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Image-based evaluation of the molecular events underlying HC11 mammary epithelial cell differentiation

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ABSTRACT

We have developed an image-based technique for signal pathway analysis, target validation, and compound screening related to mammary epithelial cell differentiation. This technique used the advantages of optical imaging and the HC11-Lux model system. The HC11-Lux cell line is a subclone of HC11 mammary epithelial cells transfected stably with a luciferase construct of the β -casein gene promoter (p-344/-1 β c-Lux). The promoter activity was imaged optically in real time following lactogenic induction. The imaging signal intensity was closely correlated with that measured using a luminometer following protein extraction ($R = 0.99$, $P < 0.0001$) and consistent with the messenger RNA (mRNA) level of the endogenous β -casein gene. Using this technique, we examined the roles of JAK2/Stat5A, Raf-1/MEK/MAKP, and PI3K/Akt signal pathways with respect to differentiation. The imaging studies showed that treatment of the cells with epidermal growth factor (EGF), AG490 (JAK2-specific inhibitor), and LY294002 (PI3K-specific inhibitor) blocked lactogenic differentiation in a dose-dependent manner. PD98059 (MEK-specific inhibitor) could reverse EGF-mediated differentiation arrest. These results indicate that these pathways are essential in cell differentiation. This simple, sensitive, and reproducible technique permits visualization and real-time evaluation of the molecular events related to milk protein production. It can be adopted for high-throughput screening of small molecules for their effects on mammary epithelial cell growth, differentiation, and carcinogenesis.

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The mammary gland undergoes a physiological cycle of lobuloalveolar development and differentiation from the virgin state to pregnancy and lactation. This cycle involves a complex interplay of both hormones and growth factors [1–3]. Abnormal development is an important factor in breast carcinogenesis. Hormones, including estrogen and prolactin, are strongly associated with an increase in breast cancer risk, with evidence of a dose-response relationship [4–6]. In addition, exposure to certain chemicals and hormone-mimicking or endocrine-disrupting compounds leads to breast carcinogenesis, precocious puberty, or inability to produce sufficient breast milk [7,8]. To date, approximately 200 chemical compounds have been recognized as risk factors in human breast carcinogenesis, but most of the chemicals to which people are routinely exposed remain to be tested for carcinogenic risk. The conventional analytic methods for molecular delineation and compound screening are generally time-consuming and need DNA, RNA, and/or protein extraction as the first step. To under-

stand the complex interplay of hormones, growth factors, and environmental compounds on the growth and differentiation of mammary epithelial cells, it is critical to develop a simple and sensitive method with the capability of high-throughput screening (HTS)¹ [9,10].

The HC11 mammary epithelial cell line was originally derived from midpregnant BALB/c mouse mammary gland tissue. HC11 cells have retained the characteristics of normal mammary epithelial stem cells. After growth to confluence in the presence of insulin and epidermal growth factor (EGF), HC11 cells become competent and respond to stimulation by the lactogenic hormones dexamethasone, insulin, and prolactin (DIP). The cells will undergo differentiation with synthesis of milk proteins such as β -casein, a marker of

¹ Abbreviations used: HTS, high-throughput screening; EGF, epidermal growth factor; DIP, dexamethasone, insulin, and prolactin; GM, growth medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DM, differentiation medium; CCD, charge-coupled device; p/s, photons per second; BCA, bicinchoninic acid; cDNA, complementary DNA; PCR, polymerase chain reaction; EtBr, ethidium bromide; UV, ultraviolet; RT, reverse transcriptase; mRNA, messenger RNA; ATP, adenosine triphosphate; MAO, monoamine oxidase.

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mammary gland terminal differentiation [7,11]. On the other hand, transplantation of HC11 cells transfected with certain oncogenes can result in tumor development in nude mice [12]. Because of these unique features, HC11 cells provide a well-established in vitro model system for studying signal transduction pathways and hormonal/growth factor regulation of mammary epithelial cell differentiation [7,13–16]. The current study describes a simple and sensitive method for optical imaging of the molecular events leading to differentiation of HC11 mammary epithelial cells. The HC11 cells are stably transfected with a β -casein promoter luciferase construct (p-344/-1 β c-Lux) (HC11-Lux). When the cells grow to confluence in the presence of EGF and insulin in the medium, they become competent and are ready to respond to DIP. The competent HC11-Lux cells show extremely high β -casein promoter activity when stimulated with DIP. The β -casein promoter activity could be detected on the basis of light emission and imaged in real time using an optical imaging system. The light signal intensity was closely correlated with the cell differentiation state. Using this system, we successfully imaged the time-dependent changes during DIP-induced differentiation. We investigated the role of several key signal transduction pathways using pathway-specific inhibitors. The image-based method maximizes the information, simplifies the traditional techniques, and is potentially useful for developing a rapid and robust HTS method for signal pathway analysis, target validation, and small-molecule screening.

