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Award Number: DAMD17-01-1-0264

TITLE: Control of Mammary Differentiation by Ras-Dependent Signal Transduction Pathways

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REPORT DATE: May 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Final (1 May 2001 - 30 Apr 2005)

4. TITLE AND SUBTITLE
Control of Mammary Differentiation by Ras-Dependent Signal Transduction Pathways

5. FUNDING NUMBERS
DAMD17-01-1-0264

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)* 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 determined the mechanism by which Ras activation, an important mitogenic signal transduction pathway that is frequently activated in breast carcinoma, inhibits mammary differentiation and apoptosis. The Ras pathway is activated by EGF stimulation of HC11 mammary epithelial cells. EGF stimulation results in activation of Erk and Akt signal transduction pathways and prevents lactogenic differentiation. Inhibition of either Ras, Erk or Akt can counter the effects of EGF on lactogenic differentiation. Expression of DNRas in HC11 cells enhances Stat5 phophorylation and DNA binding; this results in increased lactogenic differentiation as measured by elevated beta casein transcription, lipid synthesis and mammosphere formation. Using DNA microarray analysis global changes in gene expression were measured in HC11 cells undergoing lactogenic differentiation. Using the same technology genes whose expression was altered by EGF stimulation during differentiation were identified. This information provides an expression profile of gene regulation during lactogenic differentiation of HC11 cells, and identified novel targets in breast tissue exposed to mitogens.

14. SUBJECT TERMS
Breast, mammary, differentiation, Ras, signal transduction

15. NUMBER OF PAGES
55

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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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 confluency 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 inhibition 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 tested our hypothesis by constructing HC11 cell lines carrying dominant-negative (DN) Ras and HC11 cell lines expressing elevated levels of active Ras. These cell lines were used to dissect the control of differentiation using a series of markers for differentiation and cell cycle changes.

In addition, cDNA microarray analysis techniques was used to detect global changes in gene expression induced by differentiation in the HC11 cell background. We identified genes whose expression is specifically increased and decreased in these cells following induction of lactogenic differentiation. Genes whose expression was altered by exposure to EGF during lactogenic differentiation were also identified. This information provides an expression-based profile of gene regulation during lactogenic differentiation of HC11 cells, and it will be useful in identifying novel targets in breast tissue exposed to mitogens.

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 Doxicyclin (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^r) gene for antibiotic selection in mammalian cells. pRevTRE also includes the *E. Coli* Amp^r 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 promotor was used to inducibly express the genes of interest in response to varying concentrations of Doxicyclin (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 2BV12) 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 Doxicyclin (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 [7], and recent data suggests that this block required Ras and PI-3-kinase activity [11]. 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 b-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 4B, 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 4C). 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 promotor 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 promotor-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 demonstated 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, 6, 9. 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 Hepes and 10 ng/ml epidermal growth factor(EGF). Cells were maintained in T75 flasks after 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 dexamethazone(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 cm^2 flask (about 2×10^7 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 Oligtex mRNA kit.

Labeling, hybridization and analysis. Gene expression analysis was performed using mouse NIA(15K) oligonucleotide slides for microarray experiments (Agilent). In addition, 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). 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.
Statistical analysis. Normalization and analysis of the gene expression profiles was performed as follows:
Exclude the spot if red and green intensity is below 30. Normalize (center) each array using median over entire array. Truncate intensity ratios (and inverse ratios) greater than 64. Exclude a gene under any of the following conditions: Less than 20 % of expression data have at least a 1.5 -fold change in either direction from gene's median value. Percent of data missing or filtered out exceeds 50 %.
In DIP vs control experiments, there are 10813 genes that passed filtering criteria in total of 20280 genes. And the first 2479 genes are significant at the nominal 0.05 level of the paired T-test.
In EGF plus DIP vs DIP experiments, there are 1386 genes passed filtering criteria in total of 20280 genes. And the first 1129 genes are significant at the nominal 0.05 level of the paired T-test.
The results of these experiments are shown in Tables 1-5 (Wang and Cutler, 2005, appendix item #2).

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.

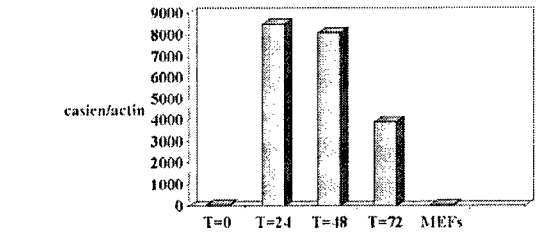
Verification of gene expression by Northern blot. HC11 cells were treated in the same way as for microarray experiment, and then induced to differentiated for 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h, respectively; undifferentiated cells at 0 h, and 144 h were used as controls. In other experiments RNA from cells induced to differentiate for 72 hours in the presence or absence of EGF (10μg/ml) were compared to RNA from undifferentiated control cells. 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. Changes in gene expression during differentiation are demonstrated in northern blots (figures 1 and 2 in Wang and Cutler, 2005, appendix item #2).

Oligonucleotide microarrays containing approximately 20,000 genes (Agilent) and 3,800 genes (Clontech) were used in this study. The gene expression patterns of the regulated genes were verified by Northern blot analysis. Data were analyzed by using a NIH program (BRB Array Tools). Microarray expression analysis and Northern blot data indicate that lactogenic differentiation induced multiple gene transcription in HC11 cells, while the presence of EGF blocked most of these transcription processes. Normalization and analysis of the gene expression profiles was perfomed. In DIP vs control experiments, there are 10813 genes that passed filtering criteria from a total of 20280 genes. The first 2479 genes are significant at the nominal 0.05 level of the paired T-test. In EGF plus DIP vs DIP experiments, there are 1386 genes passed filtering criteria in total of 20280 genes. The first 1129 genes are significant at the nominal 0.05 level of the paired T-test.

By using microarrays from different sources and performing additional northern blots the reliability of the array data has been demonstrated. Statistical analysis and the use of additional analysis tools have allowed us to draw conclusions from the data regarding the role of EGF in blocking lactogenic differentiation. Some of the conclusions include the following. Numerous ribosomal protein transcripts were induced by stimulation with lactogenic hormone even in the presence of EGF, suggesting that these genes are not a target of the mitogen-induced inhibition. Differentiation also induced gene expression changes in cell cycle regulators. Data showed that while cyclin D1 transcription was inhibited as predicted, cyclin G2 and cyclin-dependent kinase inhibitor were induced during differentiation. $P21^{CIP}$ was known to be induced following initiation of lactogenic differentiation, but no previous data was available concerning cyclin G2. In addition, the transcription element binding proteins KLF4, KLF6, KLF7 and KLF9(BTEB1) were induced during lactogenic differentiation but were inhibited by EGF. This provided, in part, an explanation of the large scale gene expression changes during lactogenic differentiation that were blocked by EGF. Interestingly, KLF6 was recently proposed to be a tumor suppressor gene. Therefore, the increased transcription and possible expression of this gene might explain the decreased morbidity of breast cancer in women who had early pregnancy and lactation. Another interesting finding from this study is that connective tissue growth factor(CTGF) is highly induced during differentiation. Our current studies are directed toward understanding the contribution of some of the genes whose expression is regulated during lactogenic differentiation.

Tasks 10 and 11 Primary mammary epithelial cells respond like HC11 cells.
Cultures of primary mammary epithelial cells were prepared form mid-pregnant FVB mice as described. The cells were stimulated with DIP, and following isolation of RNA from the cells, the expression of β-casein and whey acidic protein(WAP) were determined by realtime PCR. Real time PCR of RNA from primary mammary epithelial cell culture stimulated with DIP exhibited increased expression of the milk protein markers (fig.8). *The results revealed that the primary mammary epithelial cell cultures responded to DIP in a similar way to the HC11 cell line.*
Primary mammary epithelial cell cultures were set up and stimulated with DIP in the presence of EGF, and Mek1 inhibitors were included to determine if signal transduction through the Mek-Erk pathway could block differentiation in primary cell cultures. *The results of β-casein expression indicated that EGF stimulation at the time of DIP treatment blocked lactogenic differentiation in the primary cells and that this could be prevented by inhibiting the Mek-Erk*

pathway. In addition, primary mammary epithelia cell cultures infected with DNRas and DNMek1 adenovirus exhibited enhanced β-casein expression following stimulation with DIP.



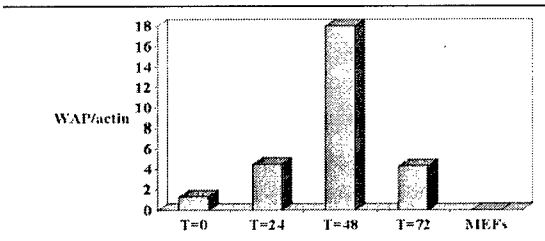


Figure 8. Lactogenic differentiation in primary mammary epithelial cell cultures. Mammary glands from mid-pregnant mice were isolated and cultures prepared. Cells from pooled cultures of glands were seeded in 6-well plates, grown to confluence in 10% FBS, 10 ng/ml EGF. The growth media was replaced with DIP, At 0, 24, 48, 72 hours of differentiation RNA was isolated and the level of β-casein expression was determined by SYBR green-based realtime PCR. The values were calculated based on normalization to β-actin. The results represent the mean of triplicate determinations.
RNA from MEF cultures prepared at the same time

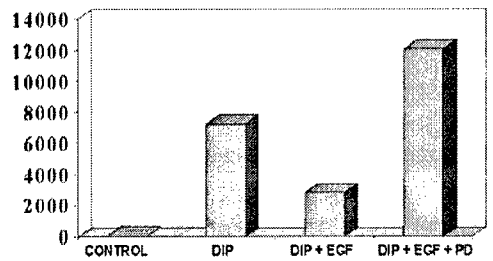


Figure 9. Effect of EGF and Mek1 inhibitors on lactogenic differentiation of primary mammary cells. Primary mammary epithelial cell cultures were seeded in 6-well plates, grown to confluence in 10% FBS, 10 ng/ml EGF. The growth media was replaced with DIP , DIP +EGF(10ng/ml), DIP+EGF(10ng/ml)+PD98059 (10μg/ml). At 48 hours RNA was isolated and the level of β-casein expression was determined by SYBR green-based realtime PCR. The values were calculated based on normalization to β-actin. The results represent the mean of triplicate determinations.

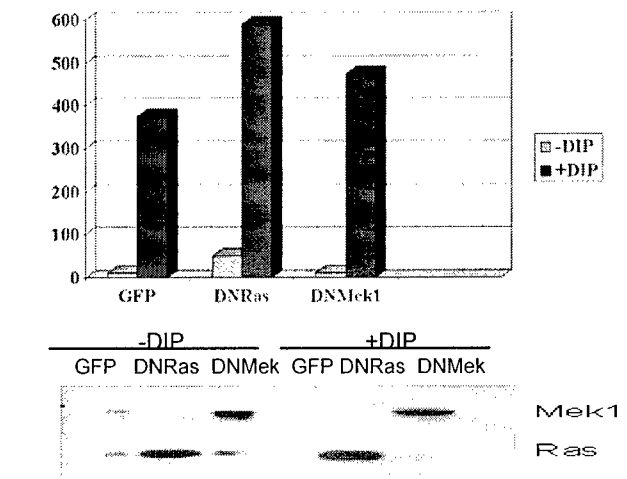


Figure 10. Effect of DNRas and DNMek1 on lactogenic differentiation of primary mammary cells. Primary mammary epithelial cell cultures were seeded in 6-well plates, grown to 90% confluence in 10% FBS, 10 ng/ml EGF. The cells were infected with 10 MOI of adenovirus encoding GFP,DNRas or DNMek1 for 5 hours. The growth media was replaced and cells were grown for 24 hours prior to addition of DIP. At 48 hours RNA and protein was isolated and the level of β-casein expression was determined by SYBR green-based realtime PCR. The values were calculated based on normalization to β-actin. The results represent the mean of triplicate determinations. The level of DNRas and DNMek1 in the cells was measured by western blotting.

Hence, we conclude that primary mammary epithelial cell cultures provide an additional model with which to study the effect of Ras pathway activation during lactogenic differentiation. In addition, the results confirm those obtained with HC11 cells demonstrating that Raf-Mek-Erk stimulation blocked lactogenic differentiation.

Expression of CTGF in HC11 lactogenic differentiation. Our expression profiling studies revealed that the level of CTGF was highly increased in DIP treated HC11 cells undergoing

lactogenic differentiation. CTGF exhibited a >12 fold increase compared to unstimulated cells. A series of experiments determined that CTGF expression was induced by dexamethasone (dex), a component of DIP and expression required only dex and not insulin or prolactin (Fig.11). In addition, the increase in CTGF induction by dex was blocked by actinomycin D. While CTGF expression could be induced by TGFβ treatment of HC11 cells, the expression of CTGF by dex was not TGFβ-dependent: the results of combined dex and TGFβ treatments were additive and TGFβ antibody treatment of dex-stimulated cultures failed to block CTGF expression. Moreover, our experiments demonstrated that neither estrogen nor progesterone treatment of cells induced expression of CTGF in HC11 cells (figure 12).

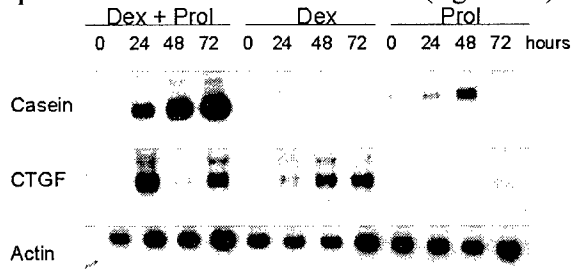


Figure 11. HC11 cells in the presence of insulin were stimulated with Dexamethasone (1μg/ml), prolactin (10μg/ml), or dexamethasone plus prolactin for 0-72 hours. RNA was extracted and examined by northern blotting for expression of β-casein, CTGF and actin.

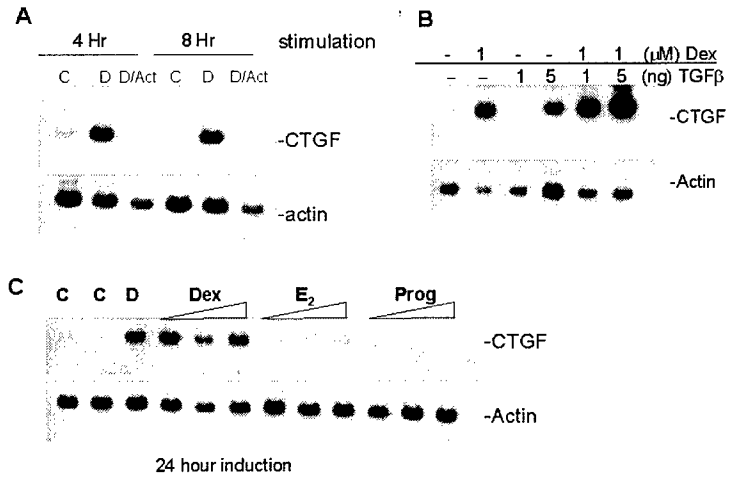


Figure 12. CTGF expression is blocked by act D, induced by TGFβ but not induced by estrogen or progesterone.
A. HC11 cells were grown to confluence then stimulated with DIP(D) or DIP + actinomycinD (1μg/ml)(D/Act) or were not treated (C). RNA was isolated at times indicated and levels of CTGF and actin RNA were determined by northern blot.
B. HC11 cells were grown to confluence then stimulated with dex or TGFβ at the indicated concentrations for 24 hours. RNA was isolated and levels of CTGF and actin RNA were determined by northern blot.
C. HC11 cells were grown to confluence then stimulated with DIP(D) or Dexamethasone (Dex), estradiol (E_2)or progesterone (Prog) at increasing concentrations of 10^{-7}, 10^{-6}, 10^{-5} M for 24 hours. RNA was isolated and levels of CTGF and actin RNA were determined by northern blot.

