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### Title and Subtitle
Control of Mammary Differentiation by Ras-Dependent Signal Transduction Pathways

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### Abstract
Mammary epithelial cells undergo periodic cycles of growth, differentiation, and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals that include mammotrophic hormones and locally-derived growth factors. This study is aimed at determining the mechanism by which an important mitogenic signal transduction pathway, which is frequently activated in breast carcinoma, inhibits mammary differentiation and apoptosis.

We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. This occurs in part via the increase in GTP-bound Ras in the cells. EGF stimulation results in activation of Erk and Akt signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras (via DNras expression) or Erk (via PD98059) or Akt (via wortmannin) can counter the effects of EGF on differentiation. The mechanism of disruption of differentiation appears to involve interference with the growth arrest that occurs prior to the induction of differentiation; the mechanism for growth arrest may require the downregulation of Mek1 expression. The expression of dominant negative Ras in HC11 cells blocked signal transduction to the Ras-Raf-Mek-Erk signal transduction pathway but not signal transduction via the PI-3-kinase pathway.

Expression studies of HC11 cells undergoing lactogenic differentiation using DNA microarrays demonstrated that there is a shift in gene expression that affects genes related to milk production as well as development and growth control.
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INTRODUCTION

Epidemiological studies indicate that the age at first pregnancy and lactation have an impact on later development of breast cancer. Mammary epithelial cells undergo periodic cycles of growth, differentiation and apoptosis during pregnancy and lactation. These processes are initiated by a complex series of signals which include mammotrophic hormones and locally-derived growth factors [1]. This study is aimed at determining the mechanism by which an important mitogenic signal transduction pathway, which is frequently activated in breast carcinoma [2,3], inhibits mammary differentiation and apoptosis.

There are limited human models of mammary differentiation available for study at the present time. However, the HC11 mouse mammary epithelial cells differentiate and synthesize β-casein following growth to confluence and stimulation with the lactogenic hormone mix, DIP (dexamethasone, insulin, prolactin) [4,5]. Regulation of β-casein expression in HC11 reflects in vivo regulation of this protein in the mammary gland [4]. Prolactin stimulation results in Jak2-mediated tyrosine phosphorylation of Stat5 a and b and nuclear translocation of the factors [6]. In HC11 cells the activation of Stat5 is not dependent on the Ras-Erk pathway [6] and, in fact, the induction of β-casein expression can be blocked by receptor tyrosine kinase signaling at the time of prolactin addition [7-10]. It is not clear which signal transduction pathways are responsible for the inhibiton of β-casein synthesis by receptor tyrosine kinase signaling.

However, the inhibition of β-casein expression by treatment of HC11 cells with EGF or Cripto [CR-1], an EGF family member, occurs through a Ras- and phosphotidylinositol-3-kinase (PI-3 kinase)-dependent mechanism [11]. Determination of the signaling mechanism(s) that are responsible for inhibiting differentiation will provide critical insight into control of this process in HC11 cells. Because inhibition of differentiation in HC11 cells appears to be dependent upon Ras, and possibly its association with PI-3-kinase, these studies focus attention on the role of Ras and its effectors in the differentiation of mammary epithelial tissue. We propose that the growth factor regulated inhibition of DIP-induced differentiation of HC11 cells results from the activation of Ras effector pathways in addition to Raf-Mek-Erk. Inhibition may require activation of the Ras-PI-3-kinase pathway and/or the Ras-RasGAP-Rho pathway.

We will test our hypothesis by constructing HC11 cell lines carrying: effector mutants of Ras which activate only a subset of effector pathways, dominant-negative (DN) mutants of proteins in the Ras-PI-3-kinase and Ras-Rho pathways, and HC11 cell lines expressing elevated levels of enhancers Ras-Raf-Mek-Erk signaling pathway. These cell lines will be used to dissect the control of differentiation using a series of markers for differentiation and cell cycle changes.

cDNA microarray analysis techniques will be used to detect global changes in gene expression induced by differentiation in the HC11 cell background. We will identify genes whose expression is specifically increased and decreased in these cells following induction of lactogenic differentiation. Temporal regulation of the expression of specific genes will be followed during a 72 hour course of lactogenic differentiation.

A complete understanding of the regulation of the differentiation process in mammary epithelial cells will aid in understanding the cellular changes and mechanisms leading to carcinogenesis in this tissue and allow evaluation of therapeutic strategies on the differentiation process.
BODY
The majority of the work completed during this period addressed the goals in the original statement of work as opposed to the revised statement of work for this project. Hence, the results reported here primarily address the original statement of work.

Task 1. Construction of vectors and cell lines. This is described in detail in the manuscript enclosed as Appendix item #1.

Construction of HC11 Tet-Off cell lines. The HC11 cell line was transfected with the pTetOff plasmid (Clontech) and the transfected cells were selected for 10 days with G418 (200-500μg/ml). Then individual colonies were picked, expanded and screened for ability to regulate a Tet-promoter. This was accomplished by transfection with a Tet-promoter luciferase construct and assay for luciferase activity with and without Doxycyclin (0-0.5-2.0μg/ml). Several of the transfected cell lines, Ax-TetOff and C6-TetOff, contained a TRE that could be regulated by Dox. These cell lines (HC11-Tet Off) were used to construct lines for the regulated expression of activated Ras or dominant negative Ras.

Production of Retroviral vector Stocks and infection of HC11 cells. pREV-TRE, a retroviral vector that expresses a gene of interest from Tet-responsive element (TRE), was derived from pLNCX, a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector. The 5' viral LTR controls expression of the transcript that contains ϕ + (the extended viral packaging signal), and the hygromycin resistance (Hyg') gene for antibiotic selection in mammalian cells. pRevTRE also includes the E. Coli Amp' gene for antibiotic selection in bacteria. The internal TRE contains seven direct repeats of the 42-bp tetO operator sequence, upstream of a minimal CMV promoter. This promoter was used to inducibly express the genes of interest in response to varying concentrations of Doxycyclin (Dox). TtA binds to the Tet-response element (TRE) and activates transcription from the minimal promoter in the absence of Dox. The plasmids pREV-TRE-RasV12 (active K-Ras 2B/V12) and pREV-TRE -RasN17 (Dominant Negative K-Ras 2B/N17) were constructed by introduction of K-Ras cDNA into pREV-TRE. Retroviral vector stocks of pRev-Tre, pRevTre-RasV12, pRevTre-RasN17 were prepared and used for retroviral infection of HC11-TetOff cells. The HC11-TetOff cell line was infected and selected in hygromycin and Doxycyclin (2μg/ml) for ten days. Six colonies were picked from Tet-Off pREV-TRE, pREV-TRE-RasV12 and pREV-TRE-RasN17 plates and seeded in 24 well plates. These cells was expanded and tested with or without Dox for the presence or absence of Ras RNA by Northern Blot.

