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Transcriptional Profiling of Krüppel-like Factor 4 Reveals a Function in Cell Cycle Regulation and Epithelial Differentiation

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Abstract

Krüppel-like factor 4 (KLF4) is an epithelially enriched, zinc finger-containing transcription factor, the expression of which is associated with growth arrest. Constitutive expression of KLF4 inhibits G1/S transition of the cell cycle but the manner by which it accomplishes this effect is unclear. To better understand the biochemical function of KLF4, we identified its target genes using cDNA microarray analysis in an established human cell line containing inducible *KLF4*. RNA extracted from induced and control cells were hybridized differentially to microarray chips containing 9600 human cDNAs. In all, 84 genes with significantly increased expression and 107 genes with significantly reduced expression due to *KLF4* induction were identified. The affected genes are sorted to several clusters on the basis of functional relatedness. A major cluster belongs to genes involved in cell-cycle control. Within this cluster, many up-regulated genes are inhibitors of the cell cycle and down-regulated genes are promoters of the cell cycle. Another up-regulated gene cluster includes nine keratin genes, of which seven are located in a specific region on chromosome 12. The results indicate that KLF4 is involved in the control of cell proliferation and does so by eliciting changes in expression of numerous cell-cycle regulatory genes in a concerted manner. Furthermore, KLF4 regulates expression of a group of epithelial-specific keratin genes in a manner consistent with a potential locus control region function.

Keywords

KLF4; cDNA microarray; transcriptional profiling; cell cycle; epithelial differentiation

Introduction

The mammalian gut epithelium is a dynamic system in which cell proliferation, occurring primarily in the crypt, is carefully balanced with differentiation and death of epithelial cells located outside the crypt.[1–4] Other epithelial tissues such as the skin exhibit this characteristic.[5–7] The homeostasis can occasionally become perturbed, leading to disease states such as cancer.[8,9] Previous studies have identified a number of key factors in regulating proliferation and differentiation of the gut epithelial cells. Examples include members of the Wnt pathway of regulators such as β-catenin[10,11] and TCF-4,[12,13] and transcription factors such as CDX2[14–16] and PDX.[17] However, the exact mechanism by which activities of these regulatory proteins are orchestrated to control intestinal epithelial proliferation and differentiation is not clearly defined.

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Krüppel is a zinc finger-containing transcription factor in *Drosophila melanogaster* that has a crucial function in controlling embryogenesis.[18] A large number of mammalian genes exhibit sequence homology to the DNA-binding domain of Krüppel. Among these, a group, called the Krüppel-like factors (KLFs), constitutes a particularly close family.[19 – 23] The prototype gene in this group is erythroid Krüppel-like factor (EKLF or KLF1), which has an essential function in regulating erythropoiesis.[24 – 26] Two additional KLFs, lung Krüppel-like factor (LKLF or KLF2[27,28]) and gut-enriched Krüppel-like factor (GKLF or KLF4[29,30]), are related to KLF1 more closely than any other members of the KLF family. In addition to sequence homology, structural-functional studies cluster these three genes into a subfamily of KLFs.[31]

KLF4 was identified originally on the basis of sequence similarity to another zinc finger-containing transcription factor, zif 268 or EGR1.[29,32] Subsequent studies indicate that its expression is enriched in epithelial tissues such as the gut and skin.[29,30,33] Moreover, the *KLF4* mRNA is found primarily in the post-mitotic, terminally differentiated epithelial cells in these organs.[29,30] *In vitro*, expression of *KLF4* is associated with the growth-arrested process.[29] Conditions that increase its expression include serum deprivation, contact inhibition, and DNA damage.[29,34] Conversely, expression of *KLF4* is decreased in conditions that involve increased proliferation such as neoplasm of the intestinal tract[35,36] and in cancer cell lines.[37] Moreover, forced expression of *KLF4* in cells by either transient or stable transfection inhibits DNA synthesis.[29,38] These studies suggest that KLF4 may have an important function in regulating cell proliferation.

We showed recently that induction in expression of *KLF4* in response to DNA damage is dependent largely on the tumor suppressor p53.[34] Following DNA damage, KLF4 binds to a specific region in the promoter of the gene encoding a key cell-cycle inhibitor, the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$,[39,40] resulting in the activation of this promoter. The ability of p53 to activate the $p21^{WAF1/Cip1}$ promoter is dependent on KLF4 and the two proteins synergize to regulate $p21^{WAF1/Cip1}$ expression.[34] Using a stably transfected cell line in which an inducible promoter controls expression of *KLF4*, we showed that induction of *KLF4* results in an arrest in the transition from the G1 to the S phase of the cell cycle.[41] More recently, using a combination of genetic and biochemical approaches, we showed that KLF4 is essential in mediating the G1/S cell-cycle effect of p53 as a consequence of DNA damage.[42] These studies demonstrate an essential role for KLF4 in cell-cycle control.

In addition to its documented effect on cell proliferation, KLF4 has been shown to be important in regulating tissue differentiation, as indicated by gene knockout studies. In one example, KLF4 is shown to be required for establishing the barrier function of the skin, as measured by penetration of external dyes and rapid loss of body fluids in newborn mice null for *KLF4*.[43] In another, KLF4 is shown to be essential for the terminal differentiation of goblet cells in the colon.[44] These studies demonstrate an important aspect of the role of KLF4 in controlling *in vivo* differentiation of specific epithelial functions.

In order to further understand the biochemical mechanism by which KLF4 regulates cell proliferation and differentiation, we initiated an effort to identify its target genes using a genomic approach. In the current study, we used the previously established inducible cell system for KLF4 and performed cDNA microarray analysis to establish the transcriptional program regulated by KLF4. This effort led to the identification of two groups of genes, one activated and the other inhibited, by KLF4. Furthermore, these genes can be sorted to a limited number of clusters on the basis of functional similarity, one of which is a group of cell-cycle regulators. Another interesting cluster in the genes up-regulated by KLF4 contains a large number of keratin genes, which are epithelial-specific. Moreover, at least seven of the identified keratin genes are located in the same region of the same chromosome. These findings suggest that KLF4 may have a dual function. One is to regulate the cell cycle by coordinating the

expression of numerous cell-cycle regulators. The other is the coordinated expression of a certain family of differentiation and epithelial-specific genes in a manner that is suggestive of a locus control region (LCR) function.

