1     -1.39     -1.90*       Unknown       AHNAK nucleoprotein     AHNAK     -1.57     -1.48       NML_01030     235281_x_at     AHNAK nucleoprotein     AHNAK     -1.57     -1.48       NML_01030     225687_at     Family with sequence similarity 83, member D     FAM83D     -1.40     -2.239*       NML_012326     225149_at     PCI domain containing 2     PCID2     -1.34     -1.35       0.5 µM BPDE       Argulation for transcription for transcription factor 3     AEBP2     -4.13     -1.24*       NML_01272     238538_at     Anyl-hydrocarbon receptor repressor     AIER     -2.00     -1.30       NML_004824     203098_at     Chromodomain protein, Y-like     CD7L     -4.35     -1.24*       NML_002388     204069_at     Meis homeobox 1     CDX1     -2.41     -2.10*       NML_002398     204063_at     Meis hom							
NM_01283   205196_s_s_at   Adaptor-related protein complex 1, sigma 1 subunit   APIS1   -1.52 *     NM_019141   20406_s_s_at   ArfGAP with GTPase domain, ankyrin repeat and PH domain 1   AGAP1   -1.39 *   -1.99 *     MM_001620   235281_X_at   AHNAK nucleoprotein   AHNAK   -1.57 *   -1.48     NM_0130919   225687_at   Family with sequence similarity 83, member D   FAM83D   -1.40 *   -2.39 *     0.5 µM BPDE   Egulation of transcription   NM_153207   225889_at   AE binding protein 2   AEBP2   -4.13 *   -1.24 *     NM_001321   229354_at   Ankyrin repeat domain 11   ANKRD11   -3.45 *   -1.74 *     NM_001674   202672_s_st   Activating transcription factor 3   ATF3   2.39 *   1.67 *     NM_001931   214743_at   Cut-like homeobox 1   CUX1   -2.41 *   -2.10 *     NM_002660   202814_s_st   Cut-like homeobox 1   MEIS1   -7.57 *   -0.06 *     NM_002671   20431_s_st   V-myc mydeoytomatosis viral oncogene homolog (avian)   MVC   -1.64 *   -1.27 *     NM_002660   202814_s_st   V-myc mydeoytomatosis viral oncogene homolog (avian	Membrane trafficking						
NNL_01914     204006_S_at     AnkAP With Lifase domain, ankytin repeat and PH domain 1     ALAPT     - 1.39     - 1.39       Unknown     -	NM_001283	205196_s_at	Adaptor-related protein complex 1, sigma 1 subunit	APISI	-1.58	-1.52*	
Unknown     AHNAK     AHNAK ucleoprotein     AHNAK     -1.57     -1.48       NM_001620     255687_at     Family with sequence similarity 83, member D     FMM82D     -1.40     -2.39*       NM_0138366     225149_at     PCI domain containing 2     PCID2     -1.34     -1.35       0.5 JM BRDE	NW_014914	204066_S_at	Aligap with Gipase domain, ankyrin repeat and PH domain i	AGAPI	- 1.39	- 1.90%	
NM_001620   235281_x_at   AHNAK nucleoprotein   AHNAK   -1.57   -1.48     NM_0030919   225687_at   Family with sequence similarity 83, member D   PGID2   -1.34   -1.33     NM_01836   225   PCID2   -1.34   -1.35     O.5 µM BPDE   -   -   -1.24*   -1.24*     NM_013207   225889_at   AE binding protein 2   AEBP2   -4.13   -1.24*     NM_01275   238538_at   Antyrin repeat domain 11   ANKRD11   -3.45   -1.74*     NM_001670   20272_s_at   Activating transcription factor 3   AITS   2.39   1.67*     NM_001670   20274_s_at   Activating transcription factor 3   AITS   2.39   1.67*     NM_001670   20274_s_at   Activating transcription factor 3   AITS   2.39   1.67*     NM_001670   20281_s_at   Hexamethylene bis-actemide inducible 1   HEXIM   2.58   1.67*     NM_002398   204069_at   Meis homeobox 1   MESI   -2.75   -2.06*     NM_002518   20540_at   Sin3A-asocitaed protein 18 kDa   SAPI8   2.02   1.61     <	Unknown						
NML 030919   225687_at   Family with sequence similarity 83, member D   FAM83D   -1.40   -2.39*     NML 018386   225149_at   PCI domain containing 2   -1.35   -1.35     0.5 µM BPDE   Regulation of transcription   AEBP2   -4.13   -1.24*     NML 05207   22589_at   A Ebinding protein 2   AEBP2   -4.13   -1.24*     NML 020731   229354, at   Anyl-hydrocarbon receptor repressor   AIRR   -2.01   -3.00     NML 013275   23853_8_at   Ankyrin repeat domain 11   ANKRD11   -3.45   -1.74*     NML 001674   20672_s_att   Activating transcription factor 3   AIF3   2.39   1.67*     NML 001674   20672_s_att   Activating transcription factor 3   AIF3   2.39   1.67*     NML 001674   20674_a, at   Cut-like homeobox 1   CUX1   -2.41   -2.10*     NML 002398   204069_at   Meis homeobox 1   MES1   -2.75   -2.06*     NML 0022467   202431_s_at   v-myc myelocytomatosis viral oncogene homolog (avian)   MYC2   -1.64   -1.31     NML 003298   224547_s_at   Nuclear receptor subfamily a, gr	NM_001620	235281_x_at	AHNAK nucleoprotein	AHNAK	-1.57	-1.48	
NM_018386     225149_at     PCI domain containing 2     PCID2     -1.34     -1.35       0.5 µM BPDE	NM_030919	225687_at	Family with sequence similarity 83, member D	FAM83D	-1.40	-2.39*	
0.5 µM BPDE     Regulation of transcription     NM_153207   225889_at   AE binding protein 2   AEBP2   -4.13   -1.24*     NM_020731   229354_at   Aryl-hydrocarbon receptor repressor   AHRR   -2.01   -3.00     NM_013275   238338_at   Ankyrin repeat domain 11   ANKRD11   -3.45   -1.74*     NM_001674   202672_s_at   Activating transcription factor 3   ATF3   2.39   1.67*     NM_004824   203098_at   Chromodomain protein, V-like   CVL   -4.35   -1.27*     NM_00460   202814_s_at   Cut-like homeobox 1   CUX1   -2.41   -2.10**     NM_002467   202431_s_at   V-myc myelocytomatosis viral oncogene homolog (avian)   MYC   -1.64   -1.31     NM_00258   204600_at   Neuroal PAS domain protein 2   NRAS2   -2.33   -1.27*     NM_002570   20840_at   Sin3A-associated protein 18 kDa   SAP18   2.02   1.61     NM_002910   214600_at   TEX domain family member 1 (SV40 transcriptional enhancer factor)   TEAD1   -2.78   -3.31*     NM_01943   203556_at   Zinc fingers and homeoboxes 2	NM_018386	225149_at	PCI domain containing 2	PCID2	-1.34	-1.35	
US_JM_BPUE       Regulation of transcription       NM_153207     22588_9_at     AE binding protein 2     AEBP2     -4.13     -1.24*       NM_020731     229354_at     Anyl-hydrocarbon receptor repressor     AHRR     -2.01     -3.00       NM_013275     238538_at     Ankyrin repeat domain 11     ANKRD11     -3.45     -1.74*       NM_004574     202672_s_at     Activating transcription factor 3     ATF3     2.39     1.67*       NM_00454     203098_at     Chromodomain protein, Y-like     CDYL     -4.35     -1.27**       NM_00460     202814_s_at     Hexamethylene bis-acetamide inducible 1     HEXIM1     2.58     1.96       NM_002467     202431_s_at     v-myc myelocytomatosis viral oncogene homolog (avian)     MVC     -1.64     -1.31       NM_002518     205460_at     Neuronal PAS domain protein 2     NR2C2     -3.23     -1.27*       NM_003290     225477_s_at     Nuclear receptor subfamily 2, group C, member 2     NR2C2     -3.23     -1.27       NM_001943     203556_at     Zinc fingers and homeoboxes 2     ZHX2     -11.06     -1.76* <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>							
Action of numerication of numericatio numerication of numerication of numericat	0.5 µM BPDE						
MM_0200731   229354_at   Anyl-hydrocarbon receptor repressor   AHR   -1.042   -1.300     NM_0120751   228358_at   Ankyrin repeat domain 11   ANKRD11   -3.45   -1.74*     NM_001674   202672_s_at   Activating transcription factor 3   ATF3   2.39   1.67*     NM_004824   203098_at   Chromodomain protein, Y-like   CDVL   -4.35   -1.27**     NM_00660   202814_s_at   Hexamethylene bis-acetamide inducible 1   HEXIM   2.58   1.96*     NM_002267   202431_s_at   v-myc myelocytomatosis viral oncogene homolog (avian)   MYC   -1.64   -1.31     NM_002518   205406_at   Neuronal PAS domain protein 2   NRAS2   -2.03   -1.27*     NM_0025870   208740_at   Sin3A-associated protein, 18 kDa   SAP18   2.02   1.61     NM_0129161   214600_at   TEA domain family member 1 (SV40 transcriptional enhancer factor)   TEAD1   -2.75   -1.80     NM_003196   214710_s_at   Cyclin B1   CCNE1   1.64   -1.64*     NM_003196   204092_s_s_at   Aurora kinase A   AURKA   -2.78   -3.31*     NM_001283 </td <td>NM 153207</td> <td>225889 at</td> <td>AF hinding protein 2</td> <td>AFRP2</td> <td>-413</td> <td>-174*</td>	NM 153207	225889 at	AF hinding protein 2	AFRP2	-413	-174*	
NNL_013275   238538_at   Ankyrin repeat domain 11   ANKRD11   -3.45   -1.74*     NNL_001674   202672_s_at   Activating transcription factor 3   ATF3   2.39   1.67*     NNL_004824   203098_at   Chromodomain protein, Y-like   CDYL   -4.35   -1.27**     NNL_004824   20398_at   Chromodomain protein, Y-like   CDX1   -2.41   -2.10**     NNL_004824   202814_s_at   Cut-like homeobox 1   CDX1   -2.43   -1.27**     NNL_00238   204069_at   Meis homeobox 1   MES1   -2.75   -2.06*     NNL_002518   205400_at   Neuronal PAS domain protein 2   NPAS2   -2.13   -1.66     NNL_002580   208740_at   Sin3A-associated protein, 18 kDa   SAP18   2.02   1.61     NNL_001943   20556_at   Zinc fingers and homeoboxs 2   ZHX2   -1.164   -1.64*     NNL_001284   214500_at   TEA domain family member 1 (SV40 transcriptional enhancer factor)   TEAD1   -2.75   -1.80     NNL_0031966   214710_s_at   Cyclin B1   CCNB1   -1.64   -1.64*     NNL_0031966   214710_s_at   Cycl	NM 020731	229354 at	Arvl-hydrocarbon receptor repressor	AHRR	-2.01	-3.00	