Task 2 and 3. Determination of the effect of dominant negative Ras expression on differentiation and Stat5 activation.

EGF blocks hormone-induced HC11 differentiation through Mek and PI-3-kinase-dependent pathways. Previous studies have demonstrated that EGF blocked lactogenic hormone-induced differentiation of HC11 cells [Hynes, 1990 #511], and recent data suggests that this block required Ras and PI-3-kinase activity [DeSantis, 1997 #507]. In the present study specific chemical inhibitors of signal transduction pathways were used to further analyze the contribution of individual signaling pathways to the block of HC11 differentiation by EGF. Because
lactogenic hormone-induced differentiation of HC11 cells is characterized by the initiation of β-casein transcription, the HC11-luci cell line, which contains a β-casein promotor linked to the luciferase gene, was used to provide a rapid readout of the differentiation process.

The HC11-luci cells were induced to differentiate with DIP in the absence and presence of EGF. Specific inhibitors of Mek, and PI-3-kinase were added to cells at the time of induction of differentiation. As expected there was a significant inhibition of β-casein driven luciferase activity in the EGF-treated samples compared to the DIP control. However, several compounds (PD98059, LY294002 and wortmannin) restored the β-casein promotor driven luciferase activity that was blocked by EGF (Figure 1A). The results demonstrated that the inhibition of Mek-Erk signaling by PD98059 and PI-3-kinase signaling by LY294002 and wortmannin disrupted the EGF signaling that inhibited lactogenic hormone-induced differentiation, as measured by the activation of β-casein promotor driven luciferase expression.

The effect of chemical inhibitors of signal transduction pathways on the synthesis of β-casein RNA was examine (Figure 1B). The results confirmed that exposure of HC11 cells to DIP activated β-casein expression and that EGF blocked the expression. However, inclusion of PI-3-kinase or Mek1 inhibitors in the induction media with EGF reversed the EGF-induced inhibition of endogenous on the β-casein promotor activity in the HC11-luci cells.

In addition, the treatment of HC11 cells with DIP resulted in increased Stat5 DNA binding, and previous studies demonstrated that the DNA binding activity of Stat5 was reduced by the simultaneous addition of EGF and lactogenic hormones to HC11 cells [Marte, 1995 #510]. EMSA was performed to examine the ability of the signal transduction inhibitors to alter Stat5 DNA binding. Nuclear extracts were prepared from HC11 cells induced to differentiate in the presence of Jak2, Mek1 or PI-3-kinase inhibitors. The results indicated that prolactin stimulation in the presence of the Mek1 and PI-3-kinase inhibitors enhanced Stat5 binding to DNA compared to the binding detected with prolactin alone (Figure 2A). In contrast, exposure of the HC11 cells to prolactin plus AG490, an inhibitor of Jak2 tyrosine phosphorylation, inhibited Stat5 DNA binding (Figure 2A, lanes 4 and 8). The results in figure 1 indicated that Mek1 and PI-3-kinase inhibitors restored the prolactin-induced Stat5 promotor activity inhibited by EGF. Moreover, the same Mek and PI-3-kinase inhibitors enhanced Stat5 DNA binding. Blocking the Mek-Erk and PI-3-kinase pathways with specific inhibitors both enhanced HC11 differentiation and prevented the EGF-dependent disruption of HC11 differentiation.

**HC11 cells expressing dominant negative (N17) Ras exhibit an enhanced differentiation response.** Ras activation likely regulates the activation of the Erk pathway by EGF and possibly contributes to the activation of PI-3-kinase. Hence, the role of Ras activation in the disruption of HC11 differentiation by EGF was examined further. HC11 cell clones expressing either activated Ki-Ras (V12) or dominant negative (DN) Ki-Ras (N17) were constructed as described in Materials and Methods. The HC11 cell lines constructed contained the Ras cDNAs under the control of a Tet-responsive promotor in a Tet-off system. Hence, the expression of Ras increased following the removal of doxycycline from the culture media. Several independent clones containing each vector were isolated and characterized for the inducibility of Ras gene expression following the removal of doxycycline from the cultures. As expected, the inducibility varied for the individual Ras(V12) and DN Ras clones. The results obtained with three independent clones derived from each vector are shown in Figure 3.

The DN Ras and the Ki-Ras(V12) HC11 cell lines were compared to the vector control cell line, REV-TRE, to determine the effect of the Ras gene expression on lactogenic hormone-
induced differentiation. HC11 transfectant cell lines expressing dominant negative Ras(N17) or activated Ras(V12) along with the vector control cell line were grown for 72 hours in the absence of doxycycline at which point the confluent cultures were incubated in DIP differentiation media. RNA was harvested from cells at 0, 48, and 72 hours post addition of DIP and used to determine the level of Ras and β casein expression by Northern blotting. The results in Figure 3 indicated that Ki-Ras(V12) expression inhibited β-casein expression by approximately 50% compared to the TRE control cell line. In contrast, the expression of dominant negative Ras(N17) enhanced β-casein induction up to two-fold compared to the control. The results demonstrated that the amount of N17 Ras expression correlated with the effect on differentiation. The HC11 cell clone expressing the greatest amount of Ras N17 (clone 12) exhibited the greatest level of β-casein expression.