Materials and methods

HC11-Lux cell culture

The HC11-Lux mammary epithelial cell line (a subclone of COMMA-1D cells) was kindly provided by David S. Salomon (National Cancer Institute, Bethesda, MD, USA) and Nancy E. Hynes (Friedrich Miescher Institute, Basel, Switzerland) [15,16]. The cells

are stably transfected with a β -casein promoter luciferase construct (p-344/-1 β c-Lux). Cells were routinely maintained in growth medium (GM) that consisted of Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, 10% heat-inactivated fetal bovine serum (FBS), 5 μ g/ml bovine insulin, 10 ng/ml EGF, and 50 μ g/ml each of penicillin, streptomycin, and neomycin. The culture medium, FBS, insulin, EGF, and antibiotics all were purchased from Invitrogen (Carlsbad, CA, USA). To induce differentiation, cells were seeded in 96-well microplates and allowed to grow to 100% confluence. The cells were maintained in GM for 1 to 2 more days to become competent. The competent cells were then stimulated with differentiation medium (DM) for various times. The DM contained DMEM/F-12, 10% heat-inactivated FBS, 5 μ g/ml bovine insulin, 1 μ M dexamethasone (Sigma, St. Louis, MO, USA), 5 μ g/ml bovine prolactin (Sigma), and 50 μ g/ml each of penicillin, streptomycin, and neomycin.

Bioluminescent optical imaging

Luciferase-based bioluminescent optical imaging was performed using a Xenogen IVIS 200 imaging system (Caliper Life Sciences, Hopkinton, MA, USA). The system is equipped with a highly sensitive, cooled charge-coupled device (CCD) camera and a light-tight specimen box. Imaging and quantification of signals were controlled by the acquisition and analysis software Living Image 3.0 (Caliper Life Sciences). To image the event of differentiation, d-luciferin was added to each well and mixed gently (final concentration of 150 μ g/ml). The microplates were then placed on the stage of the specimen box. The temperature of the stage inside the box was maintained at 37 °C. Images were captured at 5 min after adding d-luciferin. The image acquisition time was 1 min. The bioluminescent signal from the cells in each well was measured and expressed as total flux (photons per second [p/s]). At least five replicates were performed in all experiments, and each