Results

To identify potential target genes of KLF4, we treated EcR-RKO/KLF4 cells with 5 μM ponasterone A or vehicle alone for 24 hours and compared the expression profiles between the two treatment conditions using cDNA microarrays. Previous studies indicate that treatment of EcR-RKO/KLF4 cells with ponasterone A results in a significant induction in *KLF4* expression, which is accompanied by G1/S cell-cycle arrest.[41] In one experiment (experiment A), mRNAs from induced cells were reverse-transcribed and labeled with Cy3-fluorescence dye, while those from control cells were labeled with Cy5 dye. In a second experiment (experiment B), the dyes were reversed. Following hybridization to the Unigene cDNA microarray that contained 9600 human genes, the signal intensity for each of the labeled cDNAs from induced cells was measured and plotted against that from control cells for each experiment (Figure 1). In experiment A (Figure 1(A)), genes whose expression was induced by treatment with ponasterone A are found to the left of diagonal axis. Similarly, genes whose expression was decreased by treatment with ponasterone A are found to the right of the diagonal axis. Results of experiment B, in which the Cy3 and Cy5 dyes were reversed, are shown in Figure 1(B).

Figure 2 is a scatter plot analysis of balanced differential expression values for each of the genes on the microarray between experiments A and B. A change in expression of >1.7-fold (either increased or decreased) is considered significant, based on the recommendation of the manufacturer (>99.5% tolerance intervals for >99% of the elements on the microarray).[45] As expected, the majority of genes is clustered in the center of Figure 2 and represents those whose expression were unchanged after treatment with ponasterone A in the two experiments. A total of 84 genes had a differential expression value of >1.7-fold between induced and control cells in both experiments and are considered up-regulated by KLF4. Among these, 20 genes had values greater than twofold, with intestinal alkaline phosphatase (ALPI) having the largest change at 4.9-fold. A total of 107 genes had a differential expression value of >1.7-fold between control and induced cells in both experiments and are considered down-regulated. In all, 24 genes had a value of at least twofold when control cells were compared to induced cells, with KIA (proliferation related Ki-67 antigen) being the highest with a 2.99-fold change.

The group of genes up-regulated by KLF4 was further analyzed on the basis of functional similarity using the DRAGON database†. As shown in Table 1, these genes can be clustered into several groups, as genes involved in metabolism, cell-cycle control, immune response, structural integrity, signal transduction, translation control, and others. Notable examples include intestinal alkaline phosphatase (ALPI), $p57^{Kip2}$, $p21^{WAF1/Cip1}$, and numerous keratin (KRT) genes. Table 2 shows the identities of the group of genes down-regulated by KLF4. As in the case of up-regulated genes, the down-regulated genes can be clustered into several functional groups, including cell-cycle control, signal transduction, malignancy, transport, DNA replication and relaxation, mitochondrial processes, and others. Among the down-regulated genes, the group of cell cycle-related genes particularly stands out, as they represent primarily genes that are important in progression of the cell cycle with examples such as KIA, cyclin D1, CDC2/CDK1 and RAD21.

To verify the results of the microarray experiments, we performed Northern blot analysis of RNA isolated from EcR-RKO/KLF4 cells treated with ponasterone A or vehicle alone for 24

†http://pevsnerlab.kennedykrieger.org/dragon.htm

hours using several cDNA probes encoding genes that were shown to be up-regulated by KLF4 in Table 1. As seen in Figure 3(A), each of the seven genes tested showed significant induction in the mRNA levels in treated cells as compared to control. These genes represent three clusters of up-regulated genes involved in metabolism (ALPI), structural integrity (KRT18 and villin 2), and cell-cycle control ($p21^{WAF1/Cip1}$, $p57^{Kip2}$, IGFBP6, and SFN). It should be noted that each of the cell-cycle control genes tested here has been shown to be an inhibitor of the cell cycle.[40,46–49] To further verify the authenticity of the microarray experiments, we performed Western blot analysis of proteins isolated from treated and control cells. As seen in Figure 3 (B), the levels of both $p21^{WAF1/Cip1}$ and $p57^{Kip2}$ proteins were elevated significantly, as was that of KLF4. In contrast, the level of the loading control, β-actin, remained the same.

We verified the expression levels of some of the down-regulated genes obtained from the micro-array experiments by Northern blot analysis (Figure 4). As shown, all seven selected genes showed decreased levels of expression when cells were treated with ponasterone A. All seven belong to the cell-cycle group, and have been shown to control the progression of the cell cycle at various checkpoints.[50 – 59]

To further examine the kinetics of induction of several of the up-regulated genes as a result of the inducible expression of *KLF4*, we performed Northern blot analysis using RNA isolated from EcR-RKO/KLF4 cells that had been treated with ponasterone A for various periods of time (Figure 5). As shown, of the five genes tested, the induction of $p21^{WAF1/Cip1}$ and *IGFBP6* was relatively early, beginning around four hours following treatment. In contrast, the levels of transcript for *ALPI* rose relatively late. These results suggest that the time-courses of induction of the up-regulated genes by KLF4 fall into different groups.

Among the genes up-regulated by KLF4 (Table 1), we noticed that there are a large number of genes encoding a structural protein, keratin. As keratin is a major constituent protein of the epithelial tissues,[60 – 62] we analyzed the group of keratin genes that are up-regulated by KLF4 on the basis of sequence homology. Figure 6(A) shows that all nine of the keratin genes up-regulated by KLF4 are closely related, in addition to several other structural proteins such as vimentin (VIM), villin 2 or ezrin (VIL2/p81), desmoglein 2 (DSG2), and singed-like (SNL/p55). It is of note that many of the up-regulated keratin genes are located on the same region of chromosome 12. In human, there are a total of 20 genes encoding keratin, which are distributed on chromosomes 12 and 17.[63 – 65] Of the 12 keratin genes that are on the chip and located on chromosome 12, seven are up-regulated by KLF4 (Figure 6(B)). In contrast, of the seven keratin genes on the chip and located on chromosome 17, only two are up-regulated by KLF4. These results suggest that KLF4 is involved in the coordinated induction of a family of keratin genes located on a similar region of chromosome 12.

Discussion

In the present study, we report the expression profiling of the transcriptional program controlled by KLF4 using cDNA microarrays. Our study identified 84 and 107 genes whose expression are significantly up- and down-regulated, respectively, upon the inducible expression of *KLF4* in a stably transfected cell line. These results are derived after duplication of the experiments by dye reversal and extensive data filtering. Further, expression of many of the target genes was verified by Northern blot analysis and, on occasion, Western blot analysis. Examples are provided in Figure 3 for the up-regulated genes and in Figure 4 for the down-regulated genes. In these two groups of genes, there is a general agreement in fold-changes of expression between the microarray experiments and densitometric tracings of mRNA levels (Table 3). Combining the 14 genes, the Pearson correlation coefficient *r* between Northern and microarray data is 0.61. Of these, *KRT18*, *VIL2* and *CDC2* have the most discrepant correlation between microarray and Northern data. If these three genes are excluded, the Pearson

correlation coefficient *r* is raised to 0.94, indicating an excellent correlation in the remaining 11 genes. We verified the protein levels of two genes, $p21^{WAF1/Cip1}$ and $p57^{Kip2}$, and they correlate well with the microarray and the Northern data (Table 3).