In parallel experiments the effect of Ras expression on the prolactin-induced tyrosine phosphorylation of Jak2 and Stat5 was examined. HC11 TRE vector control cells as well as the Ki-Ras(V12) clone 1 and DN Ras(N17) clone 12 cells were stimulated with prolactin and the phosphorylation status of the Stat5 protein was determined by immunoprecipitation and Western blotting using anti Stat5 tyrosine 694 (Y694) phosphorylation site-specific antibodies. The results, seen in Figure 4A, indicated that the tyrosine phosphorylation of Stat5 was enhanced and sustained in the DN Ras(N17) HC11 cell line compared to the TRE vector control cell line. However, the tyrosine 694 phosphorylation was of a shorter duration in the cell lines expressing activated Ki-Ras(V12) than in the TRE control cells. These results suggested that Ras-dependent signal transduction can modulate Stat5 phosphorylation in HC11 cells in response to prolactin. The Stat5 EMSA results supported this conclusion (Figure 4B). Enhanced Stat5 DNA binding in response to prolactin stimulation was observed in the DN Ras(N17) HC11 cell lysates as compared to the vector control. In contrast, the Stat5 DNA binding activity was reduced in cells expressing activated Ki-Ras(V12). In conclusion, an increase in HC11 cell lactogenic hormone-induced differentiation is observed following blockade of the Ras signaling pathway. Moreover, in the HC11 cells that have Ras activity blocked, the enhancement of hormone-induced differentiation appeared to be attributable to an increase in Stat5 tyrosine phosphorylation and to an increase in Stat5 DNA binding resulting in enhanced transcription of β-casein, a Stat5-regulated gene.

*Infection of HC11 cells with DN Ras adenovirus enhances lactogenic differentiation.* Infection of cells with replication defective adenovirus encoding dominant negative Ha-Ras(N17) was used as another mechanism to examine the influence of the Ras pathway on lactogenic differentiation. HC11 cells and HC11-luci cells were infected with 10 MOI of either replication defective control adenovirus or adenovirus encoding DN (N17) Ras. At 24 or 48 hours post infection the cells were examined for the effect of DN Ras on Stat5 phosphorylation, β casein promoter activity and β casein RNA levels. As demonstrated in Figure 5A HC11-luci cells infected with control virus or DN Ras virus were stimulated with DIP and the level of Stat5 tyrosine 694 phosphorylation was determined. The results indicated that the expression of DN Ras (N17) increased the level of Stat 5 phosphorylation in response to DIP compared to either uninfected or vector control-infected cells. HC11-luci cells infected with either replication defective control adenovirus or adenovirus encoding DN Ras (N17) were tested for activation of β-casein promoter-driven luciferase activity (Figure 5B). There was a five-fold increase in the activation of luciferase activity in the DN Ras (N17) cells compared to the uninfected cells or the control adenovirus infected cells. In addition, there was some activation of luciferase activity in cells
infected with the DN Ras (N17) virus without DIP exposure. This result was reproducible and is not seen when uninfected cells or vector infected cells were exposed to DIP. Finally, RNA from HC11 cells infected with either replication defective control adenovirus or adenovirus encoding DN Ras (N17) was tested for expression of β-casein following exposure to DIP for 24 or 48 hours. The results in Figure 5C indicated that the infection with DN Ras (N17) virus resulted in a two-fold increase in β-casein RNA compared to the uninfected or vector infected cells exposed to DIP.

**HC11 cells expressing dominant negative (N17) Ras exhibit reduced response to EGF.** Studies were performed to determine if the DN Ras (N17) expression could block EGF-induced responses in stable transfectants of HC11 cells. HC11 cells respond mitogenically to EGF. The TRE vector control cells and the DN Ras (N17) cells were stimulated with EGF and the ability of the cells to proliferate was examined using the MTT assay. The results demonstrated that the DN Ras (N17) cell line was growth inhibited by 40% in both the absence and presence of EGF compared to the vector control cell line. This experiment was repeated using TGFα treatment of HC11 vector control and DN Ras (N17) cells. Again, the DN Ras (N17) cells exhibited a lower response to EGF and TGFα than did the vector control cell line. (Figure 6)

The ability of DN Ras to prevent the disruption of lactogenic hormone-induced differentiation by EGF in HC11 cells was examined. The cells were exposed to lactogenic hormone differentiation media in the presence and absence of EGF for varying lengths of time, RNA was extracted and the level of β-casein mRNA was analyzed by Northern blotting. The results in Figure 6 demonstrated that EGF did not inhibit the induction of β-casein transcription in response to DIP treatment in the DN Ras (N17) cell line and, hence, it appeared that differentiation proceeded in these cells even in the presence of EGF. In contrast, the vector control cell line did not express β-casein RNA in the presence of DIP plus EGF. These results demonstrated that DN Ras expression prevented the disruption of hormone-induced differentiation by EGF in HC11 cells.

**HC11 cells expressing dominant negative (N17) Ras exhibit reduced Erk activation in response to EGF.**

HC11 cells expressing DN Ras(N17) were examined to determine if expression of DN Ras prevented the activation of Mek-Erk or PI-3-kinase signaling in response to EGF. In Figure 7 the stable transfectants were removed from doxycycline and grown to confluence. The cells were starved and then stimulated with EGF for varying amounts of time. Cell lysates were prepared and analyzed by Western blot using antibodies that detect phosphorylated forms of different signaling proteins. The results revealed that stimulation of HC11 vector control cells with EGF resulted in activation of p44Erk as detected by reactivity with an antibody that recognizes the active phosphorylated form of Erk. In contrast, in HC11 cells expressing DN Ras (N17) there was no activation of p44Erk, although the Erk protein levels in the cells were similar to those in the vector control cells. The analysis of other signaling proteins revealed that Akt was activated in the control HC11 cells and partially attenuated in the DN Ras HC11 cells following treatment with EGF. This demonstrated that the PI-3-kinase pathway was not completely blocked by DN Ras expression in HC11 cells. Moreover, activation of Jun kinase and p38 kinase by EGF was not deficient in the N17 Ras HC11 cells (data not shown). These results suggest that the Mek-Erk pathway was most sensitive to inhibition by DN Ras expression.
Cells infected with the control adenovirus vector or adenovirus encoding DN Ras (N17) were examined for the effect of EGF on signal transduction pathways in an analogous fashion. The results in Figure 7 demonstrated that DN Ras (N17) adenovirus also blocked the activation of Erk but not the phosphorylation of AKT on serine 473, used as a measure of PI-3-kinase activity. The results from the DN Ras(N17) expressing cells indicates that blocking the Ras pathway in this manner in HC11 cells primarily blocks signaling to the Raf-Mek-Erk pathway. Hence, these data support the conclusion that in HC11 cells activated Ras(V12) inhibits β-casein transcription via Mek-Erk signaling, and that the effect of DN Ras(N17) expression on β-casein is primarily a result of its inhibition of the Mek-Erk pathway.

Task 5. DNA Microarray analysis of changes in gene expression following induction of lactogenic differentiation.