B. The contribution of CTGF expression to HC11 lactogenic differentiation. Following the determination that CTGF was readily induced by dexamethasone, experiments were performed to determine the contribution of CTGF to lactogenic differentiation. HC11-luci cells were transiently transfected with expression plasmid encoding CTGF and at 48 hours post transfection the cells were exposed to DIP. HC11 stable transfectants were used to test the effect of CTGF on differentiation. In HC11 cells CTGF expression enhanced the expression of β-casein and in HC11-luci cells CTGF expression enhanced β-casein-driven luciferase activity, *indicating that CTGF could positively influence the differentiation state of the cells.* However, the activity was dependent on prolactin (fig. 13).

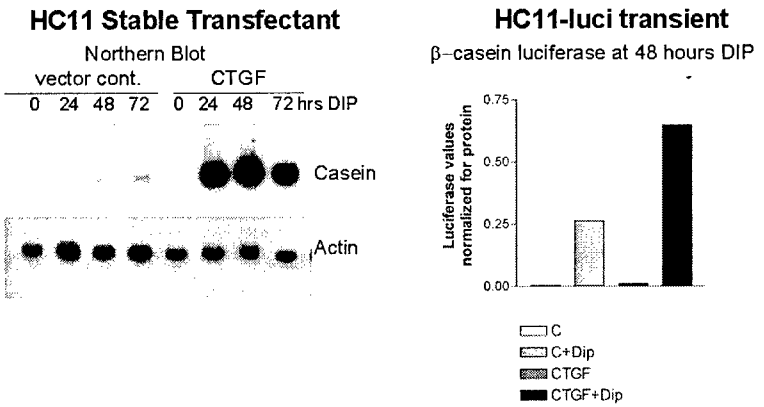


Figure 13. Expression of CTGF enhances HC11 lactogenic differentiation. **HC11 cells** were transfected with empty vector or expression plasmid encoding CTGF and selected in G418 for cells that contained the plasmids. Colonies were pooled and grown to confluence and exposed to DIP. RNA was isolated at times indicated and levels of casein and actin RNA were determined by northern blot. **HC11-luci** cells were transiently transfected with empty vector or expression plasmid encoding CTGF, and at 48 hours post transfection the cells were exposed to DIP for 48 hours. Luciferase activity was determined and expressed as relative units

Examination of the expression of CTGF in primary mammary epithelial cells revealed that CTGF is induced by DIP (data not shown). CTGF expression in mammary gland during pregnancy and lactation was analyzed. In RNA isolated from mouse mammary gland tissue CTGF expression was not detected in virgin glands but was expressed during pregnancy and lactation generally coincident with □-casein expression. CTGF RNA begins to decline by day 3 of lactation and is elevated late in involution (fig14).

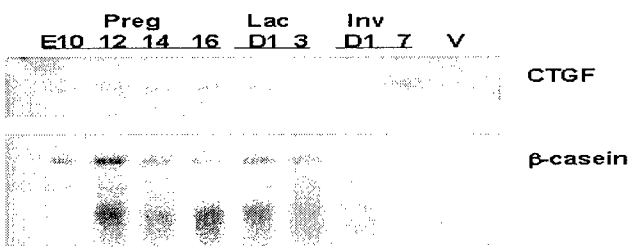


Figure 14. Expression of CTGF is detected in mammary gland during pregnancy and lactation. Northern blot of 5 μg per well of RNA isolated from mouse mammary gland at stages of pregnancy (E days 12,14,16), lactation (days 1 and 3) , involution (days 1 and 7) and vigin gland (V) was hybridized to probes for CTGF (3kb RNA) and β-casein (1.8 and 0.6 kb bands) .

KEY RESEARCH ACCOMPLISHMENTS

-Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.

-Construction of HC11 cell lines expressing RasV12 and RasN17 under the control of a regulatable promotor.

-Demonstartion that EGF disrupts differentiation via stimulation of the Erk and Akt pathways.

-Demonstration that DNRas adenovirus can be used to infect HC11 cells and that DNRas expression enhances activation of the β casein promotor.

-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 whose expression decreased 2-fold during lactogenic differentiation of HC11 cells.

-Detection of genes whose expression increased or decreased as a result of exposure to EGF during differentiation.

REPORTABLE OUTCOMES

- Construction of HC11 mouse mammary epithelial cells capable of regulating a tetracycline-inducible promotor.
- **publication**: Cerritto MG, Galbaugh T, Chopp T, Wang W, Salomon D and Cutler ML. Dominant negative Ras enhances lactogenic hormone-induced differentiation by blocking activation of the Raf-Mek-Erk signal transduction pathway. Journal of Cellular Physiology, 201:244-258, 2004.
- **publication**: Wang W and Cutler ML. Gene expression profiling in HC11 mammary epithelial cells undergoing lactogenic differentiation. *Submitted*, 2005.

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 promotor.

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. By using microarrays from different sources and performing additional northern blots the reliability of the array data has been demonstrated. Statistical analysis and the use of additional analysis tools have allowed us to draw conclusions from the data regarding the role of EGF in blocking lactogenic differentiation. Using this list and additional data from future expression profiling experiments, novel pathways important to the regulation of lactogenic differentiation will be identified.

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APPENDIX

Figures 1-7 are contained in the attached reprint, Journal of Cellular Physiology 201:244-258 (2004)

JOURNAL OF CELLULAR PHYSIOLOGY 201:244–258 (2004)

Dominant Negative Ras Enhances Lactogenic Hormone-Induced Differentiation by Blocking Activation of the Raf–Mek–Erk Signal Transduction Pathway

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Epidermal growth factor (EGF) and Ras mitogenic signal transduction pathways are frequently activated in breast carcinoma and inhibit mammary differentiation and apoptosis. HC11 mouse mammary epithelial cells, which differentiate and synthesize β-casein following growth to confluency and stimulation with lactogenic hormones, were used to study EGF-dependent signaling during differentiation. Blocking Mek–Erk or phosphotidylinositol-3-kinase (PI-3 kinase) signaling with specific chemical inhibitors enhanced β-casein promotor-driven luciferase activity. Because EGF stimulation of HC11 cells resulted in the activation of Ras, the effect of activated Ras (RasV12) or dominant negative (DNRasN17) on lactogen induced differentiation was examined. HC11 cell lines expressing RasV12 or DNRasN17 under the control of a tetracycline (tet)-responsive promotor were constructed. Activated RasV12 expression resulted in reduced tyrosine phosphorylation of Stat5 and a delay in β-casein expression in response to prolactin. However, the expression of tet-regulated DNRasN17 and adenovirus-encoded DNRasN17 enhanced Stat5 tyrosine phosphorylation, Stat5 DNA binding, and β-casein transcription. The expression of DNRasN17 blocked the activation of the Mek–Erk pathway by EGF but did not prevent the phosphorylation of AKT, a measure of activation of the PI-3-kinase pathway. Moreover, the expression of DNRasN17 prevented the block to lactogenic differentiation induced by EGF. Stimulation of HC11 cells with prolactin resulted in the association of the SHP2 phosphatase with Stat5, and this association was prevented by DNRasN17 expression. These results demonstrate that in HC11 cells DNRas inhibits the Mek–Erk pathway and enhances lactogenic hormone-induced differentiation. This occurs, in part, by inhibiting the association of the SHP2 phosphatase with Stat5. J. Cell. Physiol. 201: 244–258, 2004. Published 2004 Wiley-Liss, Inc.†

Mammary epithelial cells undergo periodic cycles of growth, differentiation, and apoptosis during pregnancy and lactation. A complex series of signals that include mammotrophic hormones, locally derived growth factors and stroma initiate and regulate these processes. In this study, we address the problem of inhibition of mammary cell differentiation by mitogenic growth factors, including epidermal growth factor (EGF), that are found locally in the mammary gland. Because elevated levels of different growth factors in the EGF family such as transforming growth factor α (TGFα) and amphiregulin have been reported in breast tumors (Dotzlaw et al., 1990; Mizukami et al., 1991; Salomon et al., 1999) this study addresses an important issue in both normal development and neoplasia.

The HC11 mouse mammary epithelial cell line used in this study was derived from the COMMA-1D cell line, which was established from a midpregnant BALB/c

Maria Grazia Cerrito and Traci Galbaugh contributed equally to this work.

Contract grant sponsor: Congressionally Directed Medical Research Program; Contract grant number: DAMD 17-01-1-0264; Contract grant sponsor: National Cancer Institute (to MLC); Contract grant number: R01CA90908.

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Received 24 July 2003; Accepted 7 January 2004

DOI: 10.1002/jcp.20077

mouse mammary gland (Danielson et al., 1984). This cell line has been employed as a model system for the study of regulation of mammary differentiation both in vitro and in vivo. HC11 cells introduced into the mammary fat pad differentiate into ductal-like structures (Humphreys and Rosen, 1997). In culture, HC11 mouse mammary epithelial cells differentiate and synthesize β-casein following growth to confluency and stimulation with the lactogenic hormone mixture, DIP (dexamethasone, insulin, prolactin) (Ball et al., 1988). β-casein expression in HC11 cells has been used as marker of differentiation, and its regulation in HC11 cells in culture reflects the in vivo regulation of expression of this protein in the mammary gland during pregnancy by prolactin (Ball et al., 1988; Peterson and Haldosen, 1998). Production of β-casein in cell culture is dependent upon both the presence of specific mitogens during the growth of the HC11 cells, cell–cell contact, deposition of extracellular matrix, and the subsequent prolactin-dependent activation of Stat5a and b when the cells have reached confluency (Taverna et al., 1991; Marte et al., 1995; Merlo et al., 1996). Prolactin stimulation results in Jak2-mediated tyrosine phosphorylation of Stat5a and b and nuclear translocation of these factors (Gouilleux et al., 1994; Marte et al., 1995; Han et al., 1997; Ali, 1998). In HC11 cells, the activation of Stat5 by prolactin is not dependent on the Ras–Erk pathway (Wartmann et al., 1996). However, the hormone-induced expression of β-casein can be blocked by the activation of different tyrosine kinase signaling pathways at the time of prolactin addition (Hynes et al., 1990; Marte et al., 1995; Merlo et al., 1996; Peterson and Haldosen, 1998). Previous studies have demonstrated that EGF prevents HC11 differentiation in response to lactogenic hormones. However, several signal transduction pathways have been implicated as responsible for the inhibition of β-casein synthesis. One study reported that the EGF-dependent inhibition of β-casein expression occurred through a Ras- and phosphoinositol-3′-kinase (PI-3 kinase)-dependent mechanism, not a Ras–Erk pathway (DeSantis et al., 1997; Salomon et al., 1999). More recently PTP-PEST, a phosphatase that can act on Jak2, was implicated as an EGF-induced protein contributing to this inhibition (Horsch et al., 2001).

Receptor tyrosine kinase (RTK) activation through different growth factor receptors leads to activation of Ras by guanine nucleotide exchange factors. The ErbB family of RTKs use this mechanism to stimulate signal transduction in the Ras pathway (Janes et al., 1994). Signal transduction that is downstream of Ras depends on the association of Ras GTPase with its effector proteins. Several proteins have been identified which associate with Ras in a GTP-dependent manner. These include Raf-1, RasGAP, p110 subunit of PI-3-kinase, AF6, Rin-1, Mek kinase 1, protein kinase C zeta, and RalGDS (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Kikuchi et al., 1994; Rodriguez-Vicania et al., 1994; Akasaka et al., 1996). Activation of Ras initiates a signaling cascade via activation of the Raf-1 and Mek-1 kinases resulting ultimately in the activation of Erk kinases (Shibuya et al., 1992; Moodie et al., 1993). The results of several studies have indicated that the activation of the Ras–Erk kinase pathway can either induce or enhance the differentiation of breast cancer cell lines (Bacus et al., 1992; Giani et al., 1998; Lessor et al., 1998). However, the activation of the Ras–Raf–Mek–Erk pathway by EGF inhibits hormone-induced differentiation in HC11 cells (Hynes et al., 1990), and the expression of v-Raf, which also activates Erk signaling, has a similar effect (Jehn et al., 1992; Happ et al., 1993).

In the present study, we have addressed the mechanism of EGF inhibition of HC11 lactogenic hormone-induced differentiation by examining the involvement of specific signal transduction pathways on differentiation. These studies indicated that the Ras–Mek–Erk pathway and, to a lessor degree, the PI-3 kinase pathway contribute to this inhibition by EGF. Moreover, the expression of DN Ras prevented the EGF-dependent disruption of HC11 differentiation indicating that Ras-signaling is central to this process. DN Ras expression blocked EGF-induced activation of the Mek–Erk signaling but not PI-3-kinase signal transduction, indicating that stimulation of the Mek–Erk pathway is the primary mechanism blocking lactogenic differentiation in HC11 cells.

MATERIALS AND METHODS

Cell culture

Mouse mammary epithelial cell lines, HC11 and HC11-luci, kindly provided by Dr. Nancy Hynes, were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μg/ml Insulin, 10 mM HEPES, and 10 ng/ml EGF as described (Hynes et al., 1990; Marte et al., 1995).

Lactogenic hormone-induced differentiation

The HC11 cells were grown to confluence and maintained for 3–5 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μg/ml Insulin, 10 mM HEPES, 10 ng/ml EGF to establish competence (Ball et al., 1988; Taverna et al., 1991). To induce lactogenic hormone-induced differentiation EGF-containing media was removed, the cells were rinsed twice and then incubated in differentiation media, i.e., serum free- or serum containing-RPMI with dexamethasone (10^{-6} M), insulin (5 μg/ml), and prolactin (5 μg/ml) referred to as DIP. The cells were harvested at the stated times after the addition of DIP. Alternatively, HC11 Tet-off cell lines were grown to confluence for 6 days in EGF-containing media in the absence of doxycycline, then maintained for 24 h in media without EGF prior to the addition of DIP. HC11 differentiation was characterized in these cells by the formation of domed structures referred to as mammospheres (Blatchford et al., 1995; Humphreys and Rosen, 1997) which were enumerated by phase contrast microscopy. The cell cultures were photographed using 20× objective with a Nikon Ix70 camera.

Construction of cell lines

The HC11 cell line was transfected with pTetOff plasmid (BD Biosciences Clontech, Palo Alto, CA) using Lipofectamine 2000 (Introvitrogen Life Technologies, Carlsbad, CA). The cells were incubated in G418 (200–500 μg/ml) selection media for 10 days, individual colonies were picked with cloning cylinders and expanded in 24-well plates. The colonies were screened

for the regulation of the Tet-promotor by transfection with a Tet-promoter-luciferase construct and incubation in medium with and without doxycycline (0–0.5–2.0 μg/ml). The promotor activity was assessed using a luciferase assay system (Promega, Madison, WI) with the light emission measured in a luminometer and expressed as light intensity/μg cell protein. Two cell lines exhibited up to 40-fold increase in a Tet-responsive promotor in response to the removal of doxycycline from the cultures. These HC11 tet-off cell lines were used for the construction of cell lines expressing specific genes under the control of the Tet-responsive element (TRE).