The present study was designed to identify target genes of KLF4 after 24 hours of its inducible expression. We therefore identified genes whose expression are either maximally or near-maximally activated or suppressed by KLF4 at this particular time. The time-course Northern experiments for some of the up-regulated genes in Figure 5 support this claim. However, there are notable differences in the kinetics of activation for the group of genes tested. For example, *ALPI* is activated relatively late in the process, whereas $p21^{WAF1/Cip1}$ is activated relatively early. We presume that our study failed to identify some of the target genes that a re activated early following induction of *KLF4* but the expression levels subsequently fall back to baseline at 24 hours. These potential "immediate-early" genes can be identified when RNA isolated from earlier time-points are included in the microarray experiments in a manner similar to a previous study that examined the transcriptional program in the response of human fibroblasts to serum.[66] We presume that such studies may identify additional KLF4 target genes that are regulatory in nature and that they may mediate the effect of KLF4.

KLF4 was identified initially as a gene whose expression accompanies growth arrest.[29] Conditions such as contact inhibition and serum deprivation stimulated *KLF4* expression.[29] Further, its *in vivo* pattern of expression mirrors that seen *in vitro*. Thus, *KLF4* mRNA is found primarily in terminally differentiated and mitotically inert epithelial cells of the intestine[29] and epidermis.[30,43] Constitutive expression of *KLF4* results in the inhibition of DNA synthesis[29] and colony formation.[38] A recent study using the inducible RKO cell line described here demonstrated that the inducible expression of *KLF4* results in an arrest in the cell cycle at the transition phase between G1 and S, and that this arrest is accompanied by the activation of expression of $p21^{WAF1/Cip1}$,[41] a potent suppressor of cell proliferation.[39] Moreover, *KLF4* is induced as a consequence of DNA damage in a p53-dependent manner, and this induction results in the transcriptional activation of $p21^{WAF1/Cip1}$ due to a direct effect of KLF4 on a specific *cis*-element in the $p21^{WAF1/Cip1}$ promoter.[34] These studies indicate that $p21^{WAF1/Cip1}$ is an important downstream mediator of KLF4. Our present study confirmed these observations and provided independent evidence that $p21^{WAF1/Cip1}$ is a target gene of KLF4. Moreover, the group of target genes up-regulated by KLF4 includes additional genes that have been proven to encode inhibitors of cell proliferation, including $p57^{Kip2}$,[46,47] IGFBP6,[48] and SFN (stratifin).[49] These findings illustrate a mechanism by which KLF4 suppresses proliferation by activating a group of cell-cycle inhibitors.

Studies have indicated that KLF4 is an inhibitor of DNA synthesis,[29] and that this effect is likely mediated at the G1/S transition of the cell cycle with a resultant arrest at this checkpoint following *KLF4* induction.[41] The results of our current study show that both $p21^{WAF1/Cip1}$ and $p57^{Kip2}$ are prominent target genes of KLF4 and are likely the explanation for its cell-cycle effect. Both $p21^{WAF1/Cip140}$ and $p57^{Kip247}$ are potent inhibitors of cyclin-dependent kinases (Cdks) in the G1 phase of the cell cycle. It is of interest to note that a similar G1 Cdk inhibitor, $p27^{Kip1}$, is regulated primarily at a post-translational level,[67,68] which may be the reason that its mRNA level was unchanged after *KLF4* induction in the current study (data not shown).

In addition to up-regulating several inhibitors of the cell cycle, KLF4 suppresses several genes that are positive regulators of the cell cycle. Examples include cyclin D1, CDC2/CDK1 (cell division cycle/cyclin-dependent kinase 1), KIA (proliferation related Ki-67 antigen), and MCM2 (minichromosome maintenance deficient 2). Many of these proteins are known to promote the cell cycle, some at the G1/S phase. Cyclin D1 is one of several D-type cyclins that couples extracellular signals to the biochemical machinery that governs progression through the G1 phase of the mammalian cell division cycle.[69] Cyclin D1 assembles with cyclin-

dependent kinases CDK4 and CDK6 to form holoenzymes that facilitate exit from G1 by phosphorylating key substrates, including the retinoblastoma protein. Previous studies showed that the inhibitory effect of KLF4 on *cyclin D1* expression is exerted at a transcriptional level.[70] Similarly, CDC2/CDK1 is responsible for controlling the transition from the G1 to the S phase, and from the G2 to the M phase of the cell cycle.[71] In addition, both KIA[50] and MCM2[72] are shown to be associated with cell proliferation. MCM2, in particular, is absolutely necessary for the initiation of DNA replication.[72] Thus, the concomitant increase of inhibitors and decrease of promoters of G1 to S phase progression upon induction of *KLF4* expression is likely to be the reason that KLF4 causes the previously observed G1/S block.[41]

Another group of cell cycle-related genes that are suppressed by KLF4 are components of the kinetochore. This group includes CENPE (centromere protein E), MAD2L1 (mitotic arrest deficient 2-like 1), BUB1B (budding uninhibited by benzimidazoles 1 homolog B), and RAD21. These proteins are required for the execution of the mitotic checkpoint in the cell cycle.[51,53,73,74] Although the significance of the down-regulation of these kinetochore-associated genes by KLF4 is not clear, it is possible that a consequence is mitotic arrest, which is the case when *CENPE* expression becomes suppressed.[73] Two additional lines of evidence link KLF4 to the G2/M phase of the cell cycle. One is its ability to down-regulate STK15 (serine/threonine kinase 5), a serine/threonine kinase mainly functioning in the G2/M phase[75] and whose amplification was shown to cause centrosome amplification, aneuploidy and transformation.[76] The other is the finding that KLF4 activates expression of *SFN* (stratifin or 14-3-3 σ), an inhibitor of G2/M progression of the cell cycle following DNA damage.[49,77] It is of interest to note that DNA damage-induced activation of *SFN* expression is dependent on p53,[49] as is *KLF4*.[34] The lone exception to the notion that KLF4 inhibits the cell cycle by activating or suppressing cell-cycle genes with opposite effects is its ability to down-regulated WEE1, which, like SFN, is an inhibitor of G2/M progression in response to DNA damage.[78] Whether WEE1 may be activated by KLF4 at a time other than the 24 hours tested in this study is unclear.

The *in vivo* expression of *KLF4* is found primarily in epithelial tissues, including the intestine[29,30] and epidermis.[30,43] Moreover, the *KLF4* mRNA is found mostly in the post-mitotic, terminally differentiated epithelial cells.[29,30,43] These findings suggest that KLF4 may play a role in the expression of differentiation-dependent genes. Indeed, KLF4 has been shown to trans-activate the promoters of several epithelial enriched genes such as *CYP1A1*,[79] *laminin α 3A*,[80] *keratin 19*,[81] and *keratin 4*.[82] Our current study identified additional genes that are targets of KLF4 and which are enriched in epithelial cells. They include *ALPI*, *VIL2* or *ezrin*, *DSG2* (desmoglein 2), and numerous genes encoding keratins (Table 1). These findings suggest that KLF4, in addition to function as a cell cycle regulator, is involved in regulating expression of differentiation-specific genes.