Cell preparation.
HC11 mouse mammary epithelial cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 5 μg/ml Insulin, 10 mM Heps and 10 ng/ml epidermal growth factor (EGF). Cells were maintained in T75 flasks at confluence for 4 days, then starve the cells in the media without EGF for 24 hours. The cells were then incubated in differentiation media (serum containing RPMI with dexamethasone (10^{-6} M), insulin (5 μg/ml) and prolactin (5 μg/ml) for 72 hours, undifferentiated HC11 cells were used as control. The cells were scraped from the flasks and precipitated for microarray RNA extraction.

RNA preparation
RNAs were extracted using Trizol reagent (Invitrogen) and RNeasy maxi kit (Qiagen). Wash the cells in the flask once with PBS. Add 5 ml of Trizol to a 75 cm² flask (about 2 x10⁷ cells) and mix by rotating. Add 2/10 volume of chloroform and shake for 15 seconds. Centrifuge at 12,000g for 15 minutes at 4°C. Take off the supernatant and add it to a polypropylene tube, recording the volume of the supernatant. Then 0.53 volumes of ethanol were added to the supernatant slowly while vortex, this step produced a final ethanol concentration of 35%. Add the supernatant from an extraction to an RNeasy maxi column, which is seated in a 50 ml centrifuge tube. Centrifuge at 2880g in a clinical centrifuge with a horizontal rotor at room temperature for 5 minutes. Pour the flow-through back onto the top of the column and centrifuge again. Discard the flow-through and add 15 ml of RW1 buffer to the column, centrifuge at 2880g for 10 minutes. Discard flow-through then add 10 ml of RPE buffer and centrifuge at 2880 g for 10 minutes. Discard flow-through and add another 10 ml of RPE buffer and centrifuge at 2880g for 15 minutes. Put the column in a fresh 50 ml tube and add 1 ml of DEPC treated water from the kit to the column and lets stand for 1 minute, centrifuge at 2880g for 5 minutes. Repeat this process once. Concentrate samples to greater than 1 mg/ml by centrifugation on a MicroCon 100 filter unit at 500g. Determine the concentration and ratio of RNA in the concentrated sample by spectrophotometry. Store at -80°C. Or purify RNA to get mRNA using Oligotex mRNA kit.

Labeling, hybridization and analysis
Gene expression analysis was performed by Atlas Glass Mouse 3.8 Microarrays (Clontech Laboratories), which include 3800 mouse DNA oligo probes, a list of these genes is available at the Clontech web site (http://www.clontech.com/atlas/genelist/index.shtml). In addition, mouse NIA(15K) slides were used for microarray experiments. Fluorescent labeling of RNAs was
performed by using an Atlas Glass fluorescent labeling kit (Clontech Laboratories) according to manufacturer's manuals. Synthesized first-strand cDNAs from RNA of HC11 cells with and without differentiation were labeled with fluorescent dyes, Cy3 and Cy5 (Amersham Pharmacia Biotech), respectively. The labeling was switched during experiment, i.e. differentiation group was labeled with Cy3 two times, and Cy5 two times; and the control group was labeled with Cy5 two times, and Cy3 two times, vise versa. The quality of the labeling and the amount of each probe used were determined by absorbance measurement for Cy3 and Cy5 probes in a Beckman DU-600 scanner. Hybridization of the microarrays was carried out in a hybridization solution for 16 hours at 50°C. Then wash the slide with wash solution for 3 times provided by manufacturer. The microarray slides were scanned and analyzed by using a GenePix 4000B scanner in both Cy3 and Cy5 channels. The differentiation induced gene up- or down-regulations were obtained by dividing differentiation value over control value. The average of Cy3 and Cy5 signals from nine house-keeping genes gives a ratio which was used to normalize the individual signals. The data is included as Figure 8.

Generation of probes
Using accession number of interested gene to find out the mRNA sequence at internet, design primers for RT-PCR about 200-500 bp gene which can be used as a probe. Use Gene Amp RT-PCR kit (Roche) to amplify the cDNA and insert the correct-sized fragment into a pCR2.1 TA cloning kit (Invitrogen), candidate clone was sent to sequencing to prove the correct sequence. Double strand DNA of the insert was digested from pCR 2.1 plasmid, gel purified as a probe. The probes were used for hybridization to Northern blots containing RNA from HC11 cells undergoing lactogenic differentiation.

Verification of gene expression by Northern blot.

For Northern blot experiment, HC11 cells were treated same as microarray experiment, and then differentiated for 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h, respectively, undifferentiated cells at 0 h, and 144 h were used as controls. RNA samples (10 µg) were electrophoresed on agarose gels and transferred to nylon filters. The filters were reacted with labeled probes in hybridization solution and incubated overnight. The blots were washed and expose to X-ray film and then quantitated on a beta scanner. Beta-actin probe was used to hybridize the same membrane and then scanned to get a normalized data. Genes that exhibit increases in expression during differentiation are shown in figure 9 and genes that are decreased in figure 10.
KEY RESEARCH ACCOMPLISHMENTS
- Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promoter.
- Construction of HC11 cell lines expressing RasV12 and RasN17 under the control of a regulatable promoter.
- Demonstration that EGF disrupts differentiation via stimulation of the Erk and Akt pathways.
- Demonstration that EGF stimulation results in accumulation of GTP-bound Ras.
- Demonstration that DNRas adenovirus can be used to infect HC11 cells and that DNRas expression enhances activation of the β casein promoter.
- Detection of a set of genes that is expressed at 2-fold or greater levels during lactogenic differentiation of HC11 cells.
- Detection of a set of genes that is expressed at 2-fold or lower level during lactogenic differentiation of HC11 cells.

REPORTABLE OUTCOMES
- Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promoter.

CONCLUSIONS
We have demonstrated that the Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. This occurs in part via the increase in GTP-bound Ras in the cells. EGF stimulation results in activation of Erk, Akt and other signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras (via DNRas expression) or Erk (via PD98059) or Akt (via wortmannin) can counter the effects of EGF on differentiation. The mechanism of disruption of differentiation appears to involve interference with the growth arrest that occurs prior to the induction of differentiation; the mechanism for growth arrest may require the downregulation of Mek1 expression. In addition, EGF mitogenic stimulation also inhibits Stat5 binding to its DNA binding site in the β casein promoter.

This data focuses on the role of two Ras effector signal transduction pathways (Erk and Akt) in preventing mammary epithelial cell differentiation. Our results indicate that inhibition of either or both of the pathways blocked the disruption of differentiation by mitogens of the EGF family. However, the block in signal transduction that resulted from dominant negative Ras expression inhibited the Mek-Erk signal transduction pathway and this inhibition is responsible for the effect on lactogenic differentiation. This approach to regulating differentiation may be useful in designing therapeutic approaches using signal transduction inhibitors (STIs).