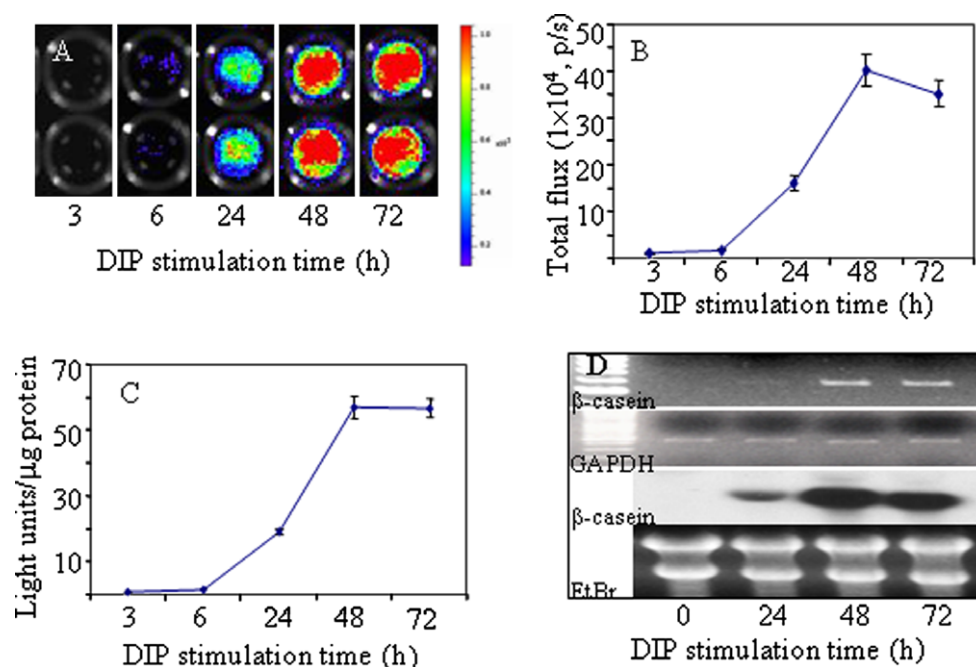


Fig. 1. Imaging of DIP-induced differentiation and comparison with traditional methods. (A) Representative images of the cells at different times following DIP induction. (B) Quantification of the light signals from the images. (C) β -Casein promoter activity measured using single-tube luminometer following protein extraction. A close correlation was obtained between image-based and luminometer measurements for β -casein promoter activity ($R = 0.99$, $P < 0.001$). (D) Semiquantitative RT-PCR and Northern blot analysis of the endogenous β -casein gene mRNA level, showing consistency with imaging findings. Cells were seeded in 96-well microplates in GM, maintained for 2 days after 100% confluence, and then stimulated with DIP and imaged. For protein and RNA extraction, 24- or 6-well plates were used. Values are the means and standard deviations of five replicates. GAPDH is used as the internal control.

experiment was repeated at least three times. The representative data are presented.

β-Casein gene promoter activity measurement using luminometric assay

HC11-Lux cells were cultured in 24-well plates and harvested at different times after DM stimulation and other treatments. The total protein was extracted from cells, and the β -casein gene promoter activity was assayed on triplicate samples using the luciferase assay system (Promega, Madison, WI, USA). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The light emission (expressed as light units) was measured on a single-tube luminometer. The β -casein promoter activity was expressed as light units/microgram protein.

Semiquantitative RT-PCR and Northern blot analysis

Cells were cultured and treated on 6-well plates. Total RNA was isolated from HC11-Lux cells using TRIzol extraction reagent (Invitrogen). From each sample, 1 μ g of total RNA was used to synthesize the first-strand complementary DNA (cDNA) in a final volume of 20 μ l using the SuperScript First-Strand Synthesis Kit (Invitrogen). Also, 0.5 μ l of the cDNA was used to amplify a segment of the β -casein gene using the primers 5'-ACTGTATCCTCTGAGACTG-3' and 5'-TCTAGGTACTGCAGAAGTC-3'. An amplified fragment of the GAPDH gene was used as an internal control, and the primers were purchased from Invitrogen. The polymerase chain reaction (PCR) was carried out for 20 cycles, with each cycle consisting of a denaturing step for 45 s at 94 °C, an annealing step for 45 s at 58 °C, and a polymerization step for 45 s at 72 °C. The PCR product was separated on 2% agarose gel containing ethidium

bromide (EtBr) and was photographed under ultraviolet (UV) illumination. For Northern blot analysis, 30 μ g of total RNA were separated on 1% agarose-formaldehyde gel. Loading of the RNA was evaluated with EtBr staining. The RNA was transferred to nylon membranes (Micron Separations, Westboro, MA, USA) and hybridized overnight to the β -casein probe. The β -casein probe was a fragment generated by reverse transcriptase (RT)-PCR as described above and was 32 P-labeled using the Ready-To-Go DNA Labeling Beads (-dCTP) (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

The results were analyzed using the statistical software Origin-Pro 7.0 (OriginLab, Northampton, MA, USA). A *P* value of 0.05 was considered as a significant correlation for the β -casein gene promoter activity between the image-based method and the luminometer measurement following protein extraction.

Results

Imaging of DIP-induced cell differentiation

To image the differentiation event, cells were seeded on 96-well microplates, allowed to grow to 100% confluence, maintained for 2 more days in the GM, and then stimulated with DM. Cells were imaged at 3, 6, 24, 48 and 72 h separately using a Xenogen optical imaging system (Alameda, CA, USA) (Figs. 1A and B). The light signal from the cells could be detected as early as 6 h, although the signal was weak. A significantly higher light signal was detected at 24 h, and the signal reached the highest levels at 48 and 72 h. When confluent cells were maintained for a relatively long period in the GM (e.g., ≥ 2 days), a stronger light signal from cells was usually detected at 48 h than at 72 h following DIP stimulation.