Evidence supporting the role of KLF4 in regulating differentiation of epithelial tissues is demonstrated by gene targeting experiments of *Klf4* that confirmed the effect of Klf4 on terminal differentiation of two epithelial cell types, epidermal keratinocytes[43] and colonic goblet cells.[44] *Klf4*-null (−/−) mice died shortly after birth due to a loss of barrier function of the skin.[43] Here, the late-stage differentiation structures of the skin, including the cornified envelope, are selectively perturbed. Accompanying *Klf4* deletion is an altered expression of *Sprr2a*, which encodes a cornified envelope protein that functions as a marker of keratinocyte differentiation.[83] In our experiments, expression of *SPRR2A* was unchanged upon *KLF4* induction (data not shown). This may be due to the fact that the RKO cell line used in the present study is intestinal, rather than epidermal in origin.

A more recent study examined the effect of *Klf4* knockout on the intestine.[44] It was shown that *Klf4*-null mice have a 90% reduction in the number of goblet cells in the colon with an

altered expression of the gene encoding a goblet cell-specific marker, MUC2.[44] It was concluded that *Klf4* plays a crucial role in the *in vivo* differentiation of colonic goblet cells. The cDNA encoding *MUC2* was not included in the chip and whether its expression is controlled by KLF4 therefore could not be determined. However, cDNA encoding *GCNT3* (Glucosaminyl (*N*-acetyl) transferase 3, mucin-type), a colonic epithelial enriched mucin,[84] was included in the chip and its level of expression was increased by 1.9-fold upon *KLF4* induction (Table 1). These findings suggest a potential mechanism by which KLF4 may regulate goblet cell differentiation by activating mucin gene expression.

A particularly interesting group among the genes up-regulated by KLF4 is one that encodes structural proteins (Table 1). Most of these structural proteins are involved in the formation of intermediate filaments including keratins, villin 2 and vimentin. Of particular interest is the activation of nine keratin genes by KLF4 (Figure 6). Keratins constitute intermediate filaments and are markers of epithelial differentiation.[61] There are 20 keratin genes in the human genome and they are divided into two classes: type I and type II. Type I keratins are relatively low in molecular mass and acidic, whereas type II keratins are larger and more basic. Filament formation usually requires heterodimerization of keratins in pairs consisting of one type I and one type II polypeptide. The two types of keratin genes are located in clusters on two separate chromosomes; type I on chromosome 17 and type II on chromosome 12.[63] It is of interest to note that the majority (seven out of nine) of keratin genes activated by KLF4 are located on chromosome 12 (Figure 6). These findings raise the possibility of the presence of a locus control region (LCR)[85] in this keratin gene cluster that is regulated by KLF4 in a manner reminiscent of the regulation of the globin gene LCR by KLF1.[86]

In summary, using expression profiling, we identified groups of genes that are up-regulated or down-regulated upon the inducible expression of *KLF4* in a colonic epithelial cell line, RKO. Functional and sequence clusterings of these genes reveal that they may mediate some, if not most, of the previously reported biochemical functions of KLF4, including inhibition of cell proliferation and promotion of terminal differentiation. The identification of the type II keratin gene cluster as a site of regulation by KLF4 raises the possibility that KLF4 may have an LCR function. Further exploration of the mechanism by which KLF4 regulates its target genes, including the keratin gene cluster, may reveal additional information on how KLF4 regulates cellular proliferation and differentiation.

Experimental Procedures

Cell culture

The inducible cell system for KLF4 has been described.[41] Briefly, a colon cancer cell line, RKO, was stably transfected with a plasmid encoding the insect hormone receptor, ecdysone receptor (EcR) and the retinoid X receptor (RXR), and a second plasmid containing KLF4 and green fluorescence protein (GFP) under the control of an ecdysone receptor response element.[41] This cell line, abbreviated EcR-RKO/KLF4, was maintained in DMEM (GIBCO, Gaithersburg, MD) supplemented with heat-inactivated 10% (v/v) fetal calf serum (Hyclone), 2 mM L-glutamine, 10 mM Hepes (pH 7.2), 100 units/ml of penicillin, 100 μg/ml of streptomycin (GIBCO), and 150 μg/ml of Zeocin for selection (Invitrogen, CA) in a 37 °C environment with 5% (v/v) CO_2-in-air. Upon reaching 80% confluence, cells were treated with 5 μM ponasterone A (PA), an ecdysone analogue, for various periods of time. To control for the experiment, the vehicle, ethanol, was added for the same periods of time.

RNA preparation and cDNA microarray analysis

Total RNA was isolated from EcR-RKO/KLF4 cells treated with ponasterone A or vehicle alone for 24 hours using Trizol™ (Invitrogen). Poly(A)-containing RNA was then purified

using the Oligotex mRNA midi-kit (Qiagen, Valencia, CA) and quantified using the Ribo-Green™ RNA quantification kit (Molecular Probes, Eugene, OR). Complementary DNAs were then generated from mRNAs obtained from treated and control cells and labeled with Cy3 and Cy5 fluorescent dyes, respectively, in one experiment and with the dyes in reverse in a second experiment. Fluorescent-labeled cDNAs were hybridized to the Human Unigene™ version 1.33 microarrays (IncyteGenomics, Palo Alto, CA), which contained cDNAs from 9600 unique human genes. Data were analyzed using the GemTools™ 2.5.0 software and expressed as a balanced differential[†]. Genes that were up-regulated and down-regulated (defined as >1.7-fold induced or suppressed) were filtered using Partek Pro (Partek, Inc., St. Charles, MO) and S-Plus™ (Insightful Corp., Seattle, WA). In addition, the genes were grouped by their function into gene clusters using the DRAGON database for human genes (http://pevsnerlab.kennedykrieger.org/dragon.htm), the Pfam database[‡], and the Swiss-Prot database[§]. The structural group of genes that were up-regulated was clustered using the MegAlign program within the LaserGene™ software package by DNASTAR (Madison, WI). The phylogenetic tree of the up-regulated structural group/intermediate filament group was done using ClustalW multiple alignment[87] and the Blosum Series protein weight matrix.[88]

Northern blot analysis

Complementary cDNA clones encoding the various genes analyzed by Northern blots were purchased from Research Genetics (Huntsville, AL). cDNA clones for $p21^{WAF1/Cip1}$ and cyclin D1 were gifts from Dr B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD) and for intestinal alkaline phosphatase (ALPI) from Dr R. Hodin (Harvard Medical School, Boston, MA). β-Actin cDNA probe was purchased from GIBCO. All cDNA clones were verified by sequencing at the Emory DNA Core Facility. The probes were radioactively labeled using the Random Primer Labeling kit (Roche). Total RNA (20 μg) prepared from ponasterone A-treated or control EcR-RKO/KLF4 cells at various time-points up to 24 hours was resolved by electrophoresis in 1.2% (w/v) agarose gels containing 2.4 M formaldehyde and transblotted onto nylon membranes (Hybond-N; Amersham). Hybridization and washing were performed under high-stringency conditions using radioactively labeled cDNA probes.