A list of genes transcriptionally regulated during lactogenic differentiation has been identified. The changes in the level of RNA for these genes has been confirmed by Northern blotting. Using this list and additional data from future expression profiling experiments, novel pathways important to the regulation of lactogenic differentiation will be identified.
REFERENCES


APPENDIX

The figures cited in the body of the report and the figure legends are contained in the attached Appendix.
Figure Legends

Figure 1. A. The effect of signal transduction inhibitors on EGF disruption of differentiation. HC11-luci cells were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media in the presence or the absence of EGF (10ng/ml). Inhibitors were added at the time of DIP induction at previously determined optimal concentrations (PD98059-20μM, LY294002-10μM, wortmannin-100nM). The luciferase activity in lysates was determined at 48 hours post induction. Luciferase activity was normalized to cell protein. The results, presented as luciferase activity in relative units and represent the mean of six determinations. *These values represent statistically significant difference (p-value .001) from the DIP + EGF condition. B. The effect of signal transduction inhibitors on EGF disruption of β-casein transcription in HC11 cells. The HC11 cells were induced to differentiate in DIP-induction media with and without EGF (10ng/ml). Inhibitors were added at the time of induction at slightly lower than optimal concentrations to avoid toxicity (PD98059-10μM, LY294002-5μM, wortmannin-50nM). Total cell RNA was harvested at 48 or 72 hours after transfer to DIP-induction media. β-casein induction was determined via Northern Blot. For quantitation β-casein expression at 48 hours was normalized to β-actin. The results are presented as relative units (R.U.).

Figure 2. EMSA. The effect of inhibitors on Stat5 DNA binding. A. HC11 cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for three days and serum-free media for 1 day. HC11 cells were pretreated with specific kinase inhibitors for 2 hours prior to DIP-induced differentiation for 15 minutes in the presence of the inhibitors. Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody. Lanes 1 and 5, control (DIP alone); lanes 2 and 6, PD 98059 (20 μM) plus DIP; lanes 3 and 7, wortmannin (20 nM) plus DIP; lanes 4 and 8, AG 490 (20 μM) plus DIP. Lane 5, 6, 7, 8 the binding was performed in the presence of anti-Stat 5 antibody for supershift. Lane 9, 10 the samples were the same as lanes 1, 2 but rabbit IgG was added. B. Gel shift (control) using Sp1 oligos as a loading control. The same protein lysates were used as in Part A, but the binding was to an Sp1 oligonucleotide. SP: supershift of Stat5.

Figure 3. HC11 cells expressing activated Ki-Ras (V12) and DN-Ki Ras (N17) under the control of the Tet-responsive promoter were utilized to evaluate the effect of Ras-based signal transduction on lactogenic differentiation. Three individual clones of HC11 cells expressing either Ras V12 or Ras N17 under the control of the Tet responsive promoter were grown to confluence, incubated in the absence of doxycycline and exposed to DIP differentiation media. The vector control cell line, TRE, was treated in parallel. RNA was harvested from cells at 0, 48, 72 and 96 hours after addition of DIP and used to determine the level of Ras and β-casein expression by Northern blotting. The Ras and β-casein expression was quantitated using a beta scanner and were normalized to the actin signal and reported in relative units.

Figure 4. The effect of RasV12 and RasN17 expression on Stat5 phosphorylation and DNA binding. A. HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12(1) or DN-RasN17(12) were grown to confluence in EGF-containing media without doxycycline to induce the expression of Ras. The cells were stimulated with DIP, and nuclear extracts were prepared from cells at 0, 15 minutes, 1 hour and 24 hours post stimulation. Total Stat5 was
immuno-precipitated and analyzed by western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 on the western blots was quantitated using a CCD camera, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. B. EMSA. HC11 TRE vector control, RasV12(1), and RasN17(12) cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for three days and serum-free media for 1 day. Treated cells were exposed to differentiation media for 15 minutes and control cells (T=0) were not exposed to DIP. (left panel) Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody as indicated. Lanes 1, 4, 7, TRE control; lanes 2, 5, 8, RasV12(1); lanes 3, 6, 9, RasN17(12). (right panel) Sp1 binding oligonucleotides were used as a loading control. Lanes 1, 4, 7 TRE control; lanes 2, 5, 8 RasV12(1); lanes 3, 6, 9 RasN17(12). Lanes 1,2,3 contain control lysate; lanes 4, 5, 6 contain lysate from DIP-treated cells; lanes 7, 8, 9 contain lysate from DIP-treated cells with the addition of 50 X cold Sp1 oligonucleotides. SP: Stat5 supershift.

Figure 5. The effect of dominant negative Ras (N17) adenovirus expression on lactogenic differentiation in HC11 cells. A. The effect of DN Ras (N17) adenovirus on Stat5 phosphorylation in response to lactogenic hormone was determined. Uninfected HC11 cells, HC11 cells infected with a control adenovirus vector and HC11 cells infected with adenovirus encoding DN Ras (N17) (at M.O.I. = 10) were incubated for 24 hours; the cells were then serum-starved overnight and stimulated with DIP for 7.5 minutes. Total Stat5 was immunoprecipitated and analyzed by western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 was quantitated using a CCD camera, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. B. HC11-luci cells infected with adenovirus vector control or adenovirus encoding dominant negative Ras (N17) were used to determine the effect of N17 Ras on β-casein driven luciferase activity. The cells were infected with the viruses described above and incubated for a period of 24 hours in media without EGF. The cells were then either stimulated with DIP for 24 hours or incubated in media without EGF for an additional 24 hours. The luciferase activity in lysates was determined and normalized to cell protein; the results, presented as luciferase activity in relative units, represent the mean of four determinations. C. The effect of dominant negative Ras adenovirus infection on HC11 expression of β-casein was determined. The HC11 cells were infected with the control or dominant negative Ras (N17) virus as described above. RNA was isolated at 0, 24 and 48 hours post induction of differentiation and used to determine the amount of β-casein transcription by Northern blotting. Hybridization of the blots with an actin probe was used as a control for RNA loading. The expression of the β-casein RNA was quantitated by measurement on a β-scanner, normalized to actin and expressed on a relative scale.