NM_001674   202672_s_at   Activating transcription factor 3   ATF3   2.39   1.67*     NM_004824   203098_at   Chromodomain protein, Y-like   CDVL   -4.35   -1.27**     NM_001913   214743_at   Cut-like homeobox 1   CUX1   -2.41   -2.10**     NM_002398   204069_at   Meis homeobox 1   MEIS1   -2.75   -2.06*     NM_002467   20231_s_at   v-myc myelocytomatosis viral oncogene homolog (avian)   MYC   -1.64   -1.31     NM_00258   225477_s_at   Nuclear receptor subfamily 2, group C, member 2   NR2C2   -3.23   -1.27     NM_005870   28740_at   Sin3A-associated protein, 18 kba   sAP18   2.02   1.66     NM_01943   203556_at   Zinc fingers and homeoboxes 2   ZHX2   -11.06   -1.76*     Cell cycle regulation   T   CCNE1   1.64   -1.64*   1.64*     NM_001238   21470_s_at   Cyclin B1   CCNE1   1.66   1.59*     NM_01243   203556_at   Zinc fingers and homeoboxes 2   ZHX2   -11.06   -1.76*     NM_001238   21400_s_at   Cyclin B1   CCNE1 <td>NM_013275</td> <td>238538_at</td> <td>Ankyrin repeat domain 11</td> <td>ANKRD11</td> <td>-3.45</td> <td>-1.74*</td>	NM_013275	238538_at	Ankyrin repeat domain 11	ANKRD11	-3.45	-1.74*	
NM_004824     203098_at     Chromodomain protein, Y-like     CDYL     -4.35     -1.27***       NM_001913     214743_at     Cut-like homeobox 1     CUX1     -2.41     -2.10**       NM_002060     202814_s_at     Hexamethylene bis-acetamide inducible 1     MEIS1     -2.75     -2.06*       NM_002398     204069_at     Meis homeobox 1     MEIS1     -2.75     -2.06*       NM_002467     202431_s_at     V-myc myelocytomatosis viral oncogene homolog (avian)     MYC     -1.64     -1.31       NM_002398     225477_s_at     Nuclear receptor subfamily 2, group C, member 2     NR2C2     -3.23     -1.27       NM_003298     225477_s_at     Nuclear receptor subfamily 2, group C, member 2     NR2C2     -3.23     -1.27       NM_005870     208740_at     Sin3A-associated protein, 18 kDa     SAP18     2.02     1.61       NM_01943     203556_at     Zinc fingers and homeoboxes 2     -11.64     -1.76*       NM_001298     204492_s_at     Aurora kinase A     Aurora kinase A     Aurora kinase A     -2.78     -3.31*       NM_001284     12523_at     Cyclin B1 <td>NM_001674</td> <td>202672_s_at</td> <td>Activating transcription factor 3</td> <td>ATF3</td> <td>2.39</td> <td>1.67*</td>	NM_001674	202672_s_at	Activating transcription factor 3	ATF3	2.39	1.67*	
NM_001913   214743_at   Cut-like homeobox 1   CUX1   -2.41   -2.10**     NM_002308   204069_at   Hexamethylene bis-acetamide inducible 1   HEXIM1   2.58   1.96     NM_002308   204069_at   Meis homeobox 1   MEIS1   -2.75   -2.06*     NM_002467   202431_s_at   v-myc myelocytomatosis viral oncogene homolog (avian)   MYC   -1.64   -1.31     NM_002518   205460_at   Nuclear receptor subfamily 2, group C, member 2   NR2C2   -3.23   -1.26     NM_005870   208740_at   Sin3A-associated protein, 18 kDa   SAP18   2.02   1.61     NM_014943   203556_at   Zinc fingers and homeoboxs 2   ZHX2   -11.06   -1.76*     Cell cycle regulation   TEA   Quint Fill   1.44   -1.64*   1.64*     NM_003196   214710_s_at   Cyclin B1   Close   -1.64   1.64*     NM_01238   213523_at   Cyclin E1   Close   1.65*   1.64*     NM_001255   202870_s_at   Cell division cycle 20 homolog (s. crevisiae)   CDC20   -1.71   -1.65*     NM_001255   202870_s_at   Cyclin-dependent ki	NM_004824	203098_at	Chromodomain protein, Y-like	CDYL	-4.35	-1.27**	
NM_006460     202814_s_at     Hexamethylene bis-acetamide inducible 1     HEXIM1     2.58     1.96       NM_002398     204069_at     Meis homeobox 1     MEIS1     -2.75     -2.06*       NM_0022467     202431_s_at     v-myc myclocytomatosis viral oncogene homolog (avian)     MYC     -1.64     -1.31       NM_002518     205460_at     Neuronal PAS domain protein 2     NPAS2     -2.13     -1.66       NM_003298     225477_s_at     Nuclear receptor subfamily 2, group C, member 2     NR2C2     -3.23     -1.27       NM_005870     208740_at     Sin3A-associated protein, 18 kDa     SAP18     2.02     -1.16       NM_019493     203556_at     Zinc fingers and homeoboxes 2     -11.06     -1.76*       Cell cycle regulation     TEAD1     -2.75     -1.80       NM_031966     214710_s_at     Cyclin B1     CCNB1     -1.64     -1.64*       NM_001238     213523_at     Cyclin E1     CCNB1     -1.64     -1.59*       NM_001250     207143_at     Cyclin-dependent kinase 6     CDK6     -2.93     -1.55*       NM_001259	NM_001913	214743_at	Cut-like homeobox 1	CUX1	-2.41	-2.10**	
NM_002398   204069_at   Meis homeobox 1   MEISI   -2.75   -2.06*     NM_002467   202431_s_at   v-myc myclocytomatosis viral oncogene homolog (avian)   MYC   -1.64   -1.31     NM_002518   205460_at   Neuronal PAS domain protein 2   NRAS2   -2.13   -1.66     NM_003298   225477_s_at   Nuclear receptor subfamily 2, group C, member 2   NR2C2   -3.23   -1.27     NM_0021961   214600_at   TEA domain family member 1 (SV40 transcriptional enhancer factor)   TEAD1   -2.75   -1.80     NM_0194943   203556_at   Zinc fingers and homeoboxes 2   -11.06   -1.76*     NM_030600   204092_s_at   Aurora kinase A   Aurora kinase A   -2.78   -3.31*     NM_031966   214710_s_at   Cyclin B1   -1.64   -1.64*     NM_001238   213523_at   Cyclin E1   CCNE1   1.86   1.59*     NM_001255   202870_s_at   Cell division cycle 20 homolog (S cerevisiae)   CDC20   -1.71   -1.65*     NM_001259   207143_at   Cyclin-dependent kinase inhibitor 1A (p21, Cip1)   CDKN1A   1.81   1.36     NM_000389   202284_s_at	NM_006460	202814_s_at	Hexamethylene bis-acetamide inducible 1	HEXIM1	2.58	1.96	
NM_002467   202431_s_at   V-myc myclocytomatosis viral oncogene homolog (avian)   MYC   -1.64   -1.51     NM_002518   205460_at   Neuronal PAS domain protein 2   NPAS2   -2.13   -1.63     NM_003298   225477_s_at   Nuclear receptor subfamily 2, group C, member 2   NR2C2   -3.23   -1.27     NM_005870   208740_at   Sin3A-associated protein, 18 kDa   SAP18   2.02   1.61     NM_021961   214600_at   TEA domain family member 1 (SV40 transcriptional enhancer factor)   TEAD1   -2.75   -1.80     NM_030600   204092_s_at   Aurora kinase A   Aurora kinase A   AURKA   -2.78   -3.31*     NM_031966   214710_s_at   Cyclin B1   CCNE1   1.86   1.59*     NM_001238   213523_at   Cyclin E1   CCNE1   1.86   1.59*     NM_001255   202870_s_at   Cell division cycle 20 homolog ( <i>S. cerevisiae</i> )   CDC20   -1.71   -1.65*     NM_001259   207143_at   Cyclin-dependent kinase 6   CDK6   -2.93   -1.55*     NM_000389   202284_s_at   Cyclin-dependent kinase inhibitor 1A (p21, Cip1)   CDKN1A   1.81   1.36 <td>NM_002398</td> <td>204069_at</td> <td>Meis homeobox 1</td> <td>MEIS1</td> <td>-2.75</td> <td>-2.06*</td>	NM_002398	204069_at	Meis homeobox 1	MEIS1	-2.75	-2.06*	
NML_002318   205400_at   Neuronal PAS domain protein 2   NRAS2   -2.13   -1.05     NML_003298   225477_ss_at   Nuclear receptor subfamily 2, group C, member 2   NR2C2   -3.23   -1.27     NM_005870   208740_at   Sin3A-associated protein, 18 kDa   SAP18   2.02   1.61     NM_021961   214600_at   TEA domain family member 1 (SV40 transcriptional enhancer factor)   TEAD1   -2.75   -1.80     NM_014943   203556_at   Zinc fingers and homeoboxes 2   ZHX2   -11.06   -1.76*     Cell cycle regulation   NM_031966   214710_s_at   Cyclin B1   CCNB1   -1.64   -1.64*     NM_001238   213523_at   Cyclin E1   CCNE1   1.86   1.59*     NM_001255   202870_s_at   Cell division cycle 20 homolog (S cerevisiae)   CDC20   -1.71   -1.65*     NM_001259   207143_at   Cyclin-dependent kinase 6   CDK06   -2.93   -1.55*     NM_000389   202284_s_at   Cyclin-dependent kinase inhibitor 1A (p21, Cip1)   CDKN1A   1.81   1.36     NM_001809   20496_s_st   Centromere protein A   CENPA   -3.95   -2.23** <td>NM_002467</td> <td>202431_s_at</td> <td>v-myc myelocytomatosis viral oncogene homolog (avian)</td> <td>MYC</td> <td>- 1.64</td> <td>- 1.31</td>	NM_002467	202431_s_at	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	- 1.64	- 1.31	