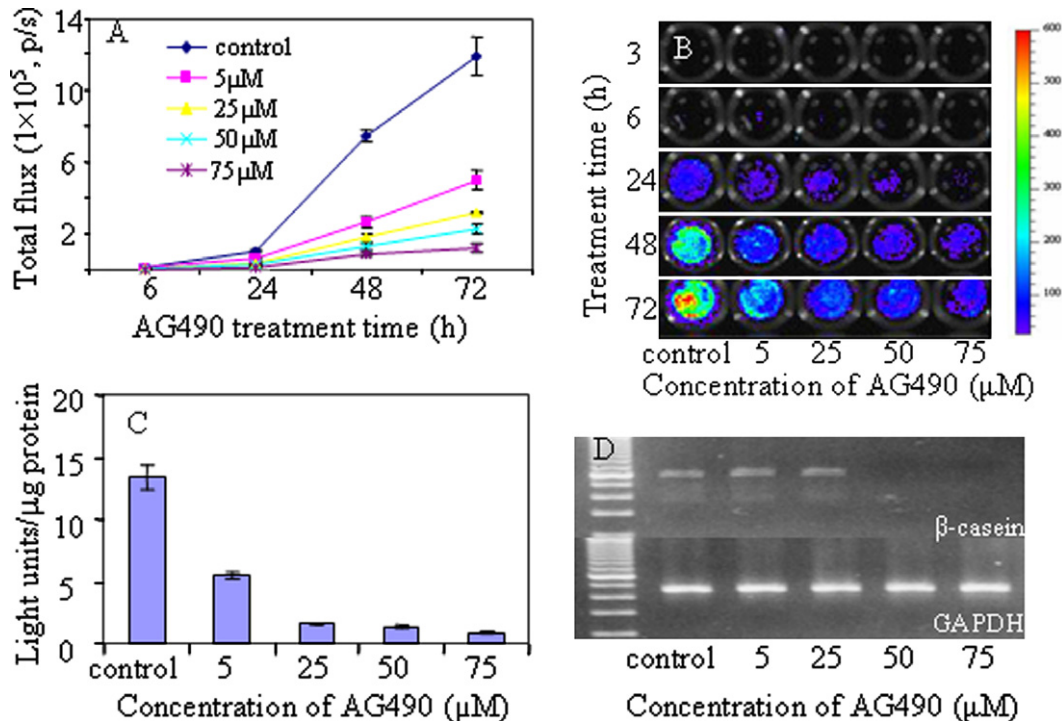


Fig. 2. Imaging of cell differentiation inhibition following inhibition of JAK2 using specific inhibitor AG490. Confluent cells were maintained for 1 day in GM and then stimulated using DM with AG490 at different concentrations. (A,B) Representative images and quantitative results showing dose-dependent decrease of light signals and indicating inhibition of cell differentiation. (C) β -Casein promoter activity at 48 h time point measured using luminometer. (D) Semiquantitative RT-PCR of endogenous β -casein gene mRNA at 48 h time point. There was a close correlation between imaging findings and luminometer measurement and consistency with the β -casein mRNA level. GAPDH is used as the internal control. Control: DM alone.

This phenomenon might be related to the different status of cell proliferation activity. To confirm the imaging results, we also measured the β -casein gene promoter activity using the luminometric assay following protein extraction (Fig. 1C). Luminometric results were similar to those of optical imaging, and a close correlation for the β -casein gene promoter activity was obtained between the two methods ($R = 0.99$, $P = 0.0008$). Both techniques demonstrated that the HC11-Lux cells underwent differentiation following DIP stimulation. To verify whether the β -casein gene promoter activity represented the differentiation status, the messenger RNA (mRNA) level of the endogenous β -casein gene was analyzed using semiquantitative RT-PCR (Fig. 1D, upper two panels) and Northern blot analysis (Fig. 1D, lower two panels). Both methods demonstrated a detectable mRNA level at 24 h, and the level became higher at 48 and 72 h following DIP stimulation. The imaging results were consistent with those from RT-PCR and Northern blot analysis. Optical imaging is simple and superior to these traditional methods for real-time visualization and evaluation of the cell differentiation. Imaging and image process could be completed within a few minutes.