Figure 6. Ras N17 expression inhibits EGF-induced proliferation and prevents EGF-dependent disruption of lactogenic differentiation. A. HC11 TRE vector control and RasN17 (12) cells were grown in absence of doxycycline and then seeded in microtiter plates in 0.5% serum-containing media with and without EGF (10μg/ml). Cell proliferation was determined at 24, 48, 72, 96 hours post addition of EGF using the MTT assay. The results are reported as the mean of four determinations. B. The HC11 TRE vector control and RasN17 (12) cells were grown as
described above and exposed to EGF (10ng/ml) or TGFα (10ng/ml). Cell proliferation was
determined using the MTT assay and the results represent the mean of four determinations. C.
HC11 TRE vector control and RasN17 (12) cells were grown to confluence in absence of
doxycycline and then exposed to DIP in the presence or absence of EGF (10ng/ml). Total RNA
was isolated after 72 hours and used for Northern blotting. The blots were hybridized to probes
for β-casein and actin. The β-Casein and actin RNA was quantitated using a beta scanner; the β-
casein RNA was normalized to the actin RNA. The % reduction of β-casein RNA by the addition
of EGF during DIP-induced differentiation was calculated using the values for normalized β-
casein expression.

**Figure 7.** The effect of dominant negative Ras (N17) adenovirus expression on signal
transduction pathways in HC11 cells. A. The HC11 TRE vector control cells and RasN17 cell
lines were grown to confluence in EGF-containing media lacking doxycycline. The cells were
incubated in media without EGF or media without EGF and serum (*) prior to restimulation with
EGF (100ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western
blotting using antibodies specific for phosphorylated and nonphosphorylated forms of the
indicated proteins. B. HC11 cells infected with control adenovirus vector or DN Ras (N17)-
encoding adenovirus at an MOI of 10 were incubated in serum-containing media for 24 hours
and incubated in EGF-free media for 20 hours prior to stimulation with EGF (100ng/ml) for the
time indicated. Lysates of cells were harvested and analyzed by Western blotting as in part A.

**Figure 8.** The gene expression pattern in differentiated HC11 cell by using DNA microarray
technique. Two different arrays of cDNAs were used for the study. The results shown here
include a list of genes expressed at >1.5 fold increased levels or >1.5 fold decreased levels
following lactogenic differentiation of HC11 cells.

**Figure 9.** Northern Blot of HC11 RNA following induction of lactogenic differentiation. RNA
was isolated from HC11 cells induced to differentiate with lactogenic hormone at various time post
hormone addition. The blots were hybridized with cDNA probes from genes that were up-
regulated as determined by array analysis. Quantitation of the hybridization results was
performed and normalized to the actin signal.

**Figure 10.** Northern Blot of HC11 RNA following induction of lactogenic differentiation. RNA
was isolated from HC11 cells induced to differentiate with lactogenic hormone at various time post
hormone addition. The blots were hybridized with cDNA probes from genes that were down-
regulated as determined by array analysis. Quantitation of the hybridization results was
performed and normalized to the actin signal.
A. **FIG. 1**

![Graph showing luciferase activity in different differentiation conditions](image)

Differentiation Conditions

B.  

**β-Casein**

**β-Actin**

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**β-Casein 48 Hours (R.U.)**

1  .13  .53  .60  .53
A. FIG. 4

P-STAT5

STAT5

0' 15' 1hr 24hr

TRE

0' 15' 1hr 24hr

V12(1)

0' 15' 1hr 24hr

N17(12)

Stat5 Phosphorylation (R.U.)

B. DIP

0 min 15 min 15 min+Ab

DIP

0 min 15 min 15 min+cold Sp1

SP

Stat5

Sp1
FIG. 6

A.  

B.  

C.  

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FIG. 7

A.

\[ \begin{array}{c|c}
\text{TRE} & \text{N17(12)} \\
\hline
\text{P-Erk} & \hspace{1cm} \\
\text{Total Erk} & \hspace{1cm} \\
\text{P-Akt} & \hspace{1cm} \\
\text{Total Akt} & \hspace{1cm} \\
\text{GSK-3\(\beta\)} & \hspace{1cm} \\
\text{Actin} & \begin{array}{cccccc}
0 & 7.5 & 7.5^* & 0 & 7.5 & 7.5^*
\end{array}
\end{array} \]

Minutes of EGF stimulation (100 ng/ml)

B.

\[ \begin{array}{c|c}
\text{Control Virus} & \text{DN-Ras Virus} \\
\hline
\text{P-Erk} & \hspace{1cm} \\
\text{Total Erk} & \hspace{1cm} \\
\text{P-Akt} & \hspace{1cm} \\
\text{Total Akt} & \hspace{1cm} \\
\text{GSK-3\(\beta\)} & \hspace{1cm} \\
\text{Ras} & \hspace{1cm} \\
\text{Actin} & \begin{array}{cccccc}
0 & 7.5 & 30 & 0 & 7.5 & 30
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Minutes of EGF stimulation (100 ng/ml)
Figure 8. Microarray Results