NML003258   22347/5_3.4t   Nuclear receptor subannity 2, group C, member 2   NM222   -3.23   -1.27     NM_0005870   208740_at   Sin3A-associated protein, 18 kDa   SAP18   2.02   1.61     NM_014943   203556_at   Zinc fingers and homeoboxes 2   ZHX2   -11.06   -1.76*     Cell cycle regulation     NM_031966   214710_s_at   Cyclin B1   CCNB1   -1.64   -1.64*     NM_001238   213523_at   Cyclin E1   CCNE1   1.86   1.59*     NM_001255   202870_s_at   Cell division cycle 20 homolog (S. cerevisiae)   CDC20   -1.71   -1.65*     NM_000389   202284_s_at   Cyclin-dependent kinase 6   CDK6   -2.93   -1.55*     NM_000389   202284_s_at   Cyclin-dependent kinase 6   CDK01/2   1.46   1.24     NM_000389   202284_s_at   Cyclin-dependent kinase inhibitor 1A (p21, Cip1)   CDKN1A   1.81   1.36     NM_000389   202284_s_at   Cyclin-dependent kinase inhibitor 1C (p57, Kip2)   CDKN1C   1.46   1.23     NM_001809   204962_s_at   Centromere protein A   CENPA   -3.95	NM_002208	205460_at	Neuronal PAS domain protein 2 Nuclear recentor subfamily 2, group C, member 2	NPAS2	-2.13	- 1.66 1.27	
NM_003070   200740_at   500740_at   500740_at   500740_at   500740_at   500740_at   500740_at   150   2.02   101     NM_021961   214600_at   TEA domain family member 1 (SV40 transcriptional enhancer factor)   TEAD1   -2.75   -1.80     NM_014943   203556_at   Zinc fingers and homeoboxes 2   ZHX2   -11.06   -1.76*     Cell cycle regulation     NM_031966   214710_s_at   Cyclin B1   CCNB1   -1.64   -1.64*     NM_001238   213523_at   Cyclin E1   1.86   1.59*     NM_001255   202870_s_at   Cell division cycle 20 homolog (S. cerevisiae)   CDC20   -1.71   -1.65*     NM_0001259   207143_at   Cyclin-dependent kinase 6   CDK6   -2.93   -1.55*     NM_000389   202284_s_at   Cyclin-dependent kinase inhibitor 1A (p21, Cip1)   CDKN1A   1.81   1.36     NM_001809   204962_s_at   Centromere protein A   CENV1C   1.46   1.23     NM_001809   204962_s_at   Centromere protein A   CENV1C   1.46   1.23     NM_005318   20886_at   H1 histone family, member 0 </td <td>NM_005870</td> <td>223477_5_dl 208740_st</td> <td>Sin3A-associated protein 18 kDa</td> <td>SAD18</td> <td>- 3.23</td> <td>- 1.27</td>	NM_005870	223477_5_dl 208740_st	Sin3A-associated protein 18 kDa	SAD18	- 3.23	- 1.27	
Initial Structure   Initis Structure   Ini	NM_021961	200740_at	TFA domain family member 1 (SV40 transcriptional enhancer factor)	TFAD1	-2.02	-1.80	
Cell cycle regulation     NM_003600   204092_s_at   Aurora kinase A   AURKA   -2.78   -3.31*     NM_031966   214710_s_at   Cyclin B1   CCNB1   -1.64   -1.64*     NM_001238   213523_at   Cyclin E1   CCNE1   1.86   1.59*     NM_051735   205034_at   Cyclin E2   CCNE2   1.67   2.18*     NM_001259   207143_at   Cyclin-dependent kinase 6   CDK6   -2.93   -1.55*     NM_000389   202284_s_at   Cyclin-dependent kinase inhibitor 1A (p21, Cip1)   CDKN1A   1.81   1.36     NM_001809   204962_s_at   Centromere protein A   CENPA   -3.95   -2.23*     NM_001818   208886_at   H1 histone family, member 0   H1F0   -2.37   -2.04*	NM_014943	203556_at	Zinc fingers and homeoboxes 2	ZHX2	-11.06	-1.76*	
Cell cycle regulation   NM_003600   204092_s_at   Aurora kinase A   AURKA   -2.78   -3.31*     NM_0031966   214710_s_at   Cyclin B1   CCNB1   -1.64   -1.64*     NM_001238   213523_at   Cyclin E1   CCNE1   1.86   1.59*     NM_057735   205034_at   Cyclin E2   CCNE2   1.67   2.18*     NM_001259   207143_at   Cyclin-dependent kinase 6   CDK6   -2.93   -1.55*     NM_000389   202284_s_at   Cyclin-dependent kinase inhibitor 1A (p21, Cip1)   CDKN1A   1.81   1.36     NM_001809   204962_s_at   Cyclin-dependent kinase inhibitor 1C (p57, Kip2)   CDKN1C   1.46   1.23*     NM_001809   204962_s_at   Centromere protein A   CENPA   -3.95   -2.23*     NM_001818   20886_at   H1 histone family, member 0   H1F0   -2.37   -2.04*     NM_014875   206364_at   Kinesin family member 14   Kirei4   -3.01   -1.49			·				
NM_003600     204092_s_at     Aurora kinase A     AURKA     -2.78     -3.31*       NM_0031966     214710_s_at     Cyclin B1     CCNB1     -1.64     -1.64*       NM_001238     213523_at     Cyclin E1     CCNE1     1.86     1.59*       NM_057735     205034_at     Cyclin E2     CCNE2     1.67     2.18*       NM_001259     207143_at     Cyclin-dependent kinase 6     CDK6     -2.93     -1.55*       NM_000389     202284_s_at     Cyclin-dependent kinase inhibitor 1A (p21, Cip1)     CDKN1A     1.81     1.36       NM_001809     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23*       NM_0018018     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KiF14     -3.01     -1.49	Cell cycle regulation						
NM_031966     214710_s_at     Cyclin B1     CCNB1     -1.64     -1.64*       NM_001238     213523_at     Cyclin E1     CCNE1     1.86     1.59*       NM_057735     205034_at     Cyclin E2     CCNE2     1.67     2.18*       NM_001255     202870_s_at     Cell division cycle 20 homolog (S. cerevisiae)     CDC20     -1.71     -1.65*       NM_001259     207143_at     Cyclin-dependent kinase 6     CDK6     -2.93     -1.55*       NM_000389     202284_s_at     Cyclin-dependent kinase inhibitor 1A (p21, Cip1)     CDKN1A     1.81     1.36       NM_0003076     213182_x_at     Cyclin-dependent kinase inhibitor 1C (p57, Kip2)     CDKN1C     1.46     1.23       NM_001809     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23**       NM_005318     20886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	NM_003600	204092_s_at	Aurora kinase A	AURKA	-2.78	-3.31*	
NIM_001238     21523_at     Cyclin E1     CCNE1     1.86     1.59*       NM_057735     205034_at     Cyclin E2     CCNe2     1.67     2.18*       NM_001255     202870_s_at     Cell division cycle 20 homolog (S. cerevisiae)     CDC20     -1.71     -1.65*       NM_001259     207143_at     Cyclin-dependent kinase 6     CDK6     -2.93     -1.55*       NM_000389     202284_s_at     Cyclin-dependent kinase inhibitor 1A (p21, Cip1)     CDKN1A     1.81     1.36       NM_00076     213182_x_at     Cyclin-dependent kinase inhibitor 1C (p57, Kip2)     CDKN1C     1.46     1.23       NM_001809     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23**       NM_005318     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	NM_031966	214710_s_at	Cyclin B1	CCNB1	-1.64	-1.64*	
NNV_057755     205034_at     Cyclin E2     CCNE2     1.67     2.18*       NM_001255     202870_s_at     Cell division cycle 20 homolog (S. cerevisiae)     CDC20     -1.71     -1.65*       NM_001259     207143_at     Cyclin-dependent kinase 6     CDK6     -2.93     -1.55*       NM_000389     202284_s_at     Cyclin-dependent kinase inhibitor 1A (p21, Cip1)     CDKN1A     1.81     1.36       NM_000076     213182_x_at     Cyclin-dependent kinase inhibitor 1C (p57, Kip2)     CDKN1C     1.46     1.23       NM_001809     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23**       NM_005318     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	NM_001238	213523_at	Cyclin El	CCNE1	1.86	1.59*	
NM_001259     20207u3_at     Cert invision cycle 20 holinolog (s. cerevisiae)     CDC20     -1.71     -1.65*       NM_001259     207143_at     Cyclin-dependent kinase 6     CDK6     -2.93     -1.55*       NM_000389     202284_s_at     Cyclin-dependent kinase inhibitor 1A (p21, Cip1)     CDKN1A     1.81     1.65       NM_00076     213182_x_at     Cyclin-dependent kinase inhibitor 1C (p57, Kip2)     CDKN1C     1.46     1.23       NM_001809     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23**       NM_005318     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	INIVI_U57735 NM_001255	205034_at	Cyclin E2 Call division cycle 20 homolog (S. correvision)	CUNE2	1.67	2.18*	
NM_000389     20284_s_at     Cyclin-dependent kinase inhibitor 1A (p21, Cip1)     CDK N1A     1.81     1.36       NM_00076     213182_x_at     Cyclin-dependent kinase inhibitor 1C (p57, Kip2)     CDKN1C     1.46     1.23       NM_000899     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23**       NM_005318     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	NM_001255	202070_S_dl	Cyclin_dependent kinase 6	CDKS	-2.03	-1.00*	
NM_00076     213182_x_at     Cyclin-dependent kinase inhibitor 1C (p57, Kip2)     CDKN1C     1.46     1.23       NM_001809     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23**       NM_005318     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	NM 000389	207145_at	Cyclin-dependent kinase inhibitor 1A (p21 Cip1)	CDK0 CDKN1A	1.81	1.35	
NM_001809     204962_s_at     Centromere protein A     CENPA     -3.95     -2.23**       NM_005318     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	NM 000076	213182 x at	Cyclin-dependent kinase inhibitor 1C (p57 Kin2)	CDKN1C	1.01	1.30	
NM_005318     208886_at     H1 histone family, member 0     H1F0     -2.37     -2.04*       NM_014875     206364_at     Kinesin family member 14     KIF14     -3.01     -1.49	NM_001809	204962 s at	Centromere protein A	CENPA	-3.95	-2.23**	
NM_014875 206364_at Kinesin family member 14 <i>KIF14</i> -3.01 -1.49	NM_005318	208886_at	H1 histone family, member 0	H1F0	-2.37	-2.04*	
	NM_014875	206364_at	Kinesin family member 14	KIF14	-3.01	-1.49	