The HC11 Tet-off cell lines were infected with retroviral vectors expressing Tet-regulated *Ki-Ras* genes. pREV-TRE (Clontech), a retroviral vector that expresses a gene of interest from TRE, was derived from pLNCX, a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector. The TRE contains seven direct repeats of the 42-bp tetO operator sequence, which can be bound by tTA transactivators, upstream of a minimal CMV promoter. The 5′ viral LTR regulates expression of the transcript that contains the viral packaging signal and the hygromycin resistance (*Hyg*^{r}) gene. The TRE is an internal promotor in this vector. pREV-TRE was used to inducibly express the Ki-Ras genes in response to removal of doxycycline (Dox).

pREV-TRE-RasV12 (active K-Ras 2B-V12) and pREV-TRE-DNRasN17 (dominant negative K-Ras 2B-N17) plasmids were constructed by introduction of K-Ras cDNA into pREV-TRE plasmid and selection on hygromycin. For the production of retroviral vector stocks 1.5×10^5 PA317 packaging cells were transfected with 1 μg of recombinant retroviral vector DNA and Lipofectamine 2000 in a 35 mm well. Twenty-four hours post-transfection the PA317 cells were split and selected in hygromycin containing media (100 μg/ml) for 10 days. Mass cultures were prepared from approximately 50–100 colonies and used to produce retroviral vectors stocks. At this point, viral titers were high enough to use for retroviral infection of HC11 Tet-off cells. The HC11-Tet-off cell line was infected with pREV-TRE, pREV-TRE-RasV12, and pREV-TRE-DNRasN17 vector stocks. Cells were selected in hygromycin (100 μg/ml) and doxycycline (2 μg/ml) for 10 days. Six colonies from each HC11 Tet-off infected cell line were isolated and expanded into cell lines. The clonal cell lines were tested for expression of vector encoded Ras RNA by Northern blot following the removal of doxycycline.

Adenovirus infection

HC11 and HC11-luci cells were infected with replication defective adenoviruses. A control vector encoding on β-galactosidase (pAd-CMV-β-gal) or a vector encoding Ha-Ras N17, kindly provided by Dr. Craig Logsdon, were used for these experiments (Nicke et al., 1999). Cells were infected with 10–50 MOI of cesium chloride gradient-purified adenovirus by incubation of cells in a low volume of virus-containing media for 5–6 h. The virus was removed and media was added to the cells for 24 h prior to additional treatment of the cells.

Luciferase assays

HC11 luci cells were induced to differentiate in DIP-induction media with and without EGF (10 ng/ml). Inhibitors were added at the time of DIP-induction. Inhibitors were added at optimal concentrations (PD98059, 20 μM; LY294002, 10 μM; wortmannin, 100 nM) determined by dose–response curves (data not shown). Cell lysates were harvested 48 h after transfer to DIP-induction media and luciferase activity was determined using a commercial kit (Luciferase Assay System, Promega) and a luminometer (Thermolab Systems, Ascent, FL). The total cell protein was determined by BCA assay (Pierce, Rockford, IL) and luciferase activity was normalized to protein for all the samples. Results are presented as relative units calculated from the mean of six determinations.

Electrophoretic mobility shift assay (EMSA)

HC11 cells were grown to confluency in media containing 10% fetal calf serum, 10 ng/ml EGF, and 5 μg/ml insulin then maintained for 3 days without EGF. The cells were then starved for 24 h in serum-free media prior to induction for 15 min with DIP as described above. Nuclear extracts were prepared according to a previously published protocol with little modification (Wartmann et al., 1996). Briefly, harvested cells were suspended in CEB (10 mM KCl, 20 mM HEPES, pH 7.0, 1 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol, 0.1 mM EGTA, 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, 50 μM β-glycerophosphate, 50 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin) and sheared with 20 strokes using a Dounce homogenizer (Wheaton, pestle B). Nuclei were pelleted by centrifugation at 800*g* for 5 min and then extracted with NEB (CEB + 300 mM NaCl) by incubating for 30 min on ice. Extracts were clarified by centrifugation for 5 min at 16,000*g*. EMSAs were performed by incubating 10 μg of nuclear protein with the Stat5 DNA binding site from the bovine β-casein promotor (5′-AGATTTCTAGGAATTCAATCC-3′) or Sp1-binding oligonucleotide, end-labeled with ^{32} P-γ-ATP, for 30 min on ice in 16 μl of EMSA buffer (10 mM HEPES, pH 7.6, 2 mM NaH_2PO_4, 0.25 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 80 mM KCl, 2% glycerol, and 100 μg/ml poly [dI-dC]). Specific binding was analyzed on 6% DNA retardation gel and pre-run for 2 h at 200 V in 0.25 × TBE (22.5 mM Tris borate, pH 8.0, 0.5 mM EDTA) at 4°C. The samples were loaded and electrophoresed for 2 h at 200 V, the gels were dried and autoradiographed. For antibody supershift assays, nuclear extracts were pre-incubated with Stat5b C17 antibody (Santa Cruz) for 20 min prior to the addition of the labeled probe.

Northern blots

Total RNA was extracted using TriPure reagent (Roche). Northern blots were prepared using 7.5 or 10 μg of total RNA separated on 1% agarose-formaldehyde gel and transferred to a nylon filter. Blots were hybridized as described previously (Masuelli et al., 1999). The probes used included: mouse β-casein, human KiRas2B, and mouse actin. Mouse β-casein probe is a 601 bp fragment (nucleotide 3–603) from the mouse β-casein cDNA, (accession number X04490.1); it was obtained by RT-PCR and TA-cloning into PCR2.1 and sequence verified. The Ki-Ras probe is a 650 bp fragment

representing the human Ki-Ras 2b cDNA, and the actin probe was obtained from Clontech. Mouse Socs-3 probe consisted of nucleotides 467-1006 (accession number NM_007707.2) and mouse Cis-1 was nucleotides 526-1046 (accession number NM_009895.2).

MTT assay

The rate of replication of HC11-TRE and HC11-DNRasN17(12) cell lines was determined by proliferation assay using MTT dye (CellTiter96 Assay by Promega). The cells were propagated for 96 h in the absence of doxycycline. The viable cells were counted by 0.4% trypan blue dye exclusion test and the cell count was adjusted of 1×10^6 cells/ml in RPMI with 0.5% FBS. Cells were plated at density of 1.5×10^3 per well in quadruplicate wells in 96-well plate with or without EGF (10 ng/ml) incubated at 37°C for 24, 48, or 72 h. For analysis of proliferation 15 μl of MTT dye solution was added to each well and the culture plate was incubate at 37°C in CO_2 incubator for 4 h. After 4 h 100 μl of solubilization-stop solution was added to each well. Following 1-h incubation at 37° C the samples were mixed by pipeting and the optical density was measured at 570 nm. The mean and standard deviation of the absorbance values for the quadruplicate wells were calculated.

Immunoprecipitations and Western blots

HC11 cell lysates were prepared in triton-glycerol buffer (1% Triton-X 100, 10% glycerol, 25 mM HEPES, 150 mM NaCl, 2 mM EDTA), NP40 buffer (1% NP40, 25 mM HEPES, 150 mM NaCl) or high salt buffer (Wyszomierski et al., 1999). All lysis buffers contained AEBSF (20 μg/ml), aprotinin, (5 μg/ml), leupeptin, (5 μg/ml), β-glycerol phosphate (100 μM), and $NaVaO_4$ (1 mM). Immunoprecipitates were prepared by incubation of 0.5 or 1 μg of primary antibody with equal amounts of protein (400 μg) and collected by binding to Protein A agarose (Invitrogen Life Technologies, Carlsbad, CA). Antibodies include anti-Stat5, sc-835 (SantaCruz Biotechnology, Santa Cruz, CA), anti-phosphoStat5 (Cell Signaling Technology, Beverly, MA). For Western blots equal amount of protein were separated by SDS–PAGE and transferred to PVDF filters. Filters were blocked with 2.5% nonfat dried milk (Blotto) in TBS-T for 1 h, then incubated with the appropriate dilution of antibody for 1 h at room temperature or 16 h at 4°C with agitation. Following the incubation with HRP-labeled secondary antibodies, blots were washed and reactivity was detected using ECL (Amersham). Blots were either exposed to Kodak XAR film or results were quantified using a CCD camera (Fuji). Films were quantitated by densitometry. Antibodies included anti-Stat5, sc-835 (SantaCruz), anti-phosphoStat5 (Cell Signaling), anti-phospho Erk, V8031 (Promega), anti-pan Erk (Transduction), anti-AKT and anti-phosphoAKT-ser 473 (Cell Signaling), anti-SHP2 (Transduction), anti-Mek1,2 (Transduction). Anti-PTP-PEST was supplied by Dr. Michael Schaller. Antibodies purchased from Santa Cruz Biotechnology were used at 1 μg/ml, and the antibodies from other suppliers were used at the dilution suggested by the manufacturer.

RESULTS

EGF blocks hormone-induced HC11 differentiation through Mek and PI-3-kinase-dependent pathways

Previous studies demonstrated that EGF blocked lactogenic hormone-induced differentiation of HC11 cells (Hynes et al., 1990), and recent data suggested that this block required Ras and PI-3-kinase activity (DeSantis et al., 1997). 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 (Fig. 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 examined (Fig. 1B). The results confirmed that exposure of HC11 cells to DIP activated β-casein expression and that EGF reduced the expression. The inclusion of PI-3-kinase or Mek1 inhibitors in the induction media with EGF reversed the EGF-induced inhibition of the endogenous β-casein promotor activity in the HC11-luci cells.

Previous studies demonstrated that the treatment of HC11 cells with DIP resulted in increased Stat5 DNA binding and that the DNA binding activity of Stat5 was reduced by the simultaneous addition of EGF and lactogenic hormones (Marte et al., 1995). Therefore, 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 of this reproducible experiment indicated that DIP stimulation in the presence of the Mek1 (PD98059) and PI-3-kinase (wortmannin) inhibitors enhanced Stat5 binding to DNA compared to the binding detected with DIP alone (Fig. 2A). In contrast, exposure of the HC11 cells to DIP plus AG490, an inhibitor of Jak2 tyrosine phosphorylation, inhibited Stat5 DNA binding (Fig. 2A, lanes 4 and 8). The results in Figure 1 indicated that Mek1 and PI-3-kinase inhibitors restored the DIP-induced Stat5 promotor activity inhibited by EGF, and the same Mek and

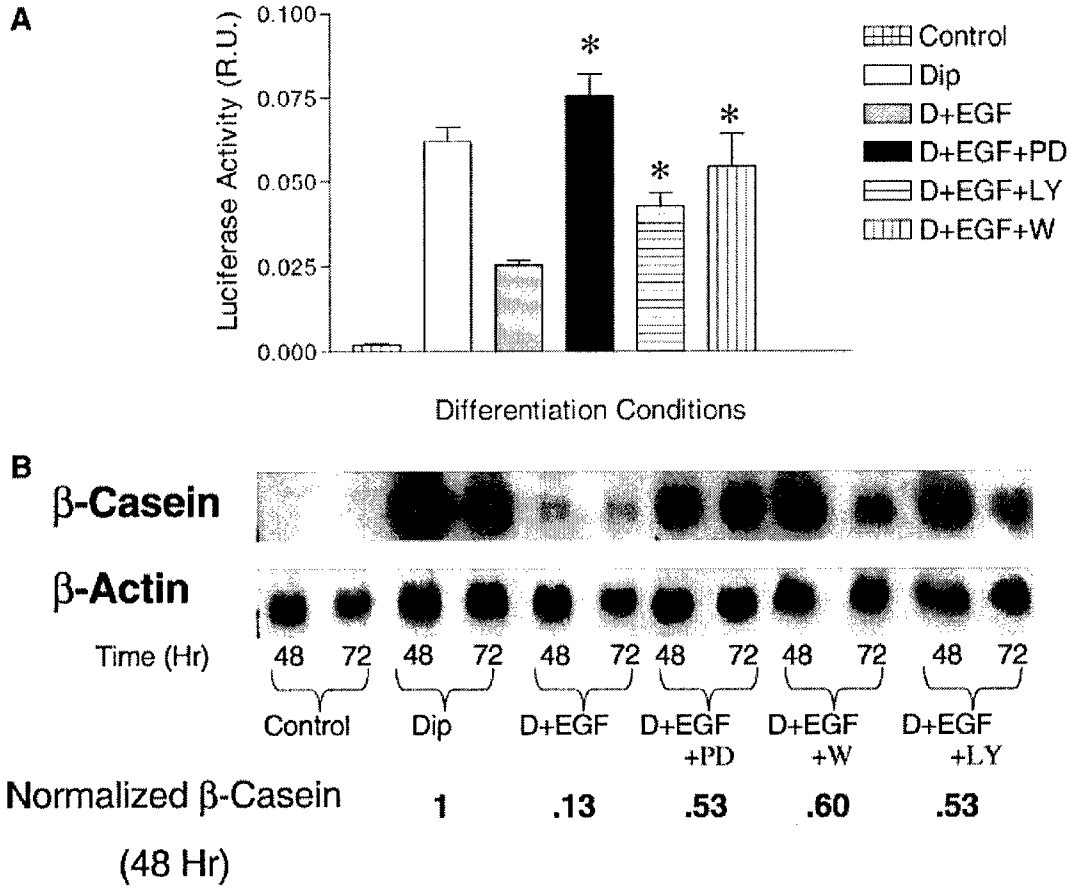

Fig. 1. **A**: The effect of signal transduction inhibitors on epidermal growth factor (EGF) disruption of differentiation. HC11-luci cells were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media with serum in the presence or the absence of EGF (10 ng/ml). Inhibitors were added at the time of DIP induction at previously determined optimal concentrations (PD98059, 20 μM; LY294002, 10 μM; wortmannin, 100 nM). The luciferase activity in lysates was determined at 48 h 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 0.001) 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 (10 ng/ml). Inhibitors were added at the time of induction at slightly lower than optimal concentrations to avoid toxicity (PD98059, 10 μM; LY294002, 5 μM; wortmannin, 50 nM). Total cell RNA was harvested at 48 or 72 h after transfer to DIP-induction media. β-Casein induction was determined via Northern blot. For quantitation β-casein expression at 48 h was normalized to β-actin. The level of expression in DIP-treated cells was set as 1.

PI-3-kinase inhibitors enhanced Stat5 DNA binding. Blocking the Mek–Erk and PI-3-kinase pathways with specific inhibitors both enhanced HC11 markers of differentiation and prevented the EGF-dependent disruption of HC11 differentiation.

HC11 cells expressing dominant negative RasN17 exhibit an enhanced lactogenic differentiation response

Because Ras activation regulates the activation of the Erk pathway by EGF and may contribute to the activation of PI-3-kinase, the role of Ras activation in the disruption of HC11 differentiation by EGF was examined. HC11 cell clones expressing either activated Ki-Ras (RasV12) or dominant negative Ki-Ras (DNRasN17) 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 RasV12 and DNRasN17 clones. The results obtained with three independent clones derived from each vector are shown in Figure 3.