Western blot analysis

Western blot analyses were performed using standard procedures. In brief, the concentrations of proteins from treated or untreated cells were measured with a spectrophotometer (Beckman) using BioRad Protein Assay with bovine serum albumin (BSA) as a standard. Total proteins (40 μg) were dissolved in loading buffer (60 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 100 mM dithiothreitol, 0.01% (w/v) bromphenol blue), heated at 100 °C for three minutes, and loaded onto an SDS/polyacrylamide gel in running buffer containing 25 mM Tris–HCl (pH 8.3), 250 mM glycine, 0.1% SDS. At the completion of electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane, which was then immunoblotted sequentially with primary antibodies followed by a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Santa Cruz). The rabbit antiserum against KLF4 was as described.[29] Anti-serum directed against $p21^{WAF1/Cip1}$, $p57^{Kip2}$ and β-actin was purchased from Santa Cruz. All blots were visualized with enhanced chemiluminescence from Amersham.

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[†]http://www.incyte.com/gem/technology/index.shtml
[‡]http://www.sanger.ac.uk/Software/Pfam/
[§]http://au.expasy.org/sprot/

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Abbreviations

ALPI
intestinal alkaline phosphatase

BUB1B
budding uninhibited by benzimidazoles 1 homolog B

CENPE
centromere protein E

CDC2/CDK1
cell division cycle 2/cyclin-dependent kinase 1

Cdk
cyclin-dependent kinase

EcR
ecdysone receptor

IGFBP6
insulin-like growth factor binding protein 6

KIA
proliferation-related Ki-67 antigen

KRT
keratin

KLF
Krüppel-like factor

MAD2L1
mitotic arrest deficient 2-like 1

MCM2
minichromosome maintenance deficient 2

PA
ponasterone A

SFN
stratifin

STK15
serine/threonine kinase 5

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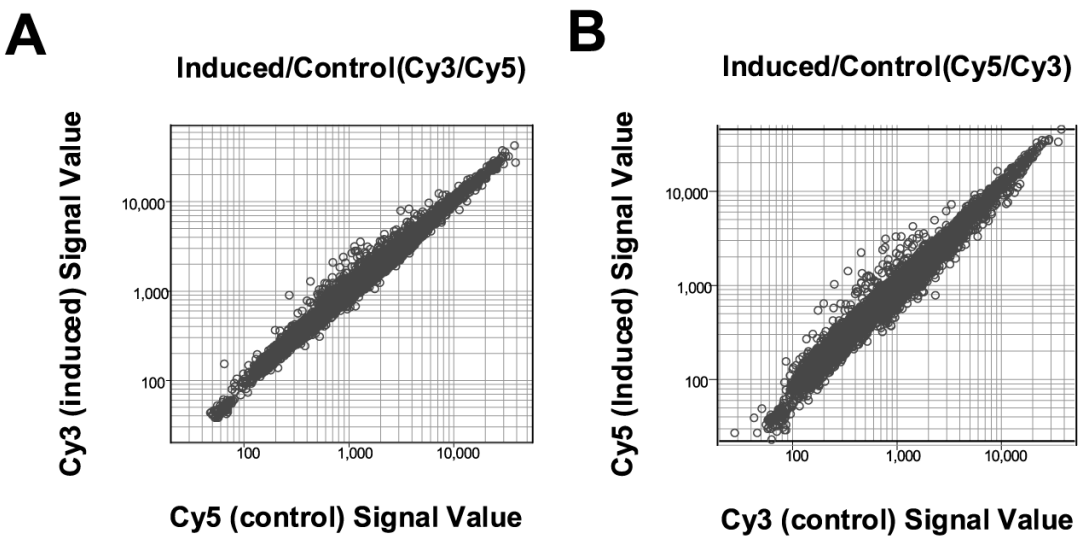


Figure 1.

Results of cDNA micro-array hybridization. EcR-RKO/KLF4 cells were treated with 5 μM ponasterone A (PA) or vehicle alone for 24 hours before subject to mRNA isolation. In experiment A, mRNAs from induced and control cells were reverse-transcribed and labeled with Cy3 and Cy5 fluorescence dye, respectively. The dyes were reversed in experiment B. Labeled cDNAs from induced and control cells were then combined and hybridized to the human cDNA microarray chip as described in Experimental Procedures. The signal value for each cDNA in the treated and control cells was measured and plotted in the Figure for both experiments A and B.