(continued on next page)

Table 1 (continued)

RefSeq ID	Probe ID	Gene title	Gene	Fold change	
			symbol	Array	PCR
0.5 μM BPDE					
Cell cycle regulation					
NM_006845	211519_s_at	Kinesin family member 2C	KIF2C	-1.60	-1.55*
NM_014572	227013_at	LATS, large tumor suppressor, homolog 2 (Drosophila)	LATS2	-3.05	-1.80*
NM_003550	204857_at	MAD1 mitotic arrest deficient-like 1 (yeast)	MAD1L1	-2.04	- 1.90
NM_004203	204267_x_at	Protein kinase, membrane associated tyrosine/threonine 1	PKMYT1	1.72	1.70*
Ci					
Signal transduction	221719 c at	$\Lambda$ kinose (DDKA) ancher protein 12	AV/AD12	4.07	2 61*
NM 172075	221710_3_dL 212410_st	A killase (FKKA) alicitor protein 15 Amuloid bota (AA) procursor protoin binding family P	ARAF 15 40000	-4.97	-2.01*
ININI_175075	213419_dt	member 2 (Fe65-like)	AFDD2	- 18.00	-3.03
NM 006888	200653 s at	Calmodulin 1 (phosphorylase kinase delta)	CALM1	125	131
NM 016441	200055 <u>s</u> _at	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	CRIM1	-2.08	-155**
NM 001554	201289 at	Cysteine-rich angiogenic inducer 61	CYR61	-191	-129*
NM 004087	202514 at	Discs, large homolog 1 (Drosonhila)	DLG1	-2.99	-1.33
NM_005228	201983_s_at	Epidermal growth factor receptor (erythroblastic leukemia	EGFR	-2.61	-2.05*
		viral (v-erb-b) oncogene homolog, avian)			
NM_004864	221577_x_at	Growth differentiation factor 15	GDF15	3.41	2.45*
NM_001945	203821_at	Heparin-binding EGF-like growth factor	HBEGF	3.14	1.25*
NM_000875	203627_at	Insulin-like growth factor 1 receptor	IGF1R	-3.16	-2.10*
NM_002222	203710_at	Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	-2.62	-1.92*
NM_005923	203836_s_at	Mitogen-activated protein kinase kinase kinase 5	MAP3K5	-3.67	-1.50
NM_003791	201620_at	Membrane-bound transcription factor peptidase, site 1	MBTPS1	-1.67	-1.50**
NM_145117	218330_s_at	Neuron navigator 2	NAV2	-3.75	-2.49*
NM_014840	204589_at	NUAK family, SNF1-like kinase, 1	NUAK1	-3.32	- 1.39
NM_002581	224940_s_at	Pregnancy-associated plasma protein A, pappalysin 1	PAPPA	-4.06	-1.68*
NM_000921	206389_s_at	Phosphodiesterase 3A, cGMP-inhibited	PDE3A	-3.95	-1.72
NM_006457	203242_s_at	PDZ and LIM domain 5	PDLIM5	-3.38	-1.83
NM_003768	200788_s_at	Phosphoprotein enriched in astrocytes 15	PEA15	1.56	1.60
NM_025179	213030_s_at	Plexin A2	PLXNA2	-3.27	-2.11
NM_002/3/	215195_at	Protein kinase C, alpha	PRKCA	-2.92	-2.1/*
NW_004249	209084_s_at	RAB28, member RAS oncogene family	KAB28	- 1.97	- 1.45
INIVI_032730	224509_S_dl	Thrombospondin 1	KIN4IPI TUDC1	-5.13	- 1.44 <sup></sup>
INIVI_003240	201108_S_dl	Triple functional domain (DTDDE interacting)	TRIO	- 1.09	- 1.51
NM 003371	200170_X_dl 205537_s_at	Vay 2 guanine nucleotide exchange factor	INIO VAV2	- 5.21	- 1.67
14141_003371	203337_3_at	vav 2 guannie nucleonue exchange factor	V/1V2	0.10	2,32
Metabolism					
NM_014324	209425_at	Alpha-methylacyl-CoA racemase	AMACR	1.54	1.81*
NM_024830	201818_at	Lysophosphatidylcholine acyltransferase 1	LPCAT1	-7.50	-1.17*
NM_000104	202434_s_at	Cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	2.95	1.57*
NM_020474	201722_s_at	UDP-N-acetyl-alpha-D-galactosamine:polypeptide	GALNT1	-1.81	-1.46
		N-acetylgalactosaminyltransferase 1 (GalNAc-T1)			
NM_017423	218313_s_at	UDP-N-acetyl-alpha-D-galactosamine:polypeptide	GALNT7	-2.08	-1.28*
		N-acetylgalactosaminyltransferase 7 (GalNAc-T7)			
NM_147175	230030_at	Heparan sulfate 6-O-sulfotransferase 2	HS6ST2	-2.91	-1.86
NM_015440	225520_at	Methylenetetrahydrofolate dehydrogenase	MTHFD1L	-2.76	-1.84
		(NADP+ dependent) 1-like		. =0	
NM_000434	208926_at	Sialidase I (lysosomal sialidase)	NEU1	1.73	1.61**
NM_020376	212/05_x_at	Patatin-like phospholipase domain containing 2	PNPLA2	1.57	1.28
NM_002970	203455_s_at	Spermidine/spermine N1-acetyitransierase 1	SALL	2.94	1.68
INIVI_024030	220167_dL	STEAP failing member 4	STEAP4	-1.74	- 1.12
DNA replication					
NM_002897	203748_x_at	RNA binding motif, single stranded interacting protein 1	RBMS1	-3.28	-1.71
DNA repair	222500	DAD22 have lar D (C annulsia)	D4D22D	2.15	1 50*
NM_002874	223598_at	RAD23 homolog B (S. cerevisiae)	KAD23B	-2.15	- 1.53*
RNA processing					
NM 005463	209067 s at	Heterogeneous nuclear ribonucleoprotein D-like	HNRPDL	-2.13	-159
NM 006362	208922 s at	Nuclear RNA export factor 1	NXF1	1.75	1.98
NM_006275	208804_s_at	Splicing factor, arginine/serine-rich 6	SFRS6	1.62	1.30
Protein synthesis					
NM_003750	200596_s_at	Eukaryotic translation initiation factor 3, subunit A	EIF3A	-1.86	- 1.10
NM_002094	215438_x_at	G1 to S phase transition 1	GSPT1	- 1.65	-1.61*
Dustain antal lan					
Protein catabolism	220101 -+	Itahu hamalag E2 uhiquitin protein linner (maure)	ITCU	10.07	1.50
NM 17/016	259101_dt	Ithiguitin protein ligase E2 component p recognin 1	IICH IIDD1	- 10.07	-1.33
11111_174310	220321_dt	obiquitin protein ngase 15 component n-recognin i	ODKI	5.55	1.34
Cytoskeleton					
NM_020806	220773_s_at	Gephyrin	GPHN	- 1.98	-2.73*
NM_005573	203276_at	Lamin B1	LMNB1	-4.17	-1.37