The DNRasN17 and the RasV12 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 DNRasN17 or activated RasV12 along with the vector control cell line were grown for 72 h 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, 72, and 96 h post-addition of DIP and used to

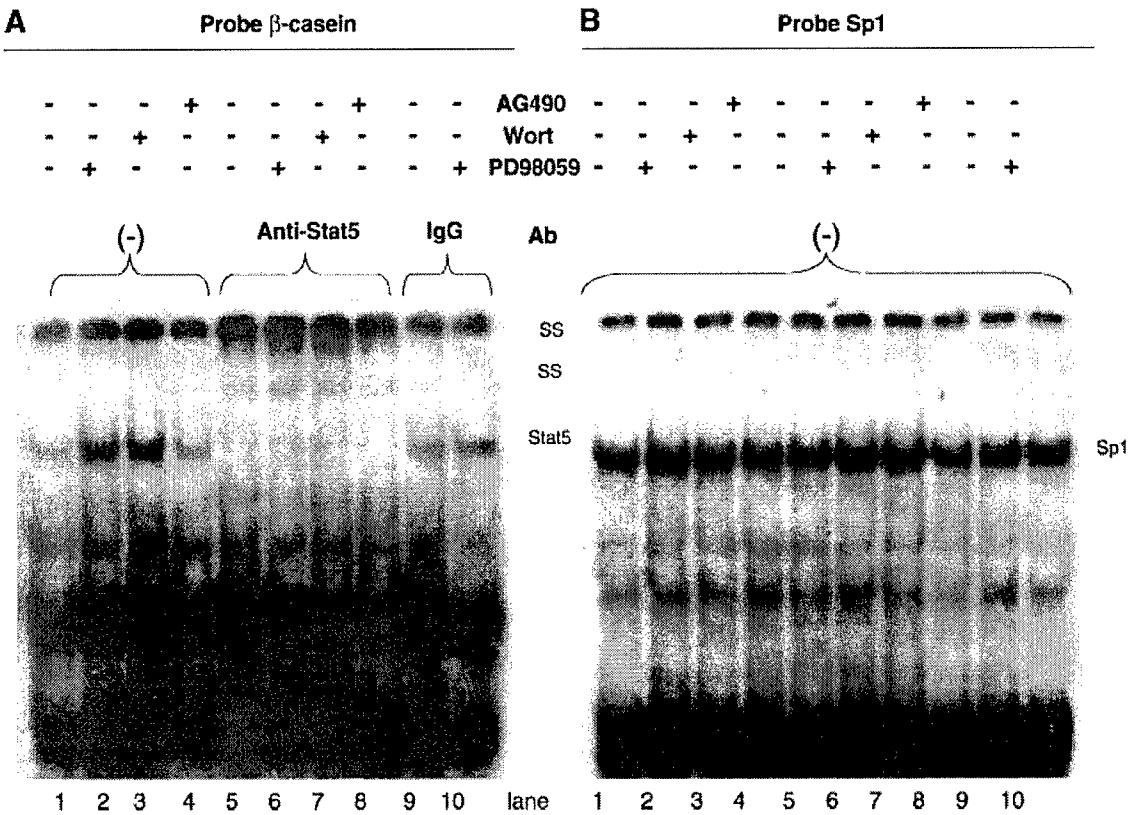

Fig. 2. The effect of inhibitors on Stat5 DNA binding by EMSA. **A**: HC11 cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for 3 days and serum-free media for 1 day. HC11 cells were pretreated with specific kinase inhibitors for 2 h prior to DIP-induced differentiation for 15 min 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. Lanes 5–8: The binding was performed in the presence of anti-Stat5 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. SS, supershift of Stat5.

determine the level of Ras and β casein expression by Northern blotting. The results in Figure 3 indicated that RasV12 expression inhibited β-casein expression by approximately 50% compared to the TRE control cell line. In contrast, the expression of DNRasN17 enhanced β-casein induction up to twofold compared to the control. The results demonstrated that the amount of DNRasN17 expression correlated with the effect on differentiation. The HC11 cell clone expressing the greatest amount of DNRasN17 (clone 12) exhibited the greatest level of β-casein expression. In contrast, all clones of expressing RasV12 inhibited β-casein expression.

The effect of Ras expression on mammosphere formation, a phenotypic measure of differentiation for primary mammary epithelial cells as well as HC11 cells, was determined. Following growth in the absence of doxycycline, EGF was removed from the cells and lactogenic differentiation was induced by the addition of DIP. The cells were photographed at 0, 72, and 120 h post-DIP and the number of domed mammospheres that appeared in each culture were enumerated (Fig. 4A). At 72 h after DIP addition, the mammospheres were easily counted, but by 120 h the size and the number in the DNRas cell line were too great to count. The results indicated that mammosphere formation was inhibited by RasV12 expression and was significantly enhanced by DNRas expression.

In parallel experiments, the effect of Ras expression on the prolactin-induced tyrosine phosphorylation of Stat5 was examined. HC11 TRE vector control cells as well as the RasV12 (clone 1) and DNRasN17 (clone 12) cells were stimulated with DIP, 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 4B, indicated that the tyrosine phosphorylation of Stat5 was enhanced and sustained in the DNRasN17 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 RasV12 than in the TRE control cells. These results suggested that Ras-dependent signal transduction can modulate Stat5 phosphorylation in HC11 cells in response to DIP. The Stat5 EMSA results supported this conclusion (Fig. 4C). Enhanced Stat5 DNA binding in response to DIP stimulation was observed in the DNRasN17 HC11 cell

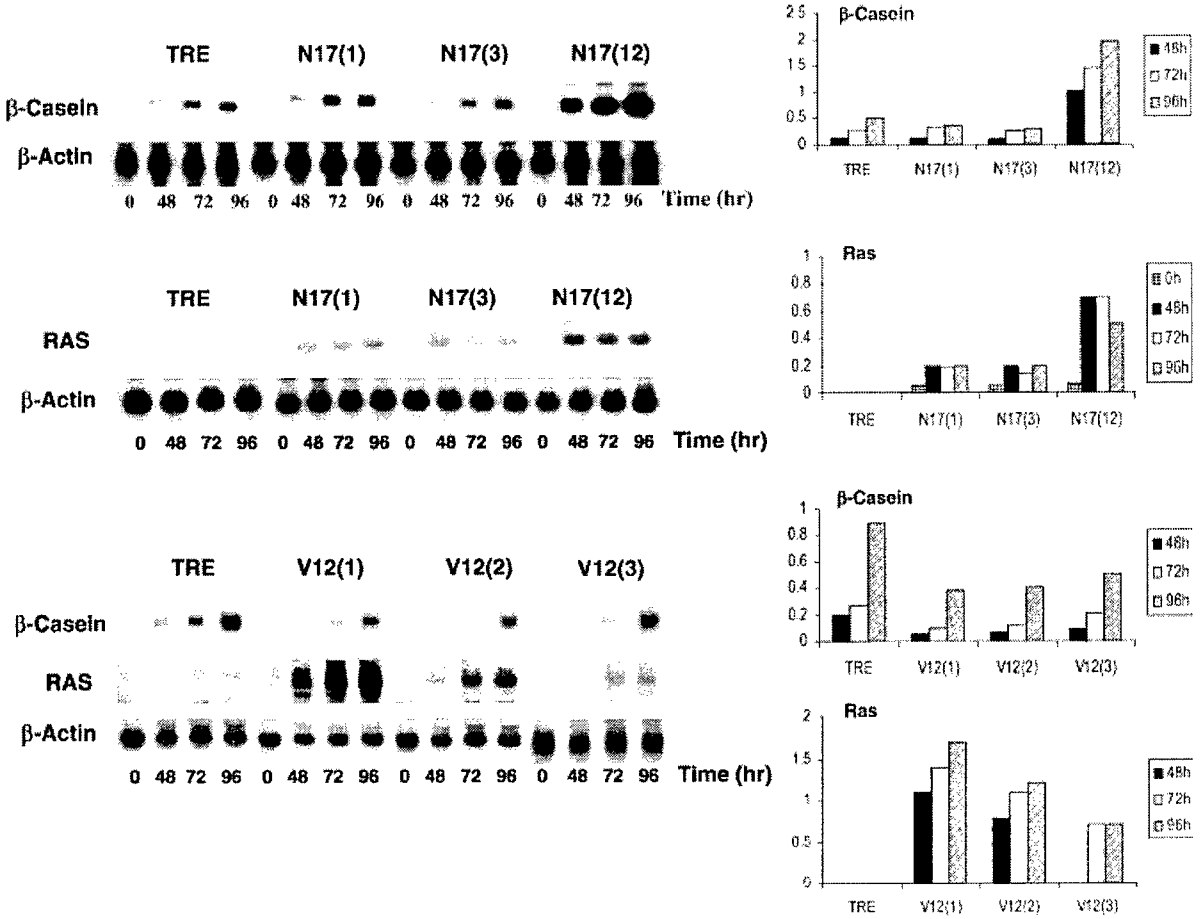

Fig. 3. The effect of RasV12 and DNRasN17 on lactogenic differentiation. HC11 cells expressing activated RasV12 and DNRasN17 under the control of the Tet-responsive promotor were utilized to evaluate the effect of Ras-based signal transduction on lactogenic differentiation. Three individual clones of HC11 cells expressing either RasV12 (clones 1–3) or RasN17 (clones 1, 3, 12) under the control of the Tet responsive promotor 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 h 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.

lysates as compared to the vector control. In contrast, the Stat5 DNA binding activity was reduced in cells expressing activated RasV12. 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.

Fig. 4. The effect of RasV12 and DNRasN17 expression on mammosphere formation, Stat5 phosphorylation and DNA binding. **A**: HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12 (clone 1) or DNRasN17 (clone 12) were grown to confluence and exposed to DIP as described in Materials and Methods. The cells were photographed at 0, 72, and 120 h post-DIP addition. The number of mammospheres per field is reported; this was determined by counting the number of mammospheres per low power field and determining the mean of five fields. **B**: HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12 (clone 1) or DNRasN17 (clone 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 min, 1 and 24 h 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, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. **C**: 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 3 days and serum-free media for 1 day. Treated cells were exposed to differentiation media for 15 min and control cells (T = 0) were not exposed to DIP. Left part: 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 part: 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 1–3: Contain control lysate; (lanes 4–6) contain lysate from DIP-treated cells; (lanes 7–9) contain lysate from DIP-treated cells with the addition of 50 × cold Sp1 oligonucleotides. SS: Stat5 supershift.

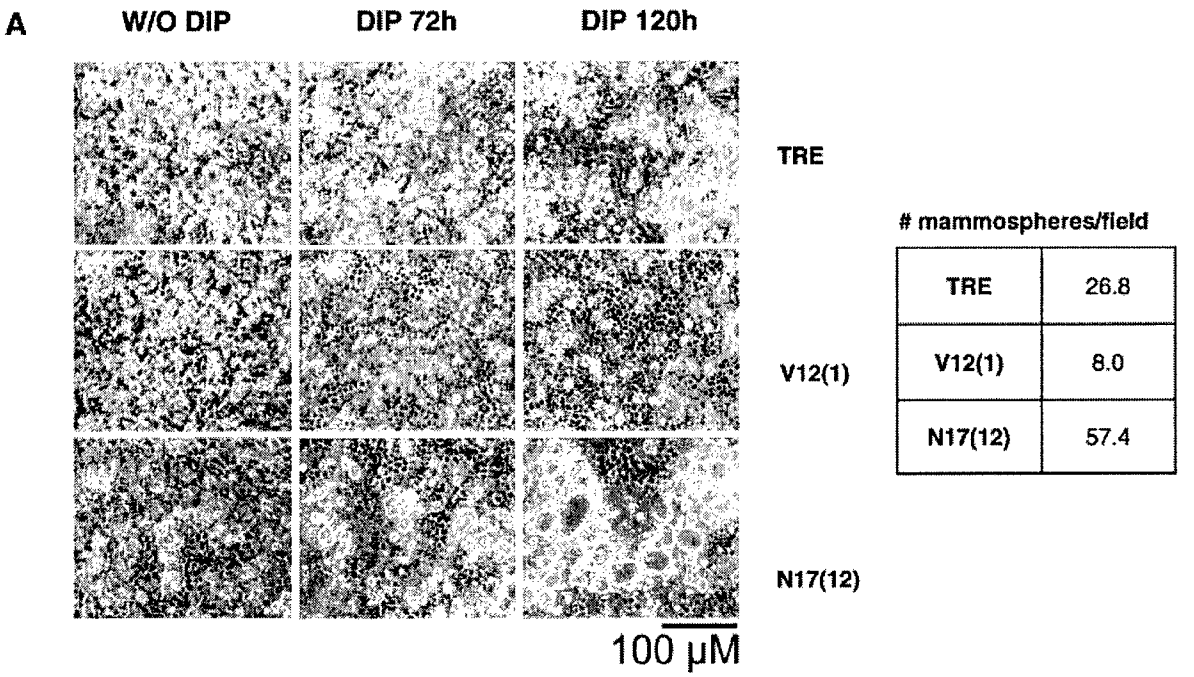
A
W/O DIP
DIP 72h
DIP 120h
TRE
V12(1)
N17(12)
100 μM
mammospheres/field
TRE 26.8
V12(1) 8.0
N17(12) 57.4
B
P-STAT5
STAT5
0' 15' 1hr 24hr
0' 15' 1hr 24hr
0' 15' 1hr 24hr
TRE
V12(1)
N17(12)
Stat5 Phosphorylation (R.U.)
1.5
1.0
0.5
0.0
15'
1hr
24hr
TRE
V12(1)
N17(12)
C
DIP 0 min 15 min 15 min+Ab
C V12 N17 C V12 N17 C V12 N17
DIP 0 min 15 min 15 min+cold Sp1
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Fig. 4.

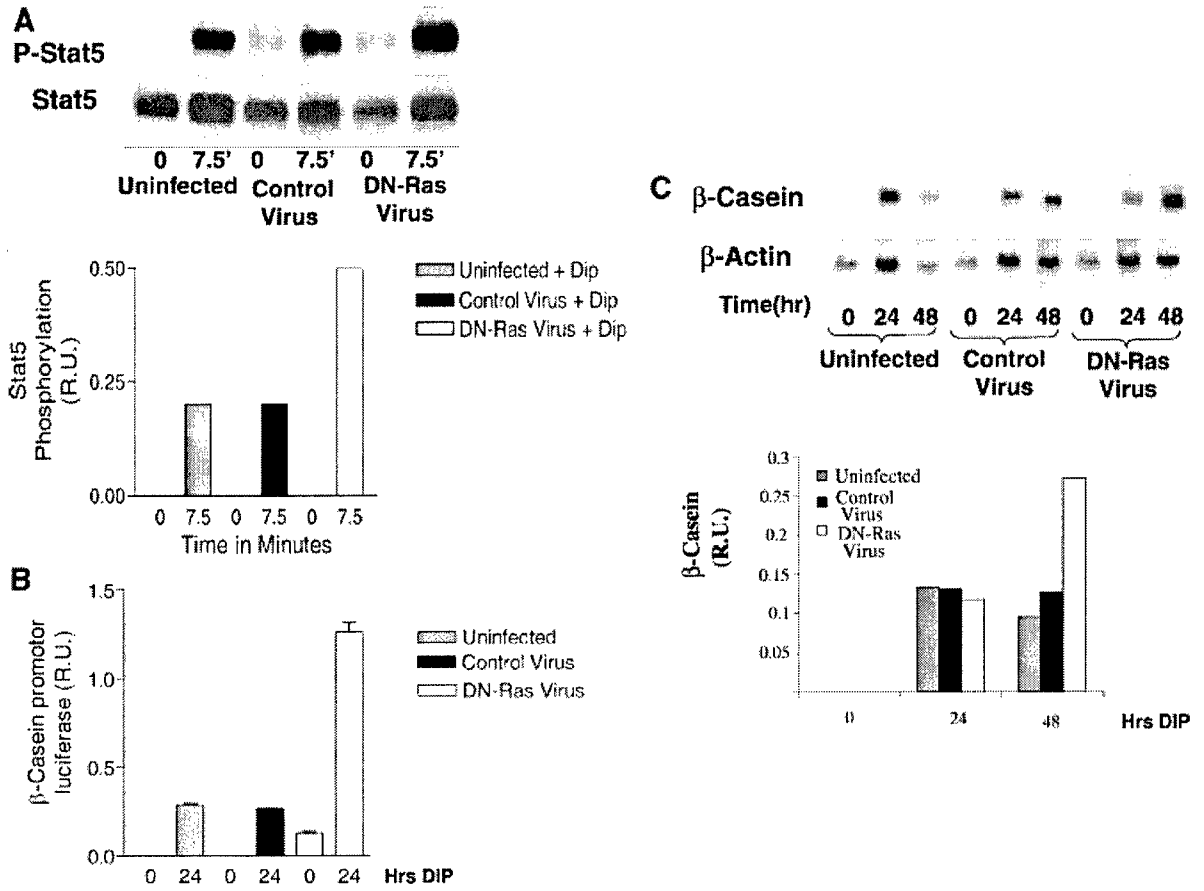


Fig. 5. The effect of dominant negative Ha-Ras (N17) adenovirus expression on lactogenic differentiation in HC11 cells. **A**: The effect of DNRasN17 adenovirus on Stat5 phosphorylation in response to lactogenic hormone was determined. Uninfected HC11 cells, HC11 cells infected with a control adenovirus vector and HC11 cells infected with adenovirus encoding DNRas (N17) (at MOI = 10) were incubated for 24 h; the cells were then serum-starved overnight and stimulated with DIP for 7.5 min. 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 RasN17 were used to determine the effect of DNRasN17 on β-casein driven luciferase activity. The cells were infected with the viruses described above and incubated for a period of 24 h in media without EGF. The cells were then either stimulated with DIP for 24 h or incubated in media without EGF for an additional 24 h. 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 DNRasN17 adenovirus infection on HC11 expression of β-casein was determined. The HC11 cells were infected with the control or DNRasN17 virus as described above. RNA was isolated at 0, 24, and 48 h 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.