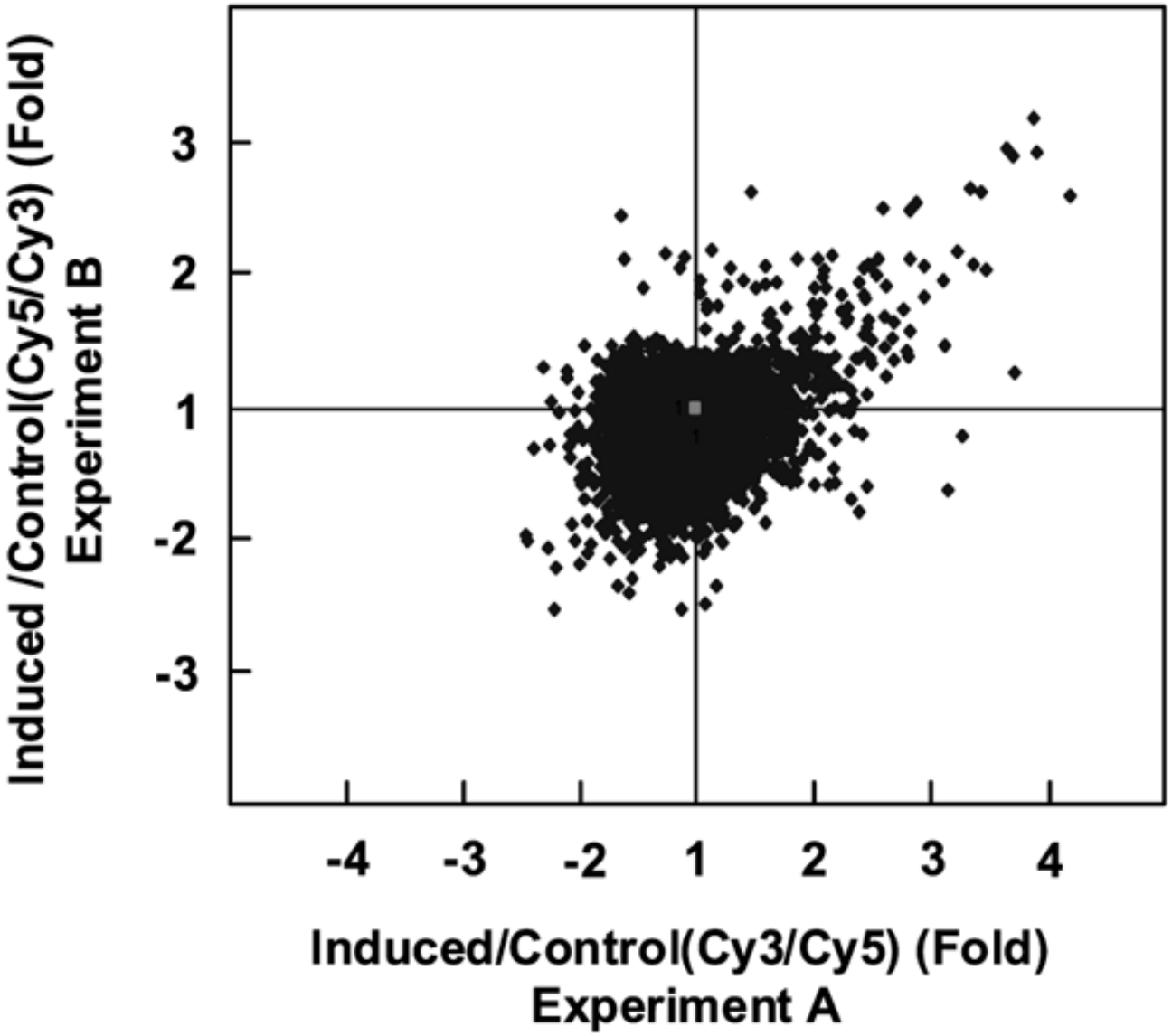


Figure 2.

Scatter plot analysis of microarray hybridization results from experiments A and B. The signal value for each cDNA from induced cells was compared to that from control cells and expressed as a balanced differential expression value. Shown is a scatter plot of balanced differential expression values for all the genes in experiments A and B. Genes in the center of the plot have a value of 1 and are unchanged in their expression by treatment with ponasterone A. Genes in the right upper quadrants are up-regulated and those in the lower left quadrants are down-regulated in both experiments.

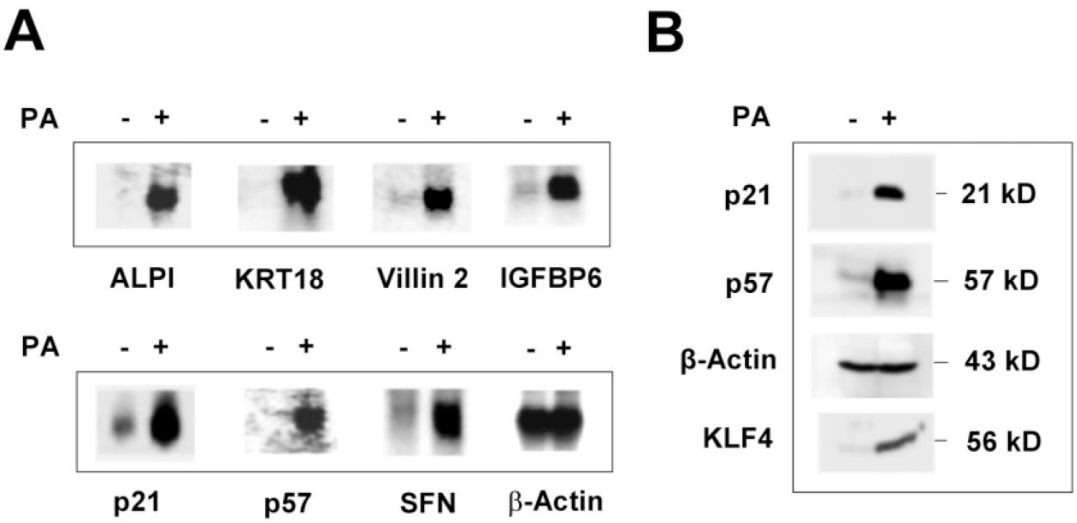


Figure 3.

Verification of expression levels of up-regulated genes identified by microarrays. (A) Total RNA was isolated from EcR-RKO/KLF4 cells treated for 24 hours with ponasterone A (PA) (+) or vehicle alone (−) treated and analyzed by Northern blot analysis using cDNA probes encoding some of the up-regulated genes identified by microarray analysis. ALPI, intestinal alkaline phosphatase; KRT18, keratin 18; IGFBP6, insulin-like growth factor binding protein 6; SFN, stratifin or 14-3-3σ. β-Actin was used as a loading control. (B) Proteins were extracted from treated or control cells and analyzed by Western blot analysis using antibodies directed against $p21^{WAF1/Cip1}$, $p57^{Kip2}$, β-actin, and KLF4.

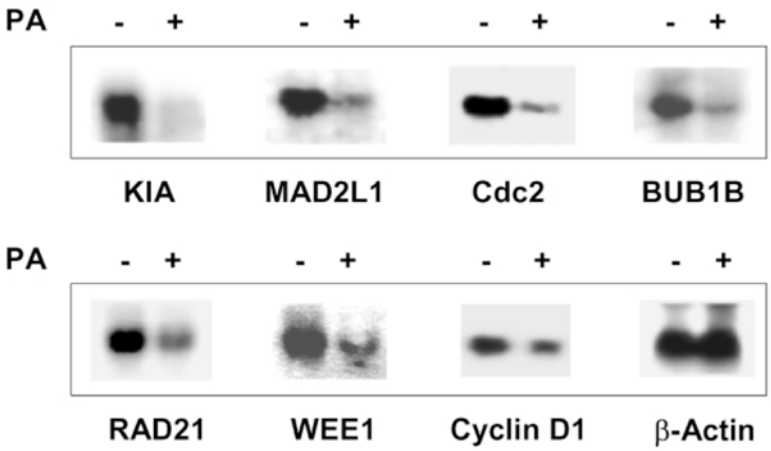


Figure 4.

Verification of expression levels of down-regulated genes identified by microarrays. Total RNA was isolated from EcR-RKO/KLF4 cells treated for 24 hours with ponasterone A (PA) (+) or vehicle alone (−) treated and analyzed by Northern blot analysis using cDNA probes encoding some of the down-regulated genes identified by microarray analysis. See Table 2 for abbreviations. β-Actin was used as a loading control.