#### Table 1 (continued)

RefSeq ID	Probe ID	Gene title	Gene symbol	Fold change	
				Array	PCR
0.5 μM BPDE					
Cytoskeleton					
NM_022818	208785_s_at	Microtubule-associated protein 1 light chain 3 beta	MAP1LC3B	1.38	1.32
NM_023009	200644_at	MARCKS-like 1	MARCKSL1	2.85	1.70
NM_001069	204141_at	Tubulin, beta 2A	TUBB2A	2.53	1.63
Membrane trafficking					
NM_001283	205196_s_at	Adaptor-related protein complex 1, sigma 1 subunit	AP1S1	-1.59	- 1.17
NM_014914	204066_s_at	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	AGAP1	-3.94	-2.99**
NM_003024	209297_at	intersectin 1 (SH3 domain protein)	ITSN1	-2.70	-1.56*
Solute transport					
NM 004694	207038 at	Solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	SLC16A6	-199	-130
NM_052885	227176_at	Solute carrier family 2 (facilitated glucose transporter), member 13	SLC2A13	-3.08	-1.38
Immune response					
NM 005516	200004 at	Major histocompatibility complex, class L F	LIAE	1.61	1 //5*
NM_002911	200904_dt	Tumor necrocic factor (licand) cunorfamily, member 0	TNECEO	1.01	1.4J
NIVI_005011	200907_dl	I unior necrosis factor (ligand) superfamily, member 9	INFSF9	1.51	1./1
INIVI_025217	238542_dl	OL 16 binding protein 2	ULBP2	1.49	2.30**
Unknown					
NM_007011	63825_at	Abhydrolase domain containing 2	ABHD2	-1.81	-1.50*
NM_019004	224682_at	Ankyrin repeat and IBR domain containing 1	ANKIB1	-2.26	-1.60*
NM_031450	221534_at	Chromosome 11 open reading frame 68	C11orf68	2.04	1.21
NM_138425	224719_s_at	Chromosome 12 open reading frame 57	C12orf57	1.67	1.38
NM_033286	225300_at	Chromosome 15 open reading frame 23	C15orf23	-2.66	-1.73**
NM_020317	209006_s_at	Chromosome 1 open reading frame 63	C1orf63	3.66	1.51
XR_017929	230251_at	Chromosome 6 open reading frame 176	C6orf176	-4.55	-2.08*
NM_152515	229610_at	Cytoskeleton associated protein 2-like	CKAP2L	-2.31	- 1.60
NM_017993	219501_at	Ecto-NOX disulfide-thiol exchanger 1	ENOX1	-2.55	-3.38**
NM_153690	227410_at	Family with sequence similarity 43, member A	FAM43A	1.84	1.50*
NM_016605	218023_s_at	Family with sequence similarity 53, member C	FAM53C	2.51	1.64*
NM_030919	225687_at	Family with sequence similarity 83, member D	FAM83D	-2.27	-1.72*
NM 022763	218618 s at	Fibronectin type III domain containing 3B	FNDC3B	-4.77	-2.38**
NM 017640	230793 at	Leucine rich repeat containing 16A	LRRC16A	-2.54	-1.98*
NM 019606	219798 s at	Methylphosphate capping enzyme	MEPCE	1.38	1.38
NM_007211	225946 at	Ras association (RalGDS/AF-6) domain family 8	RASSER	-310	-124*
NM_006997	202289 s at	Transforming acidic coiled-coil containing protein 2	TACC2	-3.88	-148
NM_020147	219596 at	THAP domain containing 10	THAP10	1.68	1.10
NM_032021	213530_at	Transmembrane protein 123	TMEM133	-9.66	-2.01
NM 031442	225555_at	Transmembrane protein 47	TMEM/7	1.00	1.50
NM 032883	209030_s_al	TOX high mobility group box family member 2	TOY2	-3.15	-150
NM 015285	220757_dt 212000_st	WD repeat domain 7	10/2	-5.15	- 1.50
NM_016061	21200U_dl	Vinnee like E (Drecenhile)	VDR7	-0.12	- 1.72
ININI_010001	21//83_8_dl	rippee-nke 5 (Drosopnia)	IPELS	1.38	1.15