Infection of HC11 cells with dominant negative Ha-Ras adenovirus enhances lactogenic differentiation

Infection of cells with replication defective adenovirus encoding dominant negative Ha-RasN17 (DNRasN17) 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 DNRasN17. At 48 h postinfection, the cells were examined for the effect of DNRasN17 on Stat5 phosphorylation, β-casein promotor activity and β-casein RNA levels. As demonstrated in Figure 5A HC11-luci cells infected with control virus or DNRasN17 virus were stimulated with DIP and the level of Stat5 tyrosine 694 phosphorylation was determined. The results indicated that the expression of DNRasN17 increased the level of Stat5 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 DNRasN17 were tested for activation of β-casein promotor-driven luciferase activity (Fig. 5B). There was a fivefold increase in the activation of luciferase activity in the DNRasN17 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 DNRasN17 virus without DIP exposure. This result was reproducible and is not seen when uninfected cells or vector infected cells were exposed to DIP. Finally, HC11 cells infected with

either replication defective control adenovirus or adenovirus encoding DNRasN17 were examined for expression of the endogenous β-casein gene following exposure to DIP for 24 or 48 h. The results of Northern blots (Fig. 5C) indicated that the infection with DNRasN17 virus resulted in a twofold increase in β-casein RNA compared to the uninfected or vector infected cells exposed to DIP.

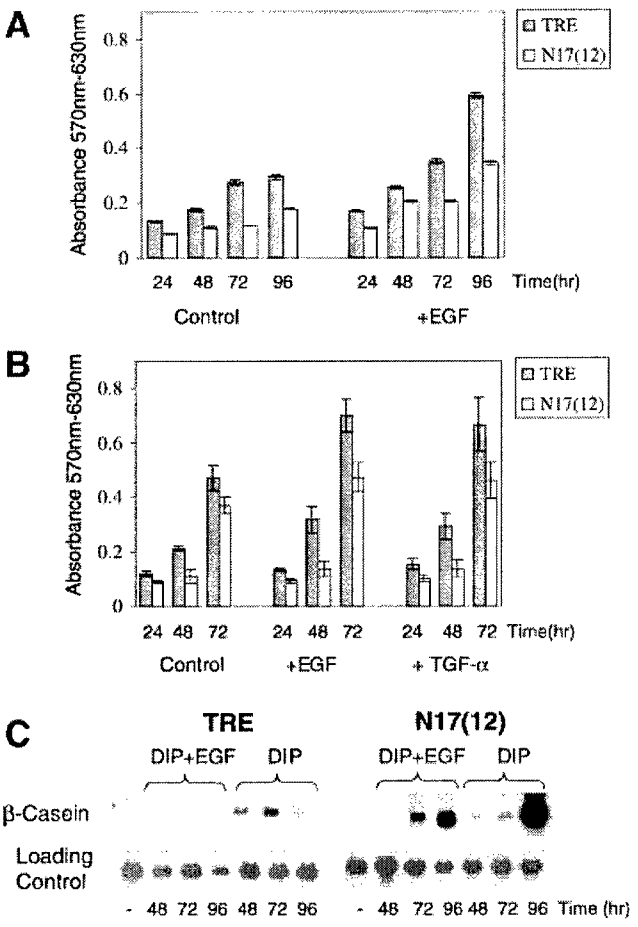
HC11 cells expressing dominant negative Ras exhibit reduced response to EGF

Studies were performed to determine if the DNRasN17 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 DNRasN17 cells were stimulated with EGF and the ability of the cells to proliferate was examined using the MTT assay. Cells were removed from doxycylcine for 96 h and then grown in reduced serum media in the absence and the presence of EGF. MTT assays were performed over the course of 4 days to follow cell proliferation. The results in Figure 6A demonstrated that the DNRasN17 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 DNRasN17 cells. Again, the DNRasN17 cells exhibited a significantly lower response to EGF and TGFα than did the vector control cell line (Fig. 6B).

The ability of DNRas to prevent the disruption of lactogenic hormone-induced differentiation by EGF in HC11 cells was examined. The HC11 TRE vector control cells and cells expressing DNRasN17 under the control of a Tet-responsive promotor were grown in the absence of doxycycline for 72 h. 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 6C demonstrated that EGF did not inhibit the induction of β-casein transcription in the DNRasN17 cell line and, hence, it appeared that differentiation proceeded in these cells even in the presence of EGF. In contrast, the expression of β-casein was blocked by EGF in the TRE vector control cell line in two separate experiments. These results demonstrated that DNRasN17 expression prevented the disruption of hormone-induced differentiation by EGF in HC11 cells.

HC11 cells expressing dominant negative Ras exhibit reduced Erk activation in response to EGF

HC11 cells expressing DNRasN17 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 7A 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, shown in Figure 7A, revealed that stimulation of HC11 vector control cells with EGF resulted in activation of p44Erk as detected by reactivity



Experiment #	Cell Line	% Reduction β-Casein RNA
1	TRE	99.1 %
	N17(12)	0 %
2	TRE	41.5 %
	N17(12)	0 %

Fig. 6. DNRas N17 expression inhibits EGF-induced proliferation and prevents EGF-dependent disruption of lactogenic differentiation. **A**: HC11 TRE vector control and DNRasN17 (clone 12) cells were grown in absence of doxycycline and then seeded in microtiter plates in 0.5% serum-containing media with and without EGF (10 μg/ml). Cell proliferation was determined at 24, 48, 72, 96 h 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 DNRasN17 (clone 12) cells were grown as described above and exposed to EGF (10 ng/ml) or TGFα (10 ng/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 (10 ng/ml). Total RNA was isolated after 72 h 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.

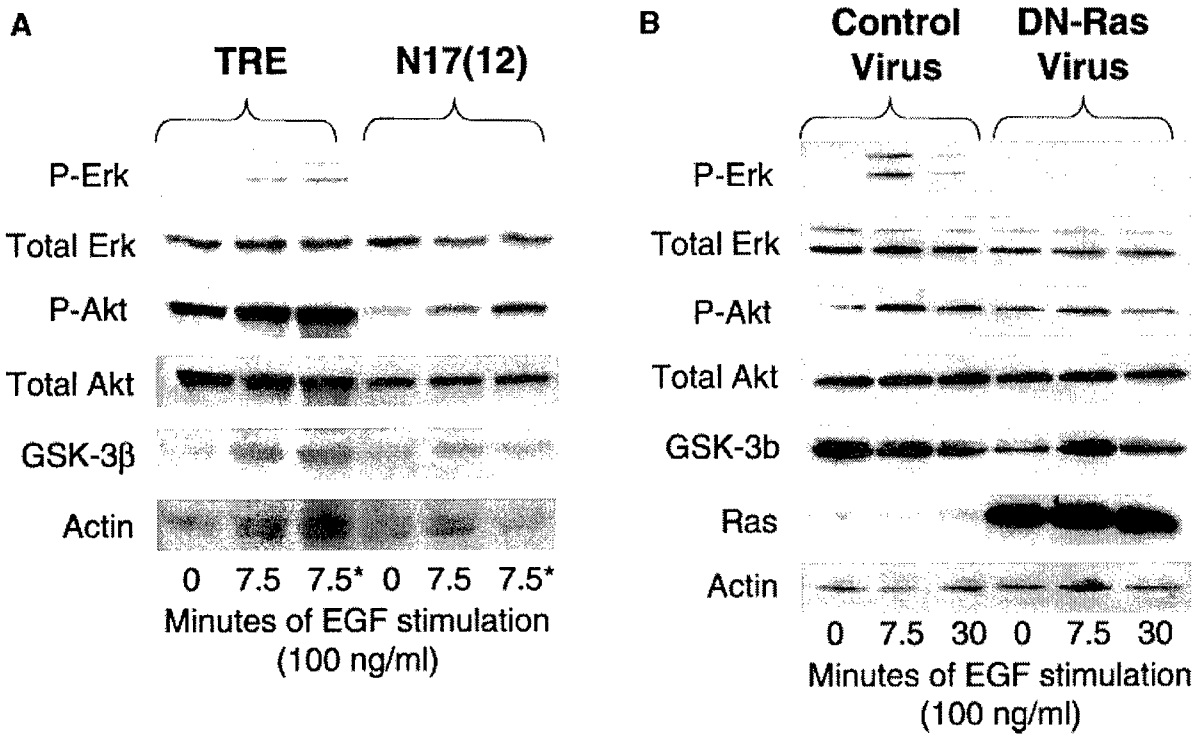


Fig. 7. The effect of DNRasN17 expression on signal transduction pathways in HC11 cells. **A**: The HC11 TRE vector control cells and DNRasN17 (clone 12) cell lines were grown to confluence in EGF-containing media lacking doxycycline. The cells were incubated in media without EGF or media without EGF and serum (*) prior to restimulation with EGF (100 ng/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 DNRasN17-encoding adenovirus at an MOI of 10 were incubated in serum-containing media for 24 h and incubated in EGF-free media for 20 h prior to stimulation with EGF (100 ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western blotting as in part A.

with an antibody that recognizes the active phosphorylated forms of Erk1, 2. In contrast, in HC11 cells expressing DNRasN17 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 little or no difference in Akt activation between the control HC11 cells and the DNRasN17 HC11 cells following treatment with EGF. This demonstrated that the PI-3-kinase pathway was not significantly blocked by DNRasN17 expression in HC11 cells. Moreover, activation of Jun kinase and p38 kinase by EGF was not deficient in the DNRasN17 HC11 cells (data not shown). These results suggest that the Mek–Erk pathway was most sensitive to inhibition by DNRas17 expression.

Cells infected with the control adenovirus vector or adenovirus encoding DNRasN17 were examined for the effect of EGF on signal transduction pathways in an analogous fashion. The results in Figure 7B demonstrated that DNRasN17 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 DNRasN17 expressing cells indicated that blocking the Ras pathway in this manner in HC11 cells primarily blocked signaling to the Raf–Mek–Erk pathway. Hence, these data support the conclusion that in HC11 cells activated RasV12 inhibits β-casein transcription via Mek–Erk signaling, and that the effect of DNRasN17 expression on β-casein is primarily a result of its inhibition of the Mek–Erk pathway.

Expression of dominant negative Ras prevents the prolactin-induced association of SHP2 with Stat5

Our results demonstrated that the expression of DNRasN17 resulted in enhanced DIP-induced activation of Stat5 as measured by tyrosine phosphorylation, DNA binding and activation of the β-casein promotor. To determine the mechanism by which this occurs, the functionality of several Stat5 regulatory pathways in DNRasN17 cells was examined. The increased activity of Stat5 likely resulted from the higher level of Stat5 tyrosine 694 phosphorylation; hence, regulation of Stat5 tyrosine phosphorylation was examined by looking for the association of tyrosine phosphatases with Jak2 and Stat5. Previous reports demonstrated that the phosphatase SHP2 interacted with Stat5; SHP2 associated with Stat5 following stimulation of the Stat5 pathway by prolactin as detected by co-immunoprecipitation of SHP2 with Stat5 (Chughtai et al., 2002). To determine if dominant negative Ras expression affected the prolactin-induced association of SHP2 with Stat5, HC11-TRE, and DNRasN17 cells were stimulated with prolactin. Cell lysates were prepared, Stat5 was immunoprecipitated, and the amount of SHP2 associated with the Stat5 was determined by Western blotting. The

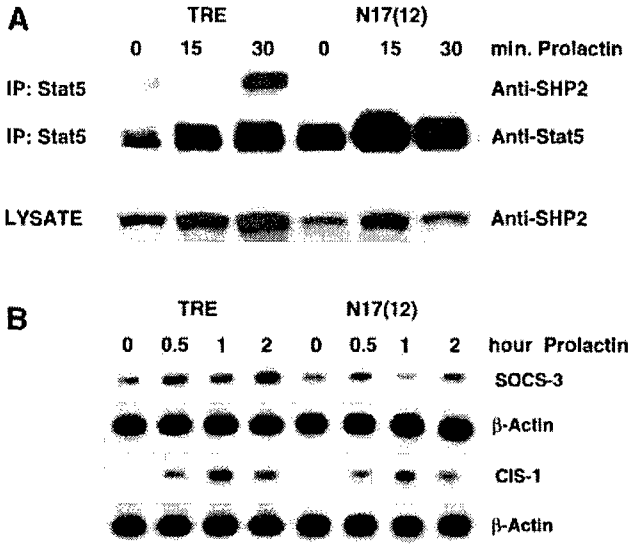


Fig. 8. Prolactin-induced binding of SHP2 to Stat5 and prolactin-induced expression of SOCS-3 and CIS-1. HC11-TRE and HC11-DNRasN17 (clone 12) cells were grown to confluency in EGF-containing media then incubated in EGF-free media for 24 h; the cells stimulated with prolactin (5 μg/ml) for the indicated time. **A**: Lysates were prepared and Stat5 was immunoprecipitated. The lysates and the immunoprecipitates were analyzed by Western blotting for Stat5 and SHP2. **B**: RNA was extracted from cells and analyzed by Northern blotting for expression of SOCS-3 and CIS-1. β-Actin was hybridized to the blots as a loading control.

results in Figure 8A demonstrated that the level of SHP2 protein expression was not reduced in HC11-DNRasN17 cells. However, in the vector control cell line, prolactin stimulation for 30 min resulted in significant association of SHP2 with Stat5, but very little SHP2 was associated with Stat5 in prolactin stimulated DNRasN17 cells (Fig. 8A). Accordingly, the degree of Stat5 phosphorylation at Y694 was significantly greater in the DNRasN17 cells than in the TRE cells.

In previous reports PTP-PEST was identified as a phosphatase that was activated by EGF and prolactin and associated with Jak2 (Horsch et al., 2001). These studies indicated that PTP-PEST was co-immunoprecipitated with Jak-2 from HC11 cells following prolactin stimulation. However, we did not observe this association in either the HC11-TRE or HC11-DNRasN17 cell lines (data not shown).