Imaging of differentiation inhibition by blocking JAK2/Stat5A signal pathway

The JAK2/Stat5A signal pathway is the major mechanism of DIP-induced mammary cell differentiation. AG490 is a JAK2-selective inhibitor [17]. To image the effect of AG490 on differentiation, AG490 was added into DM at various concentrations when GM was replaced by DM. Cells were imaged at different time points. A dose-dependent inhibition of the β -casein gene promoter activity was clearly imaged (Figs. 2A and B). The cells emitted a significantly lower light signal than the control without AG490 treatment ($P < 0.01$). At the concentration of 5 μ M AG490, the β -casein gene promoter activity decreased to less than half of the control. These results indicate that blockage of the JAK2/Stat5A signal pathway led to failure of the cell differentiation. When β -casein gene promoter activity at the 48-h time point was measured using the luminometric assay following protein extraction, similar results were obtained, with a close correlation between the two methods ($R = 0.99$, $P = 0.001$). The optical imaging results were also consistent with the mRNA level of the endogenous β -casein gene from RT-PCR analysis (Fig. 2D), indicating the reliability of optical imaging for detecting differentiation status.

Imaging the role of Raf-1/MEK/MAKP signal pathway

Activation of the Raf-1/MEK/MAKP signal pathway is responsible for mammary epithelial cell growth [16]. Previous studies demonstrated that activation of this pathway by DIP is important for optimization of DIP-induced differentiation. However, overactivation of this pathway plays an important role in mammary gland carcinogenesis. We imaged the significance of this signal pathway in the mammary cell differentiation. EGF is well known to activate this pathway. When the competent HC11-Lux cells were treated with both DIP and EGF, the imaging showed that EGF effectively inhibited the β -casein promoter activity even at a concentration as low as 0.5 ng/ml (Figs. 3A and B). This inhibitory effect in imaging was consistent with the β -casein mRNA expression by RT-PCR (Fig. 3C). These cells exhibited undetectable levels of β -casein mRNA expression at 48 h. On the contrary, the control cells without EGF showed both high β -casein promoter activity and high β -casein mRNA level. PD98059 is a specific MEK inhibitor that can selectively block MEK activation in response to different growth factors, including EGF [16]. As competent HC11-Lux cells were treated with

PD98059 and DIP together, PD98059 enhanced the DIP-induced β -casein promoter activity (Figs. 4A and B). At the concentration of 10 μ M, PD98059 showed the most effective enhancement, with twofold higher β -casein promoter activity than DIP alone. In the presence of EGF (1 ng/ml) in DM, PD98059 reversed the inhibitory effect of EGF in a dose-dependent manner (Figs. 4C and D). At the concentrations of 5 to 20 μ M, more than 50% recovery of the activity was obtained for the EGF-induced inhibition. These results suggest that EGF could block the DIP-induced differentiation and that activation of the raf-1/MEK/MAKP pathway is one of the major mechanisms of differentiation arrest.

Imaging of PI3K/Akt signal pathway

PI3K/Akt is another important signal pathway in DIP-induced HC11 differentiation [18]. We inhibited the PI3K/Akt pathway using the P13K-specific inhibitor LY294002 (Fig. 5) [18]. When LY294002 was added into the DM, the DIP-induced cell differentiation was significantly inhibited, presenting a dose-dependent inhibitory pattern. At the concentration of 10 μ M, the light signal