"\*" and "\*\*" represent *p*<0.05 and <0.01, respectively, compared with vehicle treatment (Student's paired, two-tailed *t*-test analysis).

regulation of the RTK signaling pathways by BPDE would have a negative effect on cell growth.

Two important signaling genes, *GDF15* (growth differentiation factor 15) and *PEA15* (phosphoprotein enriched in astrocytes 15), were up-regulated upon 0.5  $\mu$ M BPDE treatment. *GDF15* is a member of the TGF-beta superfamily, and induced by many stress stimuli including DNA damage [5]. Its expression was correlated with increased apoptosis in some cell types [26]. *PEA15* encodes a protein characterized with an ERK1/2 binding domain and a nuclear export sequence [27]. It can repress ERK1/2 activity by sequestration of ERK1/2 at cytoplasm, thus decreased ERK1/2-dependent transcription and slowed down cell proliferation. The altered expression of these two genes induced by BPDE would disturb cell proliferation and apoptosis.

The expression of many transcription regulators (TFs) was altered upon 0.5 µM BPDE treatment (Table 1). Among which included upregulation of *ATF3* (activating transcription factor 3) and *HEXIM1* (hexamethylene bis-acetamide inducible 1), and down-regulation of *MYC* (v-myc myelocytomatosis viral oncogene homolog (avian)). *ATF3* is induced by a wide variety of stress signals, and its induction is correlated with cellular injury [28]. Over-expression of ATF3 often leads to detrimental consequences [28]. The *HEXIM1* protein, in association with 7SK snRNA, binds and inhibits the kinase activity of P- TEFb (CDK9/CyclinT). P-TEFb activity is crucial for efficient RNA polymerase II-dependent transcription elongation. Up-regulation of *HEXIM1* mRNA and protein is a program for differentiation, and can cause growth inhibition in several cell types [29]. The *MYC* oncoprotein regulates numerous target genes involved in cell cycle, metabolism, protein synthesis, cell growth, and apoptosis [30]. Deregulation of this gene was associated with cellular apoptosis [31]. Thus, the alteration of these genes might affect cell growth, proliferation, differentiation, and apoptosis.

The metabolism gene *SAT1* (spermidine/spermine N1-acetyltransferase 1) was up-regulated upon 0.5  $\mu$ M BPDE treatment (Table 1). *SAT1* encodes a rate-limiting enzyme in polyamine catabolism. Reduction of polyamine level by *SAT1* was shown to promote apoptosis and inhibit cell growth [32]. Its up-regulation by BPDE treatment possibly indicated a tendency of apoptosis and growth suppression.

These data demonstrate that 0.5  $\mu$ M BPDE had profound impacts on many aspects of the cellular processes through regulating gene expression and related signal transduction. The overall effects of BPDE were to down-regulate the cell growth and activate apoptosis at early stage although MTT assay showed the cellular viability was only moderately affected.



Fig. 3. Cell cycle analysis. Human amnion epithelial FL cells were exposed to various concentrations of BPDE for 2 h. At 4, 13 and 22 h post exposure, cells were collected, fixed, stained with propidium iodide, and acquired on a Coulter EPICS XL flow cytometer. Triplicate experiments were carried out, and one of which was displayed as a representative. For each cell cycle profile graph, the *x* axis represented FL3-PI fluorescence value that maximized at 1024 channels, and the *y* axis represented cell counts that maximized at 512.

Transcriptional regulation of the affected genes targeted by stress signaling pathways

Analysis of the promoters of the affected genes would help to predict corresponding TFs with changes in both expression levels and activities and their related signaling pathways. 36 up-regulated genes in response to 0.5  $\mu$ M BPDE were validated with quantitative RT-PCR (Table 1). By computer-assisted prediction, TFs known to be downstream of various stress signaling pathways were picked out, thereby generated stress response-enriched TF profiles for 24 up-regulated genes (Table 2). The prominent transactivators for these affected genes were the AP-1 family TFs (AP-1, FOS and ATF2: JUN

#### Table 2

Prediction of transcription factors activated by 0.5 µM BPDE treatment<sup>a</sup>

Gene Symbol	Fold change		Predicted transcription factors	Gene function	
	Array	PCR			
ATF3	2.39	1.67*	ATF4, AP-1, FOS, REL	Transcription factor	
CDKN1A	1.81	1.36	AP-1, TP53, ATF3	Cell cycle arrest, inhibit G <sub>1</sub> /S transition	
CDKN1C	1.46	1.23	ATF6, ELK1	Cell cycle arrest, inhibit G <sub>1</sub> /S transition	
PKMYT1	1.72	1.70*	ATF4, AP-1	Cell cycle arrest, inhibit G <sub>2</sub> /M transition	
CALM1	1.25	1.31	AP-1, ATF2, FOS, ELK1	Calmodulin	
GDF15	3.41	2.45*	TP53, NFKB1	TGFβ family member	
HBEGF	3.14	1.25*	AP-1, ATF4, FOS	Growth factor	
PEA15	1.56	1.60	NFKB1, TP53	Sequestration of ERKs at cytoplasm	
CYP1B1	2.95	1.57*	CREB1	Xenobiotic and lipid metabolism	
NEU1	1.73	1.61**	FOS	Cleavage of terminal sialic acid residues	
PNPLA2	1.57	1.28	ATF6, ATF2:JUN, CREB1, ATF3, ELK1, TP53	Lipid metabolism	
SAT1	2.94	1.68	ATF2	Amino acid metabolism	
NXF1	1.75	1.98	ATF4	RNA export from nucleus	
SFRS6	1.62	1.30	ATF-1, ATF3	RNA splicing	
MAP1LC3B	1.38	1.32	CREB1	Microtubule assembly	
MARCKSL1	2.85	1.70	CREB1, NF-kappaB	Actin cytoskeleton dynamics	
TUBB2A	2.53	1.63	CREB1, AP-1, ATF2:JUN	Constituent of cytoskeleton	
TNFSF9	1.51	1.71*	NFKB1, Chop-cEBP	Immune response	
C11orf68	2.04	1.21	ATF4, CREB1, NFKB1	Unknown	
C12orf57	1.67	1.38	ELK1, ATF6	Unknown	
FAM43A	1.84	1.50*	RELA, REL, FOS	Unknown	
THAP10	1.68	1.37	ATF3	Unknown	
TMEM47	1.47	1.59	CREB1, ATF2, ATF4	Unknown	
YPEL5	1.38	1.15	CREB1, AP-1, ELK1, ATF4	Unknown	

<sup>a</sup> The Promoter Analysis Pipeline web application suite was used to identify regulatory transcription factors for up-regulated genes induced by 0.5 μM BPDE. Those transactivators related to stress response were displayed in order of their prediction probability from left to right. The "\*" and "\*\*" denote *p*<0.05 and <0.01, respectively, compared with vehicle treatment (Student's paired, two-tailed *t*-test analysis).

heterodimer), the ATF/CREB family members (CREB1, ATF4, ATF6 and ATF3), NF- $\kappa$ B with its subunits (NFKB1, RELA and REL), TP53, and ELK1.