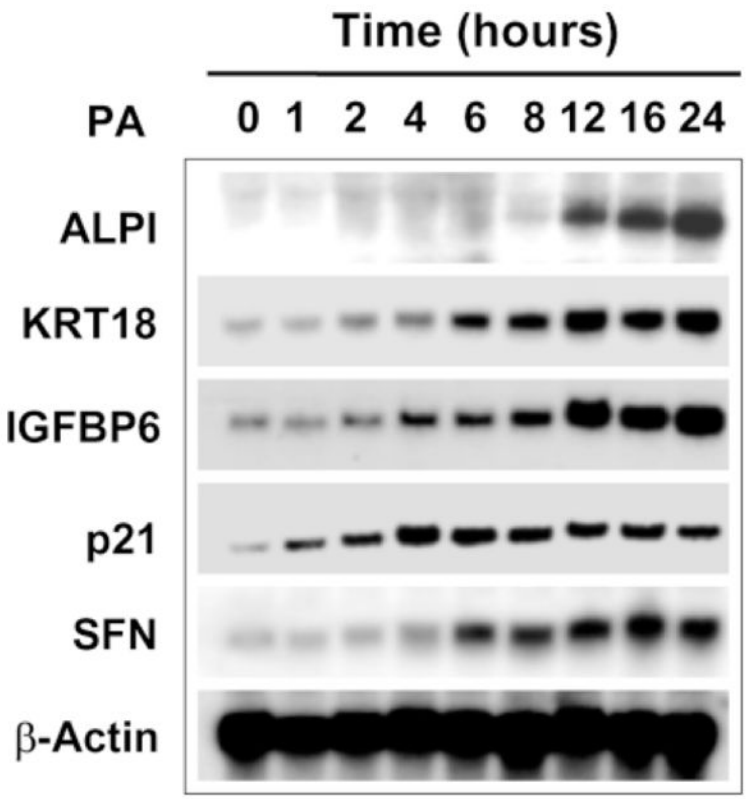
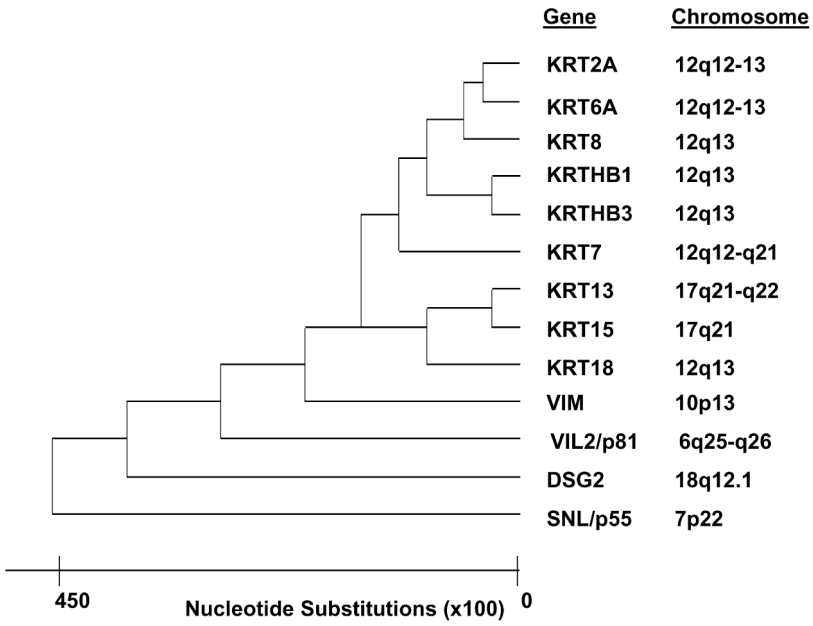


Figure 5.

Time + course of expression of up-regulated gene during induction of KLF4RNA was isolated from EcR-RKO/KLF4 cells treated with ponasterone A for the time indicated and probed by Northern blot analysis using cDNA encoding the genes in the Figure. β-Actin was used as a loading control.

A



B

	KRTs on cDNA Chip	KRTs Up-regulated by KLF4
Chromosome 12	12	7
Chromosome 17	7	2
Total	19	9

Figure 6.

A cluster of keratin and keratin-related genes is up-regulated by KLF4. (A) The sequence-relatedness of the group of keratin and keratin-related genes that are up-regulated by KLF4. Shown are also the chromosomal locations for each of the genes. KRT, keratin; VIM, vimentin; VIL2/p81, villin 2 or eczrin; DSG2, desmoglein 2; SNL/p55, singed-like. (B) The up-regulated keratin genes are sorted on the basis of their chromosomal localization.

Table 1

Gene clusters up-regulated by KLF4

Symbol	Gene name	Symbol	Gene name
Metabolism		*Structural*	
ALPI	Alkaline phosphatase, intestinal	KRT18	Keratin 18
ALPP	Alkaline phosphatase, placental	KRT13	Keratin 13
CTBS	Di-*N*-acetyl-chitobiase	VIL2	Villin 2 (ezrin)
RDP	Dipeptidase 1, renal	MCM	Methylmalonyl coenzyme A mutase
PRODH	Proline dehydrogenase 1	KRTHB1	Keratin, hair, basic 1
CAMK1	Calmodulin-dep. protein kinase 1	RTN2C	Reticulon 2C
AGPAT2	1-Acylglycerol-3-phosphate *O*-acyltransferase 2	SNL	Singed-like (fascin homolog, sea urchin)
PON	Paraoxinase 1	KRTHB3	Keratin, hair, basic 3
AAT	Antitrypsin serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1	KRT8	Keratin 8
ASS	Argininosuccinate synthetase	KRT2A	Keratin 2A
DHCR7	7-Dehydrocholesterol reductase	KRT6A	Keratin 6A
B4GALT1	UDP-Gal: β GlcNAc β 1,4- galactosyltransferase 1	KRT7	Keratin 7
GCNT3	Glucosaminyl (*N*-acetyl) transferase 3, mucin-type	KRT15	Keratin 15
KLK1	Kallikrein 1	DSG2	Desmoglein 2
CSTB	Cystatin B (stefin B)	VIM	Vimentin
Cell cycle control		*Signal transduction*	
p57/Kip2	Cyclin-dependent kinase inhibitor 1C	MT1L	Metallothionein 1L
IGFBP6	Insulin-like growth factor binding protein 6	ADSF	Adipose specific 2
p21/Cip1	Cyclin-dependent kinase inhibitor 1A	GNAI2B	Guanine nucleotide binding protein, α inhibiting activity 2
SFN	Stratifin (14-3-3 sigma)	GPRK5	G protein-coupled receptor kinase 5
PGGT1B	Protein geranyl-geranyltransferase type I β	CRIP2	Cysteine-rich protein 2
PPFIA3	Protein tyrosine phosphatase, receptor type, f polypeptide, interacting protein, α 3	MT1E	Metallothionein 1E
UFD2	Ubiquitination factor E4B	EST	Highly similar to metallothionein-IB
Immune response		*Translation*	
HBZ	Hemoglobin, zeta	TCEA2	Transcription elongation factor A (SII), 2
ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2	RNA helicase	RNA helicase-related protein
HCG II	*H. sapiens* HCG II mRNA	EST	Moderately similar to integrase
HBG1	Hemoglobin, γA		
ARHH	Ras homolog gene family, member H	*Other*	
FUT1	Fucosyltransferase 1	CDNA	cDNA FLJ13155, human
NK4	Natural killer cell transcript 4	CDNA	cDNA FLJ11658, human
PVRL1	Poliovirus receptor-related 1	CYP4F1-like	Rat CYP4F1-like
		FTL	Ferritin, light polypeptide