A previous report indicated that both EGF and prolactin stimulation induced the expression of the inhibitors of cytokine signaling, SOCS-3 and CIS-1, in HC11 cells (Tonko-Geymayer et al., 2002). To determine if changes in the regulation of SOCS-3 and CIS-1 expression was affected by dominant negative Ras expression, the level of SOCS-3 and CIS-1 expression was examined by Northern blotting at various times after the addition of prolactin to HC11-TRE and DNRasN17 cell lines. The results, shown in Figure 8B, indicated that the expression of CIS-1 and SOCS-3 was stimulated by prolactin and that the expression was similarly regulated in the HC11- DNRasN17 cells and the HC11-TRE control cell line.

These results indicated that the association of SHP2 tyrosine phosphatase activity with Stat5 was blocked by dominant negative Ras expression. The resulting block to Stat5 dephosphorylation constitutes a likely mechanism for the enhancement of Stat5 activation by DNRasN17 expression.

DISCUSSION

Members of the EGF family of peptide growth factors are found in the mammary gland and appear to play a role in growth and differentiation in that tissue (Jhappan et al., 1990). For example, EGF and amphiregulin are expressed in the ductal epithelial cells and TGFα is expressed in cap stem cells in the terminal end buds (Snedeker et al., 1992; Kenney et al., 1995). EGF and TGFα, bind to EGF receptor (ERB1) and can stimulate the proliferation of mammary epithelial cells and enhance lobular–aveolar development in the mammary gland of virgin mice (Vonderhaar, 1987). These growth factors can also prevent milk protein expression in HC11 cells and inhibit apoptosis of secretory alveolar epithelial cells in the involuting mammary gland (Smith et al., 1995). Hence, these factors play a dual role in mammary differentiation.

Growth factors of the EGF family have been detected in human breast tissue and elevated levels have been associated with breast tumors (Dotzlaw et al., 1990; Mizukami et al., 1991). The stimulation of mammary cells in culture by these growth factors activates signal transduction pathways that lead to cell survival and mitosis, and the activation of the EGF-R (ErbB1) correlates with aggressive behavior of breast tumors (Arteaga et al., 1988; Umekita et al., 1992). One of the signaling molecules activated by EGF family growth factors in breast tumors is the Ras GTPase (von Lintig et al., 2000). Previous studies have demonstrated that EGF and activated Ras inhibit differentiation in HC11 cells. Both stimulation of mammary epithelial cells with EGF and the expression of activated Ras initiate signaling through the Mek–Erk pathway. While EGF stimulation also leads to activation of the PI-3-kinase pathway, the influence of Ras on this pathway in HC11 cells has not been examined. Therefore, the present study analyzed the effects of EGF on the Ras, Erk, and PI-3-kinase pathways in HC11 cells and the contribution of those pathways to lactogenic hormone-induced differentiation.

The results confirmed the findings of several previous studies by demonstrating that EGF can block lactogenic hormone-induced differentiation in HC11 cells (Hynes et al., 1990). Chemical inhibitor studies indicated that the inhibition of β-casein promotor activity by EGF required both the Mek–Erk and PI-3-kinase pathways. While a previous study found that activation of the Erk pathway was not required for lactogenic differentiation (Wartmann et al., 1996), the contribution of Erk to the inhibition of lactogenic hormone-induced differentiation by EGF was less clear. Merlo et al. (1996) correlated the inhibition of lactogenic hormone-induced differentiation by growth factors with the ability of different growth factors to induce a high level of Erk activation. Also, expression of v-Raf, an activator of Mek–Erk signaling, inhibited lactogenic hormone-induced differentiation of HC11 cells (Happ et al., 1993). However, a

previous study by DeSantis et al. (1997) demonstrated that inhibition of Ras and PI-3-kinase blocked the inhibitory effects of EGF on β-casein synthesis. Our study extends this previous study and demonstrates that the inhibition of the Erk pathway strongly correlates with an increase in β-casein promotor activation. Moreover, in our study the stable expression of dominant negative Ki-RasN17 or the infection of HC11 cells with dominant negative Ha-RasN17 adenovirus effectively enhanced β-casein synthesis in response to lactogenic hormones, and these cells exhibited inhibition of the Mek–Erk pathway but not the PI-3-kinase signaling pathway. Hence, it appears that the Erk pathway is critical in the negative regulation of lactogenic hormone-induced differentiation by DNRas. This appears be a function of its effect on Stat5 tyrosine phosphorylation and activation. Our results are in agreement with those of Gao et al., which suggest that Erk activation alters prolactin-induced expression at a step prior to Stat5 DNA binding (Gao and Horseman, 1999). The HC11 cells expressing dominant negative Ras, which were defective in Erk activation, exhibited both an increase in Stat5 tyrosine phosphorylation and an increase in Stat5 DNA binding.

The SH2 protein tyrosine phosphatase, SHP2, has been identified in a complex with Stat5 and a role for this phosphatase in regulation of Stat5 activity has been proposed (Berchtold et al., 1998; Chughtai et al., 2002). Our results indicated that DNRas expression blocked the association of SHP2 with Stat5. The mechanism by which this occurs has not been resolved. In addition to involvement in Jak-Stat signaling, SHP2 is required for growth factor receptor activation of the Ras–Erk pathway. SHP2 plays an essential role in linking components of signal transduction pathways to growth factor receptor complexes via the scaffold protein Gab1, which targets SHP2 to the membrane (Cunnick et al., 2002). Two potential links for SHP2 to the Ras pathway have been reported. Dominant negative SHP2 expression decreased the level of activated Ras (Ras-GTP) in cells (Cai et al., 2002); this could result from a decrease in guanine nucleotide exchange factor (SOS) activity. Alternatively, it was recently reported that SHP2 regulated EGF-dependent RasGAP, but not SOS, membrane localization and increased the half-life of Ras-GTP (Agazie and Hayman, 2003). Our data demonstrated that dominant negative Ras expression, which interferes with Ras activation in part by binding and sequestering guanine nucleotide exchange factors (Lai et al., 1993), disrupts one aspect of SHP2 function. This suggests that there may exist a mechanism to regulate SHP2 by Ras via SOS or RasGAP.

While EGF stimulation of HC11 cells has been linked to the activation of PTP-PEST and dephosphorylation of Jak2 (Horsch et al., 2001), no association of PTP-Pest with Jak2 was detected following prolactin stimulation in the HC11-TRE or HC11-DNRasN17 cell lines. In addition, although expression of SOCS-3 and CIS-1 has been demonstrated in HC11 cells following exposure to prolactin (Tonko-Geymayer et al., 2002), DNRasN17 expression did not alter the transcriptional activation of SOCS-3 or CIS-1 following prolactin stimulation in our experiments. Collectively these results suggested that DNRasN17 expression enhanced Stat5 tyrosine phosphorylation primarily by blocking the association of the SHP2 phosphatase with Stat5.

The data presented here demonstrate that the addition of EGF to HC11 cells stimulates the PI-3-kinase pathway resulting in the phosphorylation of Akt and its downstream signaling pathway. The data also demonstrate that inhibition of the PI-3-kinase pathway increases β-casein promotor activity. The expression of dominant negative Ki-Ras did not prevent the activation of PI-3-kinase-Akt pathway, indicating that the activation of PI-3-kinase was primarily a consequence of the binding of the p85 subunit to the EGF receptor rather than the direct activation of p110 by activated Ras (Rodriguez-Vicania et al., 1994). These results suggest that the PI-3-kinase pathway influences a stage in Jak-Stat signaling that occurs prior to or at the level of DNA binding. A recent study has demonstrated that PI-3-kinase inhibition enhanced Stat5 activation by thrombopoitin in part by preventing nuclear export of Stat5 (Kirito et al., 2002).

There have been several studies in other tissues demonstrating that regulation of Ras-dependent signal transduction contributes to differentiation. For example, there is evidence from both in vivo systems and tissue culture systems that the Ras–Raf–Mek–Erk pathway is required for neuronal differentiation (Halegoua et al., 1991; Thomas et al., 1992; Wood et al., 1992; Cowly et al., 1994; Marshall, 1995). Also, the activation of the Mek–Erk pathway may contribute to the differentiation status of some breast cancer cell lines. For example, differentiation-linked Erk activation in breast cancer cells occurs following ligand-induced activation of RTKs, including stimulation with heregulin (NDF, Neu differentiation factor) and subsequent activation of HER-3 (Lessor et al., 1998), or following transfection and overexpression of c-erbB-2 (Giani et al., 1998). In both systems activation of the Ras–Erk pathway resulted in increased expression of $p21^{CIP}$ and enhanced differentiation. ErbB4 signaling has also been linked to prolactin-induced Stat5 activation (Jones et al., 1999). Hence, because of dual nature of Mek–Erk signaling in differentiation, it is important to understand the role of the Ras pathway in lactogenic hormone-induced differentiation. The results of this study clearly focus on signaling through the Mek–Erk pathway as a Ras-regulated disruptor of lactogenic hormone-induced differentiation. Moreover, by identifying the Mek–Erk pathway along with altered regulation of SHP2 as pathways that are inhibited by DNRasN17, these studies suggest an additional mechanism by which EGF disrupts differentiation in this cell line.

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. Maurizio Grimaldi for assistance with photography of the cell cultures.

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Gene expression profiling of the HC11 mouse mammary epithelial cell line during lactogenic differentiation

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ABSTRACT

Mammary epithelial cells undergo a series of developmental changes during pregnancy and lactation. The processes including proliferation, differentiation, secretion and apoptosis are precisely regulated. HC11 mouse mammary epithelial cells were used to follow the changes in gene expression resulting from lactogenic differentiation in mammary epithelial cells. Gene expression profiles of HC11 cells were analyzed by hybridization to DNA microarrays consisting of 20,000 genes following the treatment of the cells with dexamethasone, insulin, and prolactin (DIP) in the presence or absence of EGF. Greater than two fold changes were detected in 998 genes in the DIP versus growth control group, and 128 genes met the 2-fold change criteria in DIP versus DIP + EGF group. To validate the gene expression pattern, we used Northern blot to prove the reproducibility of the microarray data. Results showed for the first time that many genes were involved in the differentiation of mouse mammary cells and demonstrated the value of microarray analysis in defining gene transcription associated with differentiation in mammary gland.

INTRODUCTION

The mammary gland undergoes periodic cycles of growth, differentiation and regression throughout adult life. Normal mammary gland development is dependent on signals from growth factors, mammotrophic hormones, and tissue stroma. Dysregulation of these process can lead to mammary epithelial hyperplasia and ultimately to mammary tumorigenesis.

The HC11 cell line was derived from the COMMA1D cells, which originated from the mammary gland tissue of a mid-pregnancy BALB/c mouse (Danielson, Oborn et al. 1984). HC11 is an excellent model system for studying differentiation states of mammary epithelial cells. These cells retain important characteristics of normal mammary epithelial cells; they synthesize the milk protein ß-casein *in vitro* upon treatment with lactogenic hormones and exhibit normal ductal morphogenesis when injected into the cleared fat pad of syngeneic mice. A critical event for the mammary epithelial cell differentiation is the activation of the epidermal growth factor (EGF) receptor during the growth phase. In HC11 cells, activation of the EGF receptor promotes growth and is required for the establishment of competence to respond to the lactogenic hormones. Following EGF removal, HC11 cells differentiate *in vitro* in response to the synergistic actions of insulin, glucocorticoids, and prolactin (DIP) (Ball, Friis et al. 1988). The presence of EGF, other mitogens or oncogenic transformation blocks differentiation in response to DIP. The regulatory mechanisms for the signal transduction pathways that control development of the mammary gland epithelium and the molecular switch from proliferation to differentiation are not completely understood.

In previous studies proteomic techniques were used to study the changing protein pattern during mammary epithelial cell differentiation in HC11 cells (Desrivieres, Prinz et al. 2003). These studies identified about 60 proteins whose expression levels changed after 4 days of differentiation. These proteins include cytoskeletal components, molecular chaperones and regulators of protein folding and stability, calcium-binding proteins, and components of RNA-processing pathways. The proteins identified provided insights into the differentiation of mammary epithelial cells and the regulation of this process. The development of large-scale gene expression profiling by DNA microarrays allows the concurrent analysis of thousands of genes and has been applied to further the knowledge of the biochemical pathways involved in breast cancer(Hedenfalk, Duggan et al. 2001; Desai, Kavanaugh et al. 2002; Hedenfalk, Ringner et al. 2002). In this paper, oligonucleotide microarrays containing approximately 20,000 genes were used to examine the gene expression pattern during HC11 lactogeneic differentiation, and the role of some genes in differentiation and the subsequent signaling pathway were explored.

MATERIALS AND METHODS

Cell preparation. HC11 mouse mammary epithelial cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 5 μg/ml Insulin, 10 mM Hepes and 10 ng/ml epidermal growth factor (EGF)(Cerrito, Galbaugh et al. 2004). Cells were maintained in T75 flasks after 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)(DIP) for 72 hours, undifferentiated HC11 cells were used as control. In another set of experiment, control cells were incubated in differentiation media DIP for 72 hours, while experiment cells use the same treatment as in control cells except EGF is added. These 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). RNA samples were concentrated to greater than 1 mg/ml by centrifugation on a MicroCon 100 filter unit at 500g. The concentration and 260/280 OD ratio of the RNA was determined by spectrophotometry and RNA was stored at -80°C. Alternatively, the RNA was purified to recover mRNA using Oligtex mRNA kit according to manufacturer's directions.

Labeling, hybridization and analysis. Mouse (Development) Oligo Microarrays were purchased from Agilent Technologies. The content on this microarray was derived from the National Institute on Aging/National Institute of Health cDNA mouse clone set that includes genes from sequences of stem cells and very early-stage embryo cDNA libraries. It contains 20,371 60-mer oligonucleotides representing over 20,000 known mouse genes. Detailed process for labeling and hybridization were supplied in manufacturer's manual. Briefly, fluorescent cRNA was synthesized using low RNA input fluorescent linear amplification kit from Agilent Technologies. 500 ng of total RNA was used in each experiment according to manufacturer's protocol. In the differentiation experiments, five slides were used (N=5), DIP treated RNA was labeled with cyanine 5-CTP, and control RNA was labeled with cyanine 3-CTP. In differentiation blocking experiments, six slides were used (N=6), EGF treated DIP RNA was labeled with cyanine 5-CTP, while DIP treated RNA was labeled with cyanine 3-CTP. Amplified cRNA was purified using Qiagen's RNeasy mini spin columns. Hybridization was carried out using *In situ* Hybridization Kit Plus from Agilent Technologies, 750 ng of cyanine 3 and cyanine 5 labeled cRNA was used in each hybridization. Hybridization continued at 60 °C for over 17 h. Post hybridization washes includes 6 X SSC, 0.005% Triton X-102 (10 min), and 0.1 X SSC, 0.005% Triton X-102 (5 min) followed by drying with nitrogen gas and immediate scanning. Scanning was performed by GenePix 4000A scanner (Axon instruments, Inc., Foster city, CA) with Axon GenePix image acquisition and analysis software. Analysis of gene expression was performed using BRB-Array Tools Version 3 which was developed by Biometrics Research Branch, NCI (http://linus.nci.nih.gov/BRB-ArrayTools.html).

In another set of experiments 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). 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 of four experiments. The average of Cy3 and Cy5 signals from nine house-keeping genes gives a ratio which was used to normalize the individual signals.

Statistical Analysis of Microarray. Normalization and analysis of the gene expression profiles were performed as follows: Exclude the spot if red and green intensity is below 30. Normalize (center) each array using median over entire array. Truncate intensity ratios (and inverse ratios) greater than 64. Exclude a gene under any of the following conditions: Less than 20 % of expression data have at least a 1.5 -fold change in either direction from gene's median value. Percent of data missing or filtered out exceeds 50%. In DIP versus control experiments, there are 10813 genes that passed filtering criteria in total of 20280 genes. And the first 2479 genes are significant at the nominal 0.05 level of the paired T-test. In EGF plus DIP versus DIP experiments, there are 1386 genes passed filtering criteria in total of 20280 genes. And the first 1129 genes are significant at the nominal 0.05 level of the paired T-test.