1. Results of mouse 3.8K array (N=4)

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            3.034709 UNKNOWN
            3.032939 UNKNOWN
            3.012594 UNKNOWN
NM_022032.1  3.010094 Mus musculus p53 apoptosis effector related to Pmp22 (Perp-pending), mRNA
            2.973903 UNKNOWN
NM_008489.1  2.968955 Mus musculus lipopolysaccharide binding protein (Lbp), mRNA
            2.95416 UNKNOWN
V00711.1  2.951182 Mus musculus mitochondrial genome
            2.886258 UNKNOWN
            2.872857 UNKNOWN
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            2.802611 UNKNOWN
NM_010816.1  2.765922 UNKNOWN: Similar to Mus musculus microchordia (Morc), mRNA
            2.757215 UNKNOWN
XM_126337.1  2.74768 Mus musculus similar to KIAA0774 protein (2210021E03Rik), mRNA
XM_129146.1  2.72966 Mus musculus RIKEN cDNA 2410004D18 gene (2410004D18Rik), mRNA
            2.702827 UNKNOWN
S79304.1  2.669833 UNKNOWN: Similar to Rattus sp. cytochrome oxidase subunit I mRNA, partial cds; and I
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            2.620403 UNKNOWN
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AC108508.2  2.530429 Genomic sequence for Mus musculus, clone RP23-437014, complete sequence
BC012020.1  2.495217 Mus musculus, Similar to cytochrome c oxidase III, mitochondrial, clone IMAGE:4500967
            2.486124 UNKNOWN
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NM_145632.1  2.384494 Mus musculus similar to PROBABLE DNA-DIRECTED RNA POLYMERASES I, II, AND
            2.377329 UNKNOWN
AB042432.1  2.369087 UNKNOWN: Similar to Mus musculus domesticus mitochondrial DNA, complete genome
AL117595.1  2.365093 UNKNOWN: Similar to Homo sapiens mRNA; cDNA DKFZp564C2063 (from clone DKFZ
AB042523.1  2.359226 UNKNOWN: Similar to Mus musculus mitochondrial DNA, complete genome, strain:SAM
XM_153383.1  2.33976 UNKNOWN: Similar to Mus musculus LOC217571 (LOC217571), mRNA
NM_019714.1  2.338437 Mus musculus B lymphocyte gene 1 (Bce1-pending), mRNA
            2.330001 UNKNOWN
XM_181325.1  2.328126 Mus musculus RIKEN cDNA 2310063P06 gene (2310063P06Rik), mRNA
AB042432.1 2.327995 Mus musculus domesticus mitochondrial DNA, complete genome
BC029250.1 2.324261 Mus musculus, clone IMAGE:4225337, mRNA
AF214115.1 2.314126 UNKNOWN: Similar to Peromyscus maniculatus H19 mRNA, complete cds
XM_138680.1 2.309929 UNKNOWN: Similar to Mus musculus similar to Zinc finger protein 208 (LOC212569), mR
XM_178097.1 2.306275 UNKNOWN: Similar to Mus musculus similar to zinc finger protein 97 [Mus musculus] (L
2.300626 UNKNOWN
2.300514 UNKNOWN
BC005741.1 2.298709 UNKNOWN: Similar to Mus musculus, clone MGC:11932 IMAGE:3599820, mRNA, comp
AB042432.1 2.290126 Mus musculus domesticus mitochondrial DNA, complete genome
AF378830.1 2.280251 Mus musculus cytochrome c oxidase subunit II (Cox2) mRNA, complete cds; mitochondri
NM_133689.1 2.269036 Mus musculus RIKEN cDNA 4930579J09 gene (4930579J09Rik), mRNA
NM_010937.1 2.263036 Mus musculus neuroblastoma ras oncogene (Nras), mRNA
NM_0111340.1 2.258001 Mus musculus serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigi
NM_019946.1 2.255483 Mus musculus microsomal glutathione S-transferase 1 (Mgst1), mRNA
Y17323.1 2.251001 UNKNOWN: Similar to Rattus norvegicus CDK109 mRNA
X70496.1 2.242086 UNKNOWN: Similar to R. norvegicus mRNA for Mss4 protein
2.231822 UNKNOWN
2.229957 UNKNOWN
BC031203.1 2.229787 Mus musculus, clone IMAGE:5066398, mRNA
NM_010638.1 2.226703 UNKNOWN: Similar to Mus musculus Kruppel-like factor 9 (Klf9), mRNA
2.222709 UNKNOWN
XM_129811.1 2.222592 Mus musculus RIKEN cDNA 231006109 gene (231006109Rik), mRNA
NM_009681.1 2.218333 Mus musculus adaptor-related protein complex AP-3, sigma 1 subunit (Ap3s1), mRNA
XM_150228.2 2.208729 UNKNOWN: Similar to Mus musculus LOC235584 (LOC235584), mRNA
2.20785 UNKNOWN
BC006888.1 2.198633 UNKNOWN: Similar to Mus musculus, clone MGC:6272 IMAGE:2647757, mRNA, comple
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2.184586 UNKNOWN
XM_134035.1 2.176983 Mus musculus similar to hypothetical protein FLJ11305 [Homo sapiens] (LOC234069), r
NM_146163.1 2.175514 Mus musculus hypothetical protein MGC11654 (MGC11654), mRNA
2.175471 UNKNOWN
2.170787 UNKNOWN
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2.158989 UNKNOWN
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2.151456 UNKNOWN
2.147605 UNKNOWN
2.146966 UNKNOWN
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2.129659 UNKNOWN
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2.107172 UNKNOWN
XM_112239.1 2.097092 UNKNOWN: Similar to Mus musculus similar to zinc finger protein 40 (LOC195533), mRf
NM_009266.1 2.095076 Mus musculus selenophosphate synthetase 2 (Sps2), mRNA
NM_031386.1 2.091199 Mus musculus testis expressed gene 14 (Tex14), mRNA
XM_158024.1 2.084281 UNKOWN: Similar to Mus musculus LOC214101 (LOC214101), mRNA
XM_144763.1 2.082466 UNKOWN: Similar to Mus musculus similar to anti-poly(dC) monoclonal antibody kappa
NM_009263.1 2.080536 Mus musculus secreted phosphoprotein 1 (Spp1), mRNA
NM_011258.1 2.067001 Mus musculus replication factor C, 140 kDa (Recc1), mRNA
XM_129401.2 2.066203 Mus musculus RIKEN cDNA 4933411J24 gene (4933411J24Rik), mRNA
XM_138798.1 2.056712 UNKOWN: Similar to Mus musculus similar to pol protein [Sus scrofa] (LOC238787), mRNA
NM_007621.1 2.049525 Mus musculus carbonyl reductase 2 (Cbr2), mRNA
NM_024185.2 2.039376 Mus musculus RIKEN cDNA 2310047O13 gene (2310047O13Rik), mRNA
NM_013449.1 2.037027 UNKOWN: Similar to Homo sapiens bromodomain adjacent to zinc finger domain, 2A (I)
NM_015263.1 2.031245 Homo sapiens rabconnectin-3 (RC3), mRNA
NM_007621.1 2.020431 Mus musculus carbonyl reductase 2 (Cbr2), mRNA
NM_007570.1 2.018471 Mus musculus B-cell translocation gene 2, anti-proliferative (Btg2), mRNA
XM_128441.2 2.007491 Mus musculus similar to Zinc finger protein 118 [Mus musculus] (LOC224591), mRNA
NM_005113.1 2.005113 Mus musculus transferrin receptor (Trfr), mRNA
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NM_011595.1 0.494771 Mus musculus tissue inhibitor of metalloproteinase 3 (Timp3), mRNA
NM_008894.1 0.493414 Mus musculus polymerase (DNA directed), delta 2, regulatory subunit (50 kDa) (Pold2), mRNA
NM_011638.1 0.493036 Mus musculus transferrin receptor (Trfr), mRNA
NM_013493.1 0.492277 Mus musculus G protein-coupled receptor 56 (Gpr56), mRNA
NM_013870.1 0.492239 Mus musculus smoothelin (Smtn), mRNA
NM_134189.1 0.491 Mus musculus UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2, beta (Galfact), mRNA
NM_019813.1 0.490641 Mus musculus drebrin 1 (Dbn1), mRNA
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NM_019521.1 0.483139 Mus musculus growth arrest specific 6 (Gas6), mRNA
NM_146098.1 0.482955 Mus musculus hypothetical protein MGC28180 (MGC28180), mRNA
NM_013704.1 0.481381 UNKOWN
XM_137043.1 0.480612 Mus musculus similar to squamous cell carcinoma antigen recognized by T cells 2 [Homines]
NM_013737.1 0.478506 Mus musculus phospholipase A2 group VII (platelet-activating factor acetylhydrolase, plasminogen activator) (Pla2g7), mRNA
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NM_024427.1 0.455214 Mus musculus tropomyosin 1, alpha (Tpm1), mRNA
NM_007564.1 0.455199 Mus musculus zinc finger protein 36, C3H type-like 1 (Zfp36l1), mRNA
NM_013541.1 0.454435 Mus musculus glutathione S-transferase, pi 2 (Gstp2), mRNA
NM_133185.1 0.453914 Mus musculus RIKEN cDNA 0610011C19 gene (0610011C19Rik), mRNA
XM_123426.1 0.450573 Mus musculus RIKEN cDNA 2700063G02 gene (2700063G02Rik), mRNA
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NM_007680.1 0.447048 UNKNOWN
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NM_007899.1 0.444352 UNKNOWN: Similar to Mus musculus extracellular matrix protein 1 (Ecm1), mRNA
XM_129745.2 0.443464 Mus musculus procollagen, type III, alpha 1 (Col3a1), mRNA
XM_181394.1 0.443004 Mus musculus procollagen, type IX, alpha 3 (Col9a3), mRNA
NM_009929.1 0.442813 Mus musculus procollagen, type XVII, alpha 1 (Col18a1), mRNA
BC006061.1 0.442602 Mus musculus, clone IMAGE:3591705, mRNA
NM_023118.1 0.441519 Mus musculus disabled homolog 2 (Drosophila) (Dab2), mRNA
NM_007833.1 0.440749 UNKNOWN: Similar to Mus musculus decorin (Dcn), mRNA
NM_025378.1 0.44 Mus musculus RIKEN cDNA 1110004C05 gene (1110004C05Rik), mRNA
XM_128198.1 0.439966 Mus musculus expressed sequence Al316867 (Al316867), mRNA
XM_126577.2 0.439568 Mus musculus mitogen activated protein kinase kinase 6 (Map2k6), mRNA
NM_013467.1 0.439396 Mus musculus aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1), mRNA
NM_007831.1 0.438683 UNKNOWN
NM_037093.1 0.438683 UNKNOWN
XM_132709.1 0.436531 Mus musculus RIKEN cDNA 2310008J16 gene (2310008J16Rik), mRNA
AL161729.27 0.435648 UNKNOWN: Similar to Human DNA sequence from clone RP11-435OS on chromosome
XM_132915.2 0.430084 Mus musculus similar to retinoic acid inducible protein 3 [Mus musculus] (LOC232431), r
NM_146098.1 0.426666 Mus musculus hypothetical protein MGC28180 (MGC28180), mRNA
NM_146102.1 0.414826 Mus musculus hypothetical protein MGC28084 (MGC28084), mRNA
NM_008160.1 0.41452 Mus musculus glutathione peroxidase 1 (Gpx1), mRNA
NM_007831.1 0.407415 UNKNOWN
NM_019791.1 0.406249 Mus musculus melanoma antigen, family D, 1 (Maged1), mRNA
XM_134149.2 0.406008 Mus musculus similar to FAT tumor suppressor (Drosophila) homolog [Rattus norvegicus]
XM_137731.2 0.402814 UNKNOWN
XM_137731.2 0.397649 Mus musculus similar to Component of gems 4 (Gemin4) (p97) (LOC237853), mRNA
NM_010500.1 0.396203 Mus musculus immediate early response 5 (Ier5), mRNA
M95495.1 0.394583 UNKNOWN: Similar to Dog Na/Ci-dependent taurine transporter mRNA, complete cds
NM_144865.1 0.389533 Mus musculus hypothetical protein MGC28827 (MGC28827), mRNA
XM_034188.1 0.386053 Mus musculus, clone IMAGE:4976037, mRNA
NM_138685.1 0.37926 Mus musculus elfin-like protein 1 (SWAM1), mRNA
NM_053178.1 0.377991 Mus musculus lipidosis-related protein lipidosin (lpd), mRNA
AB085694.1 0.367999 Mus musculus bpag1-e mRNA for bullous pemphigoid antigen 1-e, partial cds
3. Comparison of common genes from two arrays for reproducibility. Results from two arrays were compared for reproducibility, data showed that the regulation of genes have the same direction (up- or down-regulation), although the mean numbers were different.