Among these predicted TFs, previous studies had confirmed that AP-1, ATF3, NF- $\kappa$ B, TP53, and ELK1 could be activated by BPDE in various cell types [12,33–35]. The upstream signaling pathways for activation of these TFs were the MAPKs (ERKs, JNKs, p38) pathways for activating AP-1, the JNK/SAPK pathway for ATF3 accumulation and activation, the phosphorylation and degradation of I $\kappa$ B $\alpha$  for stimulating NF- $\kappa$ B, the ATM/ATR, ERKs, and p38 MAPKs for activating TP53, and the ERKs pathway for activating ELK1. The targeted genes included *CDKN1A*, *CDKN1C*, *PKMYT1*, *ATF3*, *SAT1*, *GDF15*, *PEA15*, and etc. (Table 2). As mentioned above, the up-regulation of these genes would impede cell cycle progression, inhibit cell growth, and promote apoptosis.

The predicted activation of ATF/CREB family members (such as CREB1, ATF4, and ATF6) by BPDE has not been reported before. CREB1 is a phosphorylation-dependent TF that can be activated by MAPKs, CaMKs, Akt, and protein kinase A (PKA) [36]. Four up-regulated genes upon 0.5 µM BPDE treatment were predicted to be targeted by CREB1 regulators, including three cytoskeleton-related genes, i.e. *MARCKSL1* (MARCKS-like) [37], *MAP1LC3B* (microtubule-associated protein 1 light chain 3 beta) [38], and *TUBB2A* (tubulin, beta 2A) [39], and one metabolism gene, i.e. *CYP1B1* (cytochrome P450, family 1, subfamily B, polypeptide 1). These cytoskeleton-related genes were not known involved in cellular response to BPDE exposure before. *CYP1B1* is an enzyme involved in metabolizing procarcinogens including polycyclic aromatic hydrocarbons [40]. The induction of *CYP1B1* by benzo[a] pyrene (BaP) was well-known [40], and BPDE is one of the ultimate carcinogens of BaP.

Both ATF4 and ATF6 were known involved in endoplasmic reticulum (ER) stress [41]. Following the initiation of ER stress, ATF4 is translationally activated by EIF2AK3 (PERK) kinase-mediated phosphorylation of eukaryotic initiation factor 2 alpha (eIF2 $\alpha$ ), while ATF6 is posttranslationally capacitated following sequential cleavage by MBTPS1 (membrane-bound transcription factor peptidase, site 1) and MBTPS2. After activation, ATF4 regulates the expression of genes involved in oxidative stress resistance, and ATF6 enhances the production of molecular chaperons such as HSPA5 (GRP78/BiP), HERPUD1 (Herp), and DNAJC3 (P58<sup>IPK</sup>), thereby mediating protective roles for cells. Both TFs also induce the expression of the pro-apoptotic protein DDIT3 (GADD153/CHOP). Our lab previously found that the protein level of HSPA5 and DDIT3 was up-regulated upon BPDE treatment [13]. These evidences support that BPDE may trigger ER stress response, in which ATF4 and ATF6 are involved. In this study, we found that several ATF4- and ATF6- targeted genes, such as ATF3, PKMYT1, CDKN1C, and etc. were activated in response to BPDE treatment, which could lead to down-regulation of cell cycle and cell growth (Table 2). Furthermore, we previously showed that another DNA-damaging alkylating agent, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), could activate a cAMP-PKA-CREB signaling pathway in vero cells [42] and ER stress in FL cells [14], implying that the activation of ATF/CREB TFs and ER stress could be a common mechanism of cellular response to chemical genotoxic agents.

In this study, we have investigated genome-wide transcriptional response by DNA microarray at an early stage after exposure to different levels of the mutagen and carcinogen BPDE in human amnion epithelial FL cells. More than a hundred of gene expression changes were validated with quantitative real-time RT-PCR. These early responsive genes belong to multiple functional categories. The 0.5  $\mu$ M BPDE elicited much more extensive transcriptional changes compared with 0.05 and 0.005  $\mu$ M BPDE. Also the 0.05  $\mu$ M BPDE elicited more changes in gene expression than the 0.005  $\mu$ M dose such as in cell cycle genes. In correlation, the high-dose BPDE generated moderate cytotoxicity and provoked severe S and G<sub>2</sub>/M phase cell cycle arrest, while the two lower doses did not impair cell viability evidently, but the medium dose still evoked apparent S and G<sub>2</sub>/M phase cell cycle arrest in contrast to the low dose. Previously, Akerman

et al. showed a high-dose (1  $\mu$ M) BPDE that resulted in more than 50% cytotoxicity within 24 h induced tens of transcriptional changes in TK6 cells of several hundred genes spotted on a cDNA array, while two lower doses (0.1 and 0.01  $\mu$ M) of BPDE with low cytotoxicity almost did not induce any transcriptional changes of these genes, though both of which also induced adducts and mutation [7]. These observations regarding cellular differential response to various levels of BPDE indicate that gene expression and phenotypic changes are tightly linked, and higher doses of toxicants would have deeper impact on both of which.

Cell division cycle is a highly coordinated machinery accompanied by complex transcriptional programs, which involve periodical expression of nearly one thousand genes (>850) according to the cell cycle phases [43]. A prominent observation of this study is that the expression of more than 150 of these cell cycle phase-related genes was modulated, and adapted to the cell cycle arrest phenotype after BPDE exposure (Table 1 and Supplementary Table 1). For example, histone genes are highly expressed in S phase under normal conditions [43], which implies that a naturally growing culture with more S phase cells in proportion would have higher expression of these genes. However, the expression of H1F0 histone gene was less in 0.05 and 0.5 µM BPDE-treated cultures than control culture, though there were higher proportions of S phase cells in treated cultures. This down-regulation most likely accommodated with the S phase arrest induced by BPDE. A number of other genes such as that function in G<sub>2</sub>/M phase were also regulated at early stage and adapted to the cell cycle arrest as described before. Other labs have also observed that expression of a few cell cycle genes including histone genes and cyclin genes were altered by BPDE damage and correlated with S and  $G_2/M$  phase arrest in different treatment and cell models [7,10–12]. Thus, disturbance of the periodically expressed cell cycle genes might be a common mechanism when establishing cell cycle arrest upon DNA damage.

BPDE was previously shown not to induce a  $G_1/S$  transition arrest in both normal and transformed cell types [10,12,44], as also found in a normal cell type in this study. Such a nature would presumably promote mutagenesis and genome instability due to permitting DNA synthesis on damaged templates [44]. An important finding in this study was that the expression of *CCNE1* and *CCNE2* was up-regulated upon 0.5  $\mu$ M BPDE treatment. This modulation would overcome the negative effect of altered expression of *CDK6*, *CDKN1A* and *CDKN1C*, and promote the damaged cells entry into S phase. Thus, *CCNE1* and *CCNE2* may play a role in the  $G_1$ -arrest deficiency, and act as oncogenes in BPDE-induced mutagenesis and carcinogenesis.

Abundant genes related to cell growth and apoptosis were affected by the 0.5 µM BPDE treatment, e.g., the up-regulation of *GDF15*, *PEA15*, *ATF3*, *HEXIM1* and *SAT1*, and the down-regulation of *EGFR*, *IGF1R*, *PRKCA*, *ITPR1* and *MYC*, which generally indicated cellular damage, growth inhibition, and apoptotic tendency. The up-regulation of *ATF3* [8,12], *HEXIM1* [11] and *SAT1* [7], and down-regulation of *MYC* [7], had also been found in previous studies, indicating common mechanisms can exist in response to BPDE in different cell types.

Bioinformatic analysis of up-regulated genes has predicted the activation of stress response-related TFs including AP-1, ATF3, NF-κB, TP53, ELK1, CREB1, ATF4, ATF6, and etc. after BPDE exposure. Among which, activation of AP-1, ATF3, NF-κB, TP53 and ELK1 as well as related stress signaling pathways, e.g. MAPKs (ERKs, JNKs, and p38 MAPK), NF-κB, and ATM/ATR, by BPDE was previously reported [12,33–35], while the predicted activation of CREB1, ATF4, and ATF6 by BPDE, and its possible relation with ER stress pathway were not known before. The up-regulated genes were implicated in multiple functions including cell cycle, cell survival, signal transduction, RNA processing, cytoskeleton and metabolism, etc. These transcriptional changes and cross-talks among their related signaling pathways reflected the complexity of gene regulation in cellular response to chemical insults.