Generation of probes. In order to generate probes, the accession number of interested gene was used to obtain the mRNA sequence in PubMed, and then primers were designed for RT-PCR to produce DNA fragment (about 500 bp). Use Gene Amp RT-PCR kit (Roche) to amplify the cDNA and insert the PCR fragment into a pCR2.1 TA cloning vector (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, and was gel purified as a probe.

Northern blot. For Northern blot experiments, 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 was extracted for Northern blot by using Trizol reagent. In other Northern blot experiments, the same RNA used in Agilent Microarrays were utilized. Briefly, 1% agarose gel was prepared in MOPS buffer. RNA samples (10 μg) were mixed with

deionized formamide, formadehyde, and MOPS buffer, then heat at 55 °C for 15 min, load gel and run at 75 V for 2 hours. Blot the gel for 16 hours and the blots were UV fixed for 2 min. The filter was pre-hybridize at 42 °C for more than 1 hour. The probe was labeled with ^{32}P-dCTP (ICN), and was purified using a spin column(Amsharm Biotech). The probe was heated to 95 °C in 0.5 ml salmon sperm DNA for 5 min, and cooled rapidly on ice. Add probe to hybridization solution and incubate overnight. Membranes were washed 3 times at 52 °C, expose the membranes to X-ray film and then scan on beta scanner. Beta-actin probe was used to hybridize the same membrane and then scanned for the normalization purpose.

RESULTS

Regulation of gene expression by DIP in HC11 cells. To determine the global changes in gene transcription in HC11 cells undergoing lactogenic differentiation, we have used slide-based oligonucleotide microarrays containing 20,280 genes from Agilent Technologies. RNA was extracted from HC11 cells maintained in growth media as well as from cells induced to differentiate with DIP. Total RNA was purified and fluorescent cRNA was synthesized using low RNA input fluorescent linear amplification kit. In the differentiation experiments (N=5), DIP treated RNA was labeled with cyanine 5-CTP, and control RNA was labeled with cyanine 3-CTP. Hybridization was carried out using a commercial kit (In situ Hybridization Kit Plus) and reactions were quantitated using an Axon microarray reader. Analysis of gene expression was performed using BRB-Array Tools Version 3, which was developed by Biometrics Research Branch, NCI.

In the DIP versus control experiments, there are 10,813 genes that passed the filtering criteria from a total of 20,280 genes. The changes in expression of the first 2479 genes are significant at the nominal 0.05 level of the paired T-test, and 998 genes met the 2-fold change criteria. Table 1 shows the partial list of genes up- or down-regulated during lactogenic differentiation of HC11 cells. A complete list of all 998 genes is included in appendix 1.

In another set of differentiation experiments a smaller oligonucleotide based array, Clontech 3.8 K arrays, were used. Table 2 shows the partial list of genes that increased or decreased in expression during lactogenic differentiation based on hybridization to the Clontech arrays. A complete list of all genes that met the 2-fold criteria is included in appendix 2.

Regulation of gene expression by EGF during lactogenic differentiation in HC11 cells. RNA was extracted from HC11 cells induced either induced to differentiate with DIP or treated with DIP containing 10 ng/ml EGF. Total RNA was purified and fluorescent cRNA was synthesized as described above. RNA from DIP plus EGF treated cells was labeled with cyanine 5-CTP, and RNA from DIP-treated cells was labeled with cyanine 3-CTP. Hybridization was carried out and the results were quantitated as described. Analysis of gene expression was performed using BRB-Array Tools Version 3. In EGF plus DIP versus DIP experiments, there are 1,386 genes passed filtering criteria in total of 20,280 genes. The first 1,129 genes are significant at the nominal 0.05 level of the paired T-test, and 128 genes met the 2-fold change criteria. Table 5 shows the partial list of genes up- or down-regulated by EGF. A complete list of all genes that met the 2-fold criteria is included in appendix 3.

Northern blot and real-time PCR confirmation of microarray data. Twelve of the up- or down- regulated genes were selected for confirmation by Northern blot; a list of these genes is provided in Table 2. After the first round hybridization with each specific probe, the results were quantitated using a beta scanner and the same membrane was hybridized with beta-actin probe. The fold changes of each gene were normalized to beta-actin expression and are shown in figures 1 and 2. The Beta-actin normalized data revealed a good correlation between the fold changes obtained in the microarray and Northern blot

(Figure 1). We compared real-time PCR results with Northern blot results using same samples and the results demonstrated the reproducibility of these experiments.

DISCUSSION

A number of microarray studies on breast cancer have focused on human breast tissue and mouse models (Hedenfalk, Duggan et al. 2001; Desai, Kavanaugh et al. 2002; Hedenfalk, Ringner et al. 2002; Kelly, Bachelot et al. 2002; Guo, Russo et al. 2003), and several have examined mouse epithelial cells or human breast cancer cell lines such as MCF-7(Guo, Russo et al. 2004), and T47D cells(Mrusek, Classen-Linke et al. 2005). Our study analyzed mammary epithelial cell lactogenic differentiation using for the first time the microarray profiles from HC11 cells undergoing differentiation. In this study oligonucleotide-based microarrays were used to identify the differentiation pattern in HC11 cells. To qualify the accuracy of this microarray chips both northern blot and real-time PCR techniques were used, and results showed very good correspondence.

While numerous genes were up or down regulated by DIP (Table 1), several categories of genes are of particular note. Genes involved in regulation of mitogenesis and cell cycle progression are normally reducued during differentiation. For example, Cyclin D1(CCND1) was reduced during DIP-induced differentiation and expressed in response to the addition of EGF to the differentiation media. EGF stimulates mitogenesis in this system so that the expression of CyclinD1 is serves as a marker for cells in cell cycle. Cyclin D1 plays a pivotal role in the regulation of progression from the G_1 to the S phase through the formation of active enzyme complexes with cyclin-dependent kinases Cdk4 and Cdk6. CyclinD1 was implicated in tumorigenesis as a result of localization to chromosome 11q13, a region of the genome that is commonly amplified in a range of human carcinomas including breast cancer, and dysregulation of cyclin D1 gene expression or function contributes to loss of normal cell cycle control during carcinogenesis (Sutherland and Musgrove 2002; Sutherland and Musgrove 2004). In MCF-7 cells, the inhibition of cell growth was associated with a decrease in the expression of cyclin D1 and a reduction of cyclin D1 promoter activity (Sanchez-Rodriguez, Kaninda-Tshilumbu et al. 2005). This result is similar to our results which showed decreased cyclinD1 mRNA production in differentiated HC11 cells.

Genes involved in lactogenesis, that were predicted to increase in response to DIP, appeared as molecular markers for the differentiation state in our assay. This group includes the milk protein β-casein, serum glucocortecoid kinase and enzymes that can contribute to the secretory pathways. In addition, several transcription factors, including krupple like factors and SpiC increased during lactogenesis. These factors have been cited as markers of a more differentiated state in several cancers including breast and prostate. Hence, the array analysis results reported here support the idea that general markers of breast differentiation are identifiable by this method.

Several unexpected results associated with this assay are note worthy. The most highly up-regulated gene in response to DIP was connective tissue growth factor (CTGF, CCN2), a CCN protein. CTGF is a cysteine-rich protein that can modulate numerous cellular responses including proliferation, chemotaxis, adhesion, migration, and extracellular matrix production. CTGF promotes endothelial cell growth, migration, adhesion, and survival in vitro, and CTGF action in angiogenesis is mediated at least

partly through interactions with integrins. CTGF regulates the activity and production of other angiogenic proteins. Profibrotic activity of CTGF is interesting because high levels of CTGF were detected in many fibrotic lesions, (skin, kidney, lung, liver). CTGF is induced by and acts as a co-factor for transforming growth factor β in the induction of fibrogenesis. Transforming growth factor β, via CTGF, mediates extracellular matrix activity (Gao and Brigstock 2004; Perbal 2004). In our study, CTGF is highly up-regulated in differentiated HC11 cells, suggesting that it might play an important role in stromal signals required for differentiation and carcinogenesis.

Another protein that plays a potential role in adhesion was identified in the array experiments. Osteopontin(OPN) or secreted phosphoprotein 1(SPP1) is a secreted glycoprotein that contains functional domains for calcium-binding, phosphorylation, glycosylation, and extra-cellular matrix adhesion . OPN has multiple molecular functions that mediate cell adhesion, chemotaxis, angiogenesis, prevention of apoptosis, and anchorage-independent growth of tumor cells. Recent studies linked OPN with the regulation of metastatic spread by tumor cells. While the exact molecular mechanisms that define the role of OPN expression in tumor metastasis are not known, OPN activates PI-3-kinase signaling in breast epithelia. A variety of stimuli, including EGF- as shown in our assays, as well as phorbol 12-myristate 13-acetate, 1,25-dihydroxyvitamin D, basic fibroblast growth factor, tumor necrosis factor-α, interleukin-1, interferonγ and lipopolysaccharide, increase OPN expression (Mi, Guo et al. 2004; Nagatomo, Ohga et al. 2004). Our data showed that OPN was also up-regulated in DIP differentiated HC11 cells and that this expression was enhanced by EGF.

In summary, microarray techniques were used to study the differentiation of mouse HC11 cells undergoing DIP-induced lactogenic differentiation, 998 genes were up- or down regulated with greater than two fold changes in DIP versus control group. Analysis of changes in expression in DIP+ EGF versus DIP treated HC11 cells was employed to identify genes that could potentially participate in EGF-induced block to differentiation. In this experiment 128 genes met the 2-fold change criteria. Northern blot and real-time PCR confirmed the accuracy of the microarray results.

ABBREVIATIONS

DIP, dexamethasone, insulin, prolactin.

ACKNOWLEDGEMENTS:

This work was supported by grant DAMD-17-01-1-0264 to Mary Lou Cutler.

Table 1. Partial list of genes differentially transcript in HC11 cells after DIP.(DIP versus Control, Agilent arrays)

mean of log ratio	p-value	GB acc	Description
up-regulated genes			
17.0692248	0.0000098	NM_010217.1	connective tissue growth factor
13.4421959	0.0004945	NM_138314.1	non-metastatic cells 7, protein expressed in
11.14873952	0.0002881	XM_150141.2	LOC234574
9.522688101	0.0000609	NM_009263.1	secreted phosphoprotein 1
7.69516999	0.0006805	BC009155.1	microsomal glutathione S-transferase 1
7.643536442	0.0002742	NM_022032.1	p53 apoptosis effector related to Pmp22
6.986319761	0.0013017	NM_011326.1	sodium channel, nonvoltage-gated 1 gamma
6.439168301	0.0018506	NM_009681.1	adaptor-related protein complex AP-3, sigma 1 subunit
6.335661401	0.0005149	NM_029083.1	RIKEN cDNA 5830413E08 gene
5.923853709	0.0003607	BC036990.1	metallothionein 1
5.847013528	0.0028077	XM_123496.1	Tcfcp2-related transcriptional repressor 1
5.775174223	0.0006173	NM_007621.1	carbonyl reductase 2
5.773951091	0.001373	NM_138578.1	Bcl2-like
5.759602371	0.0006522	NM_012519.1	calcium/calmodulin-dependent protein kinase II, delta
5.719155211	0.0004807	NM_009976.1	cystatin C
5.416841983	0.0001758	NM_010286.1	glucocorticoid-induced leucine zipper
5.147111567	0.0021434	NM_009128.1	stearoyl-Coenzyme A desaturase 2
5.14084859	0.000177	NM_025610.1	RIKEN cDNA 2410004D18 gene
5.134731027	0.0041275	NM_011361.1	serum/glucocorticoid regulated kinase
4.87738793	0.0008795	AK009928.1	RIKEN cDNA 2310051E17 gene
4.82017634	0.0043761	NM_011313.1	S100 calcium binding protein A6 (calcyclin)
4.786828777	0.0020808	AF434663.1	immunoglobulin superfamily, member 4
4.749355426	0.0173842	NM_010730.1	annexin A1
4.597554071	0.0012304	NM_026217.1	autophagy 12-like (S. cerevisiae)
4.590577465	0.0015269	NM_025436.1	sterol-C4-methyl oxidase-like
4.516116188	0.0016936	NM_010884.1	N-myc downstream regulated 1
4.477740665	0.0015348	XM_177182.1	expressed sequence C85344
4.439487743	0.0023195	NM_011400.1	solute carrier family 2 (facilitated glucose transporter)
4.411231384	0.0006233	NM_011803.1	core promoter element binding protein
4.407841901	0.000225	NM_010907.1	nuclear factor of kappa light chain gene enhancer
4.337880257	0.0030802	NM_013470.1	annexin A3
4.325822371	0.0020775	NM_145942.1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
4.212031455	0.0050377	AL080093.1	expressed sequence AU067636
4.164223867	0.0036234	XM_132485.1	RAS-related C3 botulinum substrate 1
4.109387934	0.0104497	NM_010762.1	myelin and lymphocyte protein, T-cell differentiation
4.100802658	0.0010927	NM_009983.2	cathepsin D
4.077570861	0.0073333	AF378830.1	prostaglandin-endoperoxide synthase 2
4.047969236	0.0008687	NM_145977.1	RIKEN cDNA 2210413P12 gene
4.011798665	0.006926	NM_146120.1	gelsolin
3.977737052	0.002251	BC020081.1	RIKEN cDNA 2310016C08 gene
3.950990159	0.0011512	BC019207.1	low density lipoprotein receptor
3.947260885	0.0023132	NM_053623.1	expressed sequence AU018108
3.931095403	0.0033135	NM_019971.1	platelet-derived growth factor, C polypeptide
3.92174486	0.0052617	NM_013492.1	clusterin
3.885899446	0.00773	NM_008084.1	glyceraldehyde-3-phosphate dehydrogenase
3.874324298	0.0026403	BC038392.1	ATPase, H+ transporting, lysosomal 70kD, V1
3.850382989	0.0112736	NM_033079.1	DNA segment, Chr 6, Miriam Meisler 5, expressed
3.837534457	0.0024586	BC037116.1	adducin 3 (gamma)
down-regulated genes			
0.570000993	0.0309545	NM_025836.1	RIKEN cDNA 1300012C15 gene
0.56666635	0.0076789	Mm.214924	ESTs
0.565456881	0.0220481	Mm.11987	ESTs, Moderately similar to POL2_MOUSE
0.539313291	0.0237038	Mm.173695	ESTs, Moderately similar to cofactor required for Sp1
0.444009365	0.0185901	M_008086.1	growth arrest specific 1
0.377664008	0.0011905	C012724.1	insulin-like growth factor binding protein 2

Table 2. Partial list of genes differentially transcript in HC11 cells after DIP.(DIP versus Control, Clontech arrays)

mean	GB acc	Description
up-regulated genes		
6.02275	NM_010217	connective tissue growth factor
4.232	NM_008843	prolactin induced protein
4.11725	NM_013467	alcohol dehydrogenase family 1, subfamil
3.9215	NM_008489	lipopolysaccharide binding protein
3.54625	NM_008952	peroxisomal sarcosine oxidase
3.34025	NM_013515	erythrocyte protein band 7.2
3.318	NM_009721	ATPase, Na+/K+ transporting, beta 1 poly
2.9665	NM_011338	small inducible cytokine A9
2.87525	NM_012046	sporulation protein, meiosis-specific, S
2.85725	NM_011461	Spi-C transcription factor (Spi-1/PU.1 r
2.6545	NM_008065	GA repeat binding protein, alpha
2.54975	NM_007607	carbonic anhydrase 4
2.399	NM_011361	serum/glucocorticoid regulated kinase
2.05575	NM_019496	Alport syndrome, mental retardation, mid
1.96225	NM_009681	adaptor-related protein complex AP-3, si
1.95425	NM_009775	benzodiazepine receptor, peripheral
1.92625	NM_009779	complement component 3a receptor 1
1.849	NM_013634	peroxisome proliferator activated recept
1.8485	NM_013799	arginine-tRNA-protein transferase 1
1.8335	NM_011057	platelet derived growth factor, B polypeptide
1.8125	NM_009127	stearoyl-Coenzyme A desaturase 1
1.80025	NM_007918	eukaryotic translation initiation factor
down-regulated genes		
0.599	NM_009320	solute carrier family 6 (neurotransmitter)
0.5975	NM_008536	transmembrane 4 superfamily member 1
0.5115	NM_011271	ribonuclease 1, pancreatic
0.41725	NM_008764	tumor necrosis factor receptor superfamily
0.331	NM_010701	leukocyte cell derived chemotaxin 1

Table 3. Names and abbreviations of genes used for Northern blot probes.