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0.79175 | NM_008619 | 0.864599 | Moloney leukemia virus 10 |
0.79175 | NM_008619 | 0.555623 | Moloney leukemia virus 10 |
0.7585 | NM_008211 | 0.730108 | H3 histone, family 3B |
0.7585 | NM_008211 | 0.68806 | H3 histone, family 3B |
0.7565 | NM_019774 | 0.984505 | A kinase anchor protein 8 |
0.7145 NM_008160  0.41452 glutathione peroxidase 1
0.69325 NM_008410  0.620398 integral membrane protein 2 B
0.69325 NM_008410  0.570781 integral membrane protein 2 B
0.629 NM_008557  0.315307 FXYD domain-containing ion transport regulator
0.59975 NM_009335  0.609136 transcription factor AP-2, gamma
Fig. 9 Up-regulated genes

Casein

Actin

Pip

Actin

Kif9

Actin

Sgk

Actin

Ap3

Actin

Spic

Actin

0.6 Kb

1.7 Kb

2.5 Kb

1.2 Kb

2.3 Kb
Fig. 10 Down-regulated genes

Lane 1 to 9: control, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 144 h control; Left: Northern blot of different genes and their correspondant beta-actin expression, right: fold changes of gene expression normalized by beta-actin. Abbreviation Klf9 is changed to Bteb1 in the new version of NCBI web.