Gene ontology analysis of the microarray data using GSEA has identified the enrichment of genes implicated in cell cycle regulation, proliferation, apoptosis, signal transduction, transcription, RNA processing, protein metabolism, transport, cytoskeleton and DNA repair etc. (Supplementary Table 2). The alteration of these genes primarily indicated down-regulation of cell cycle, proliferation, transport, cytoskeleton and DNA repair after 0.5 µM BPDE exposure (Supplementary Fig. 1). In addition to above-mentioned quantitative RT-PCRvalidated genes, other enriched gene categories identified by microarray may also have important relevance with cellular response to BPDE. Many cytoskeleton genes were also involved in cell cycle G<sub>2</sub>/M phase, e.g., KIF2C, KIF11, CENPF, MID1, TPX2, BUB1, and etc. [43], indicating that down-regulation of cytoskeleton might be related to the cell cycle arrest induced by BPDE. The repression of transport genes such as AP1S1, AP1G1, AGAP1, EEA1, KPNA1, KPNA3, KPNA6, KPNB1, and etc., which played roles in vesicle-mediated and nucleocytoplasmic transport [45,46], was previously not noticed. The down-regulation of DNA repair genes such as RAD23B, GTF2H1, GTF2H4, RAD50, RAD51C, TDG, and etc. that function in nucleotide excision repair, homologous recombination or base excision repair [47], would possibly promote mutagenesis after BPDE-DNA damage [11]. All these expression changes and related biological meanings deserve further investigations.

In summary, this study revealed that BPDE exposure could induce early transcriptional changes and disturb multiple cellular processes, which parallel with cell cycle arrest and growth inhibition in a dosedependent manner. Analysis of the function and regulation of the affected genes helped to understand how stress signaling pathways, transcription factors, and their target genes were coordinated in the cellular response to BPDE at early stage. The gene expression change levels induced by the dosage of BPDE used in this study were relatively low, and independent RNA samples were applied for the DNA microarray and real-time RT-PCR measurements, both of which may account for why only a part of differentially expressed genes observed by the microarray analysis was verified by the RT-PCR measurements. Further biological experiments are needed to elucidate what exact roles these differentially expressed genes and related pathways play in this chemical-induced effect. In addition, more meticulous analysis of gene expression patterns in a variety of cell types, and time-course comparison will help to generate a general model for cellular responses to genotoxic stress.

#### Materials and methods

#### Cell culture and chemical treatment

Benzo[a]pyrene diol epoxide  $((\pm)$ -anti-BPDE) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Kansas City, MO). BPDE stock solution was prepared in anhydrous dimethyl sulfoxide (DMSO) and used immediately. Human amnion epithelial FL cells were cultured in minimal essential medium (MEM, Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% newborn bovine serum (Gibco-Invitrogen), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. For treatment, FL cells in logarithmic growth were exposed to various doses of BPDE or a DMSO solvent control in serum-free medium for 2 h. All cultures received an equal volume of DMSO (0.1%). Following the chemical exposure, the cultures were rinsed with Hank's buffer solution, and incubated in fresh serum-supplemented medium for additional time before harvest. The cytotoxicity of BPDE was determined with a MTT reduction assay using CellTiter  $96^{\textcircled{R}}$ AQueous One Solution Cell Proliferation Assay Kit (Promega).

## Microarray profiling and data analysis

For microarray study, duplicate cultures were treated with various concentrations (0, 0.005, 0.05, 0.5  $\mu$ M) of BPDE for 2 h, respectively. At

4 h post treatment, cells were rinsed once with ice-cold phosphate buffered saline and lysed, total RNA was isolated with Trizol reagents (Invitrogen), and further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). The quality and quantity of RNA were assessed by denaturing agarose gel electrophoresis and spectrophotometric ultraviolet absorbance at 260/280 nm, respectively. Each of the generated RNA samples was measured by one Human Genome U133 Set array, which covers more than 39,000 transcripts and variants representing 33,000 well-substantiated human genes and expressed sequence tags. Microarray hybridization and detection was performed as described in the Affymetrix GeneChip Expression Analysis Technical Manual.

Microarray data analysis was performed according to the Affymetrix GeneChip® Expression Analysis (Data Analysis Fundamentals). The probe level data was processed using the MAS5.0 algorithm, and the average intensity of each array was globally scaled to a target of 500. The followed single-array and two-array comparison analysis generated detection call and change call for each probe set, respectively. For duplicated control and treatment microarrays, each probe set would possess four detection calls arisen from each microarray, and four change calls produced by the orthogonal comparisons between the control and treatment microarrays. The fold change of a probe set was calculated based on the signal log ratios resulted from two-array comparison analysis. The criteria of significantly differential expression for a probe set was: 1) it possessed at least one "Present" detection call in the single-array analysis, 2) it showed four consistent "Increase" or "Decrease" change calls (100% standard) or three consistent "Increase" or "Decrease" change calls plus one "No change" call (75% standard) in the two-array comparison analysis, and 3) the magnitude of fold change was more than 1.2.

The genes differentially expressed in at least one of the three doses of BPDE treatment were subjected to Cluster 3.0 software (Stanford University) for hierarchical cluster analysis using Pearson correlation (uncentered) distance metrics and average linkage cluster method. The cluster result was visualized with Java TreeView 1.0.13 software (Stanford University). Gene Set Enrichment Analysis (GSEA) was used to analyze the functional categories enrichment in the differentially expressed genes by comparing to the mSigDB gene ontology gene set collection (v2.5) (http://www.broad.mit.edu/gsea/msigdb/index.jsp) [48]. Individual gene sets were filtered out when enough of the members (by default 15–500 genes) were contained in the differentially expressed gene list. The enrichment degree of a filter-out gene set was denoted by the ratio relative to the gene set original size.

## Quantitative real-time RT-PCR

Triplicate RNA samples were independently prepared for quantitative real-time RT-PCR analysis. Total RNA was isolated with Trizol reagents (Invitrogen), and all RNA samples were DNase-treated using the DNA-free™ Kit (Ambion, Austin, TX) to eliminate DNA contamination. RNA integrity was verified by 1% agarose gel electrophoresis and the quantity was determined by spectrophotometry. Singlestrand cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR was performed using ABI TagMan Low Density Arrays, which is a 384-well microfluidic card pre-loaded with optimised probes and matching primers for customer-selected genes. The reaction system in each well was about 2 µL, containing 1 ng cDNA template, 250 nM probe, 900 nM each of the primers, and TaqMan<sup>®</sup> Universal PCR Master Mix. The thermal cycling process was performed on an ABI Prism 7900HT Sequence Detection System, starting with 50 °C for 2 min and 94.5 °C for 10 min, and continuing with 40 cycles of 97 °C for 15 s and 59.7 °C for 30 s. Relative quantification analysis was performed with ABI Prism SDS 2.1.1 software. The endogenous control 18s rRNA was used to normalize differences in the input amount of total cDNAs. Normalized ∆Ct values were then used to determine the statistical significance (p < 0.05) of differential expression between BPDE-exposed and control samples using Student's paired, two-tailed t-test. Fold changes were calculated using the comparative  $C_T$  method as described in the ABI Prism 7900HT SDS User Guide.

#### Cell cycle analysis

Cells in 6-well plate were treated with various concentrations (0, 0.005, 0.05, 0.5  $\mu$ M) of BPDE. At different times (4, 13 and 22 h) post exposure, cells were harvested by trypsinization, fixed in 70% ice-cold ethanol at 4 °C for overnight, then washed and resuspended in PBS containing propidium iodide (20  $\mu$ g/mL), RNaseA (50  $\mu$ g/mL), and TritonX-100 (0.1%) at 37 °C for 30 min. Samples were then measured on a Coulter EPICS XL flow cytometer, and the post-acquisition list mode data was analyzed with WinMDI 2.8 software.

#### **Bioinformatic analysis**

The PAP (Promoter Analysis Pipeline) web application suite (http:// bioinformatics.wustl.edu/webTools/portalModule/PromoterSearch. do) was used to analyze the TFBSs (transcription factor binding sites) in the promoter regions of the up-regulated genes [49]. It identifies specific TFBSs by calculating overrepresentation of characterized transcription factor binding profiles in the promoter sequences using weight matrices from TRANSFAC and JASPER databases. In current study, the identified TFBSs (p<0.05) were reciprocally used for determination of regulatory transcription factors for each gene.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2008.12.007.

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