Gb acc	name	abbr.
NM_010217	connective tissue growth factor	ctgf
NM_012519	calcium/calmodulin-dependent protein kinase II, delta	camk2d
NM_027015	ribosomal protein S27	rps27
NM_010884	N-myc downstream regulated 1	ndr1
NM_009263	secreted phosphoprotein 1	spp1
NM_022891	ribosomal protein L23	rpl23
NM_007631	cyclin D1	ccnd1
NM_008843	prolactin induced protein	pip
NM_010638	Kruppel-like factor 9, basic transcription element binding protein 1	klf9(bteb1)
NM_011361	serum/glucocorticoid regulated kinase	sgk
NM_009681	adaptor-related protein complex AP-3, sigama 1 subunit	ap3
NM_011461	Spi-C transcription factor (Spi-1/PU.1 related)	spic
NM_008557	FXYD domain-containing ion transport regulator 3	fxyd3
NM_009335	transcription factor AP-2, gamma	ap2

Table 4. Functional division of up- and down-regulated genes

Upregulated genes

A. Markers of lactogenic differentiation	
NM_008843	prolactin induced protein
NM_012046	sporulation protein, meiosis-specific, S
NM_009972	casein beta
NM_011461	Spi-C transcription factor (Spi-1/PU.1 r
B. regulatory, but related to dex not prolactin	
NM_011361	serum/glucocorticoid regulated kinase
NM_010286	glucocorticoid-induced leucine zipper
NM_010217	connective tissue growth factor
C. regulatory and useful markers between control and EGF, DN Ras and Ras	
NM_009681	adaptor-related protein complex AP-3, si
NM_008065	GA repeat binding protein, alpha
NM_011057	platelet derived growth factor, B polype
NM_011944	mitogen activated protein kinase kinase
NM_009263.1	secreted phosphoprotein 1
NM_012519.1	calcium/calmodulin-dependent protein kinase II, delta
XM_123496.1	Tcfcp2-related transcriptional repressor 1
XM_132485.1	RAS-related C3 botulinum substrate 1
NM_011803.1	core promoter element binding protein
AF378830.1	prostaglandin-endoperoxide synthase 2
NM_010907.1	nuclear factor of kappa light chain gene enhancer
NM_022032.1	p53 apoptosis effector related to Pmp22
D. interesting as an extension of growth control	
NM_008489	lipopolysaccharide binding protein
NM_008952	peroxisomal sarcosine oxidase
NM_013515	erythrocyte protein band 7.2
NM_011338	small inducible cytokine A9, chemokine
NM_007607	carbonic anhydrase 4
NM_019496	Alport syndrome, mental retardation, mid
NM_013467	alcohol dehydrogenase family 1, subfamil
NM_009721	ATPase, Na+/K+ transporting, beta 1 poly
NM_013492.1	clusterin
BC009155.1	microsomal glutathione S-transferase 1
NM_010884.1	N-myc downstream regulated 1
NM_019971.1	platelet-derived growth factor, C polypeptide
NM_007621.1	carbonyl reductase 2
NM_138578.1	Bcl2-like
NM_011313.1	S100 calcium binding protein A6 (calcyclin)
NM_009128.1	stearoyl-Coenzyme A desaturase 2
BC036990.1	metallothionein 1

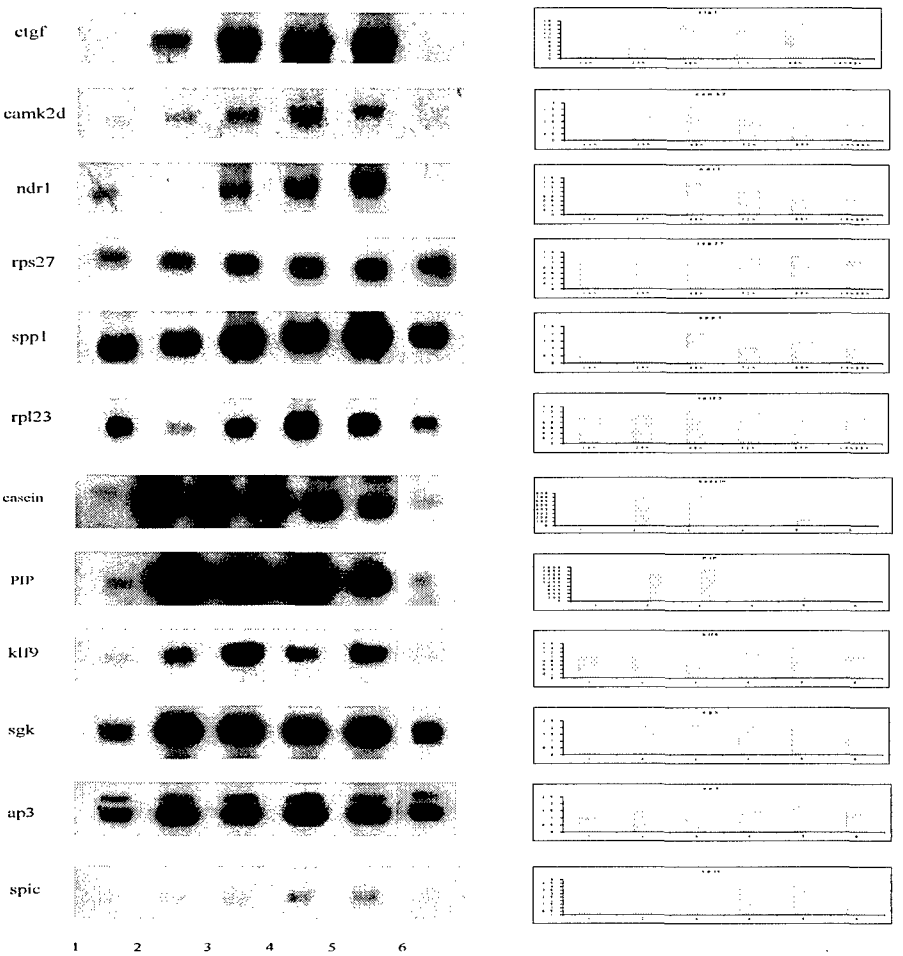
Downregulated genes

C. regulatory	
NM_009335	transcription factor AP-2, gamma
NM_008536	transmembrane 4 superfamily member 1
NM_008594	milk fat globule-EGF factor 8 protein
Mm.173695	ESTs, Moderately similar to cofactor required for Sp1
NM_007631	cyclin D1
D. extension of growth control	
NM_010701	leukocyte cell derived chemotaxin 1
NM_008764	tumor necrosis factor receptor superfami
NM_009320	solute carrier family 6 (neurotransmitte
M_008086.1	growth arrest specific 1
NM_008557	FXYD domain-containing ion transport regulator 3

Table 5. Partial list of genes whose transcription changes after EGF ((DIP+EGF) versus DIP, Agilent arrays).

mean	p-value	GB acc	Description
up-regulated genes			
2.629325346	0.0049573	NM_009263.1	secreted phosphoprotein 1
2.54281691	0.0008723	NM_016980.1	ribosomal protein L5
2.522356968	0.0000113	AF378830.1	prostaglandin-endoperoxide synthase 2
2.456467561	0.0000237	NM_008972.1	prothymosin alpha
2.347383128	0.0010689	NM_009121.1	spermidine/spermine N1-acetyl transferase
2.33154908	0.0004548	NM_019682.1	dynein, cytoplasmic, light chain 1
2.318271338	0.0000407	BC006739.1	catenin beta
2.307858951	0.0000824	NM_133777.1	RIKEN cDNA 6720465F12 gene
2.299748625	0.0001515	NM_023372.1	RIKEN cDNA 0610025G13 gene
2.294296857	0.0203708	NM_007631.1	cyclin D1
2.280497971	0.0022307	BC010581.1	stathmin 1
2.279510217	0.0046942	XM_134967.2	eukaryotic translation elongation factor 1 alpha 1
2.274293628	0.0011008	NM_031165.1	heat shock protein 8
2.274131004	0.0006492	NM_022891.1	ribosomal protein L23
2.264831199	0.0046178	NM_008305.1	perlecan (heparan sulfate proteoglycan 2)
2.260996285	0.000296	NM_025592.2	ribosomal protein L35
2.260724408	0.000086	NM_009608.1	actin, alpha, cardiac
2.256595314	0.0049616	BC034257.1	epithelial membrane protein 1
2.25147238	0.0001414	NM_019703.1	phosphofructokinase, platelet
2.246214176	0.0000793	XM_178595.1	chromobox homolog 3 (Drosophila HP1 gamma)
2.230281272	0.0001232	NM_133834.1	expressed sequence AA407306, Mus musculus similar to
2.22879665	0.0009146	NM_009255.1	serine (or cysteine) proteinase inhibitor, clade E, member 2
2.224673263	0.0011449	BC010726.1	phospholipase A2, group VII (platelet-activating factor
2.222725739	0.0003309	NM_013261.1	peroxisome proliferative activated receptor, gamma, coactivator 1
2.218331019	0.0056255	XM_129211.2	phosphoserine aminotransferase
2.210294911	0.0003704	NM_010480.1	heat shock protein, 1
2.205449432	0.0001814	NM_025919.1	RIKEN cDNA 2010203J19 gene
2.203718197	0.0007032	XM_128846.2	similar to 60S RIBOSOMAL PROTEIN L17 (L23) (AMINO
2.200748267	0.0004923	BC012508.1	SET translocation
2.198605922	0.0003785	NM_145142.1	HNK-1 sulfotransferase
2.191165553	0.00075	NM_009076.1	hypothetical pseudogene ribosomal protein L12
2.188916599	0.0005466	NM_007961.1	ets variant gene 6 (TEL oncogene)
2.172805823	0.0004297	AK021352.1	RIKEN cDNA D730048J04 gene
2.166441483	0.0001855	NM_028044.1	calponin 3, acidic
2.159682559	0.0000483	BC003308.1	casein kinase 1, delta
2.153471062	0.0002713	NM_011400.1	solute carrier family 2 (facilitated glucose transporter), member 1
2.139667816	0.0003473	NM_008774.1	poly A binding protein, cytoplasmic 1
2.138032076	0.0003554	XM_135387.1	similar to 40S RIBOSOMAL PROTEIN S19
2.137441105	0.0001705	BC002046.1	ephrin A1
2.134511179	0.0001161	NM_011218.1	protein tyrosine phosphatase, receptor type, S
2.130928293	0.0000937	XM_126364.1	similar to ATP synthase, H+ transporting, mitochondrial F0
down-regulated genes			
0.559978646	0.0024771	NM_008372.2	interleukin 7 receptor
0.557859374	0.0016467	U44955.1	gap junction membrane channel protein alpha 3
0.553438674	0.0004679	ESTs	related to Mus musculus T-cell receptor alpha/delta
0.55288258	0.0026569	ESTs	related to Homo sapiens insulin-like growth factor 2 receptor
0.552475761	0.0036117	NM_020496.1	T-box 20
0.550021959	0.0003715	XM_130944.2	zinc finger protein 36
0.540875929	0.0010332	ESTs	no related protein was found
0.525350484	0.0090139	NM_172740.1	RIKEN cDNA B230312I18 gene,similar to Mus musculus mRNA
0.503499338	0.0037439	ESTs	no related protein was found
0.495838452	0.0014903	BC033410.1	eukaryotic translation initiation factor 4E nuclear import factor 1

Fig. 1


ctgf
camk2d
ndr1
rps27
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rpl23
casein
PIP
klf9
sgk
ap3
spic
1
2
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Fig. 2

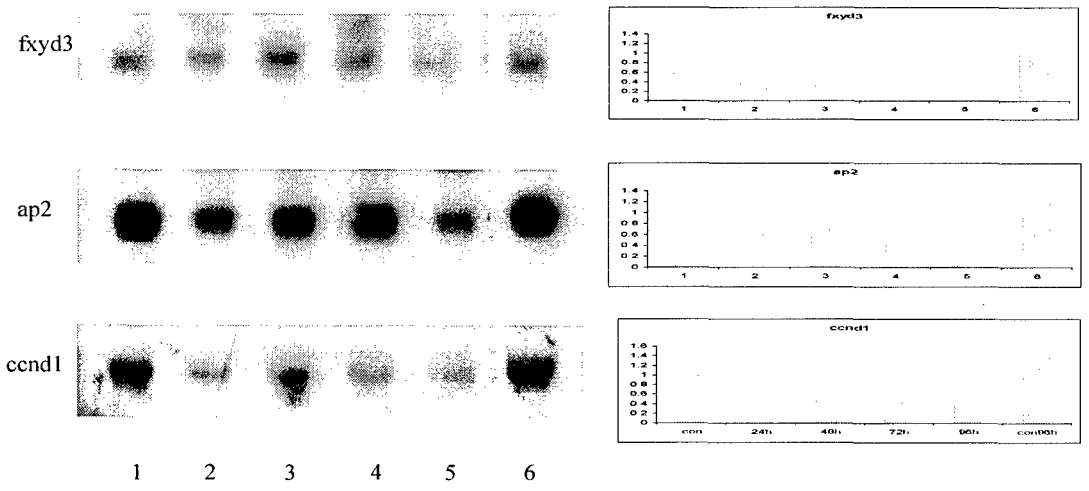
fxyd3
ap2
ccnd1
1 2 3 4 5 6
fxyd3
ap2
ccnd1
con
24h
48h
72h
96h
con(96h)

FIGURE LEGENDS

Fig. 1. Northern blot results of selected up-regulated genes, DIP versus control. HC11 mouse mammary epithelial cells were cultured in complete RPMI 1640 medium with EGF after confluence for 4 days, cells were starved 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)(DIP) for 0, 24, 48, 72, 96 hrs and 96 hrs control (as in lanes 1 to 6) undifferentiated HC11 cells were used as control. RNAs were extracted and used for Northern blot. Left: Northern blot of different genes. Right: fold changes of gene expression normalized to beta-actin.

Fig. 2. Northern blot results of selected down-regulated genes, DIP versus control. HC11 mouse mammary epithelial cells were cultured in complete RPMI 1640 medium with EGF after confluence for 4 days, cells were starved 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)(DIP) for 0, 24, 48, 72, 96 hrs and 96 hrs control (as in lanes 1 to 6) undifferentiated HC11 cells were used as control. RNAs were extracted and used for Northern blot. Left: Northern blot of different genes. Right: fold changes of gene expression normalized by beta-actin.

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