Table 2

Gene clusters down-regulated by KLF4

Symbol	Gene name	Symbol	Gene name
Cell cycle		*Malignancy*	
KIA	Proliferation related Ki-67 antigen	CBFB	Core-binding factor, β subunit
FOXM1	Forkhead box M1	PURB	Purine-rich element binding protein B
CENPE	Centromere protein E	TXNIP	Thioredoxin interacting protein
THBS1	Thrombospondin 1	STK15	Serine/threonine kinase 15
JAG1	Jagged 1	TTK	TTK protein kinase
MAD2L1	Mitotic arrest deficient-like 1	ZWINTAS	ZW10 interactor anti-sense
CDC2/CDK1	Cell division cycle 2/cyclin-dependent kinase 1	BMI-1	B lymphoma Mo-MLV insertion region
BUB1	Budding uninhibited by benzimidazoles 1 homolog B	ALDOA	Aldolase A, fructose-bisphosphate
RAD21	RAD21 homolog (S. pombe)	PPP2CA	Protein phosphatase 2A, catalytic, alpha
Wee1	WEE1 + homolog (S. pombe)	PTEN	Phosphatase and tensin homolog
RRM1	Ribonucleotide reductase M1 polypeptide	DEK	DEK oncogene (DNA binding)
TMPO	Thymopoietin	BAGE	B melanoma antigen
KNSL6	Kinesin-like 2	NUP98	Nucleoporin 98kD
MCM2	Minichromosome maintenance deficient 2	ZNF220	Zinc finger protein 220
CCND1	Cyclin D1		
HAT1	Histone acetyltransferase 1		
XPO1	Exportin 1 (CRM1 homolog, yeast)		
NEDD5	Neural precursor cell expressed, developmentally down-regulated 5		
KAP1	KRAB-associated protein 1		
Signaling		*Transport*	
NPR3	Atrionatriuretic peptide receptor, type C	MAN1B1	Mannosidase, α, class 1B, member 1
KIAA0008	KIAA0008 gene product	SLC12A2	Solute carrier family 12, member 2
VRK1	VRK1	SLC4A3	Solute carrier family 4, member 3
F2	Coagulation factor II (thrombin) receptor	SEC24C	SEC24 related gene family, member C
CALCRL	Calcitonin, receptor-like	COPE	Coatomer protein complex, subunit epsilon
IDE	Insulin-degrading enzyme	SORT1	Sortilin 1
ARL6	ADP-ribosylation factor-like 6 interacting protein	KPNA2	Karyopherin alpha 2
NESP55	Neuroendocrine secretory protein 55	SLC15A1	Solute carrier family 15, member 1
FLJ10955	Hypothetical protein FLJ10955		
GUCYB3	Guanylate cyclase 1, soluble, beta 3		
FZD1	Frizzled (Drosophila) homolog 1		
Mitochondria		*DNA-repair/relaxation*	
HMT1L1	hnRNP methyltransferase-like 1	TOP2	Topoisomerase (DNA) II, alpha

Symbol	Gene name	Symbol	Gene name
CDNA FLJ13016	Moderately similar to *Rattus norvegicus*	FEN1	Flap structure-specific endonuclease 1
fis	mRNA for SECIS binding protein 2		
ART4	ADP-ribosyltransferase 4	H2AX	H2A histone family, member X
Protein phospha tase 1D	Protein phosphatase 1D magnesium-dependent, δ isoform	HEX1	Exonuclease 1
CDNA FLJ10293	Clone NT2RM1000280, highly similar to vacuolar ATP synthase subunit D	H2AFZ	H2A histone family, member Z
MpV17	Transgene, murine homolog, glomerulosclerosis		
MTMR7	Myotubularin-related protein 7		
CLPP	Caseinolytic protease, ATP-dependent, proteolytic subunit, *E. coli* homolog		
MTND2	NADH-ubiquinone oxidoreductase, subunit ND2		
IDO	Indoleamine-pyrrole 2,3 dioxygenase		
EIF3S5	Eukaryotic translation initiation factor 3, subunit 5		
ERCC6	Excision repair cross-complementing rodent repair deficiency, group 6		
Metabolism		*DNA-binding/transcription*	
OCLN	Occludin	TERF1	Telomeric repeat binding factor (NIMA-interacting) 1
PAPSS2	3′-Phosphoadenosine 5′-phosphosulfate synthase 2	DBP	D site of albumin promoter (albumin D-box) binding protein
CTH, gammalyase	Cystathionine γ-lyase	ATF2	Activating transcription factor 2
RANBP2L1	RAN binding protein 2-like 1	NR1D2	Nuclear receptor subfamily 1, group D, member 2
PCTK2	PCTAIRE protein kinase 2	SF3B3	Splicing factor 3B, subunit 3
PRKAA2	Protein kinase, AMP-activated, α 2 catalytic		
Adhesion		*Other/EST*	
MMP10	Matrix metalloproteinase 10 (stromelysin 2)	ESTs	ESTs
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	Incyte EST	Incyte EST
LAMC2	Laminin, γ 2	ALDH7A1	Aldehyde dehydrogenase 7 family, member A1
LUM	Lumican	P311 protein	P311 protein
MMP13	Matrix metalloproteinase 13 (collagenase 3)	RNA binding motif protein	RNA binding motif protein, X chromosome
MMP10	Matrix metalloproteinase 10 (stromelysin 2)	Incyte EST	Incyte EST
		PRKAR2A	PRKAR2A
		KIAA0074 protein	KIAA0074 protein

Table 3

Comparison of expression ratios between microarray experiments and Northern blot analysis

Up-regulated genes				Down-regulated genes		
Gene	**Microarrays[a]**	**Northern blots[b]**	**Western blots[b]**	**Gene**	**Microarrays[c]**	**Northern blots[d]**
ALPI	4.89	3.2		KIA	2.99	2.6
P57	4.08	3.0	4	MAD2L1	2.12	1.9
KRT18	3.33	5		CDC2	2.09	2.8
IGFBP6	3.23	2.4		BUB1B	2.06	1.8
Villin2	2.94	3.9		RAD21	2.06	1.9
P21	2.22	2.3	3	WEE1	2.01	1.9
SFN	2.13	2		Cyclin D1	1.70	1.6

[a] Numbers indicate means of fold-induction of two experiments.

[b] Fold-induced by densitometric tracing.

[c] Means of fold-decreased of two experiments.

[d] Fold-decreased by densitometric tracing.