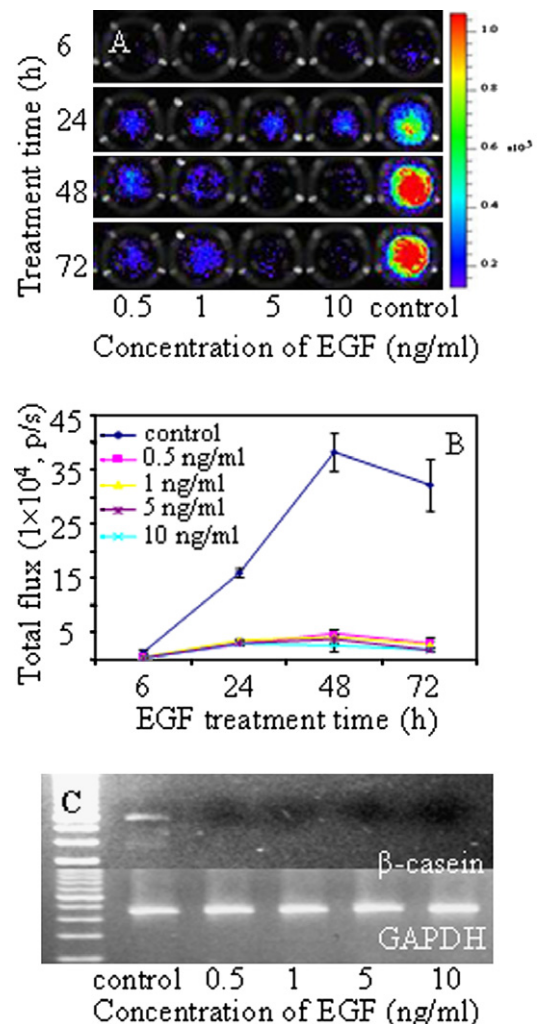


Fig. 3. Imaging of cell differentiation arrest following overactivation of Raf-1/MEK/MAKP pathway by adding EGF into DM. (A,B) Representative images and quantitative results showing complete differentiation arrest by EGF. (C) Semiquantitative RT-PCR of endogenous β -casein gene mRNA at 48 h time point showing undetectable β -casein mRNA that was consistent with imaging findings. GAPDH is used as the internal control. Control: DM alone.

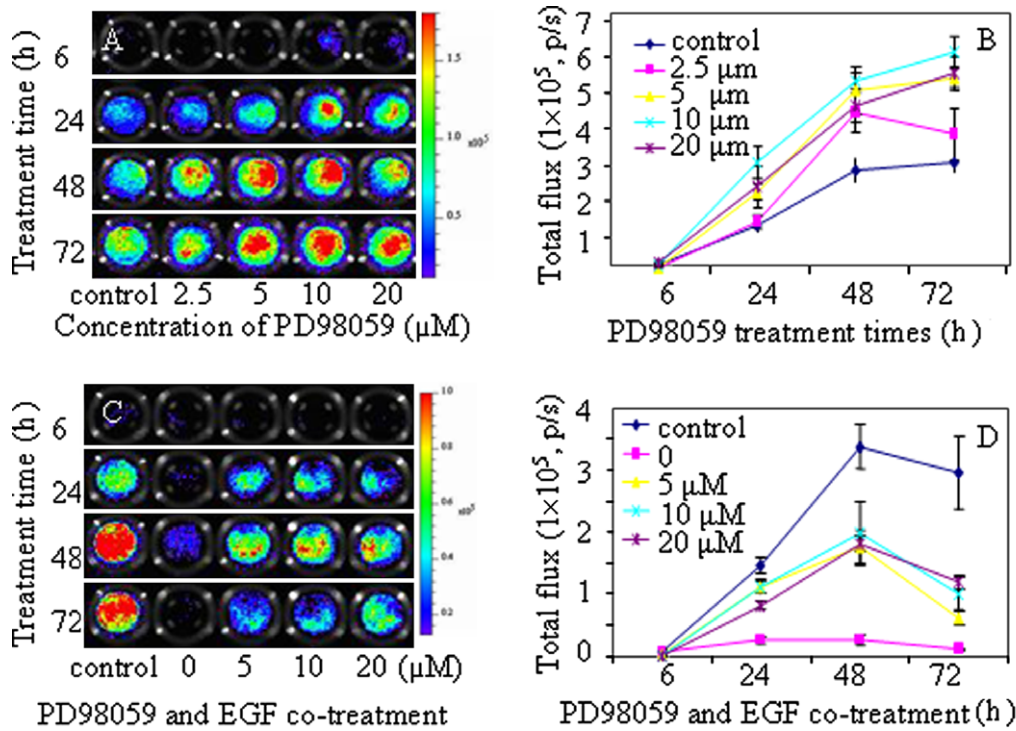


Fig. 4. Imaging of the cell differentiation alteration following inhibition of Raf-1/MEK/MAKP pathway using MEK-specific inhibitor PD98059 and its reversal effect of EGF-induced differentiation arrest. (A,B) Representative images and quantitative results showing enhancement of DIP-induced differentiation by PD98059. (C,D) Cotreatment of cells using PD98059 and EGF showing partial reversal of EGF-induced arrest. Control: DM alone. In other samples, EGF was added at the concentration of 1 ng/ml and PD98059 was added as indicated in panels C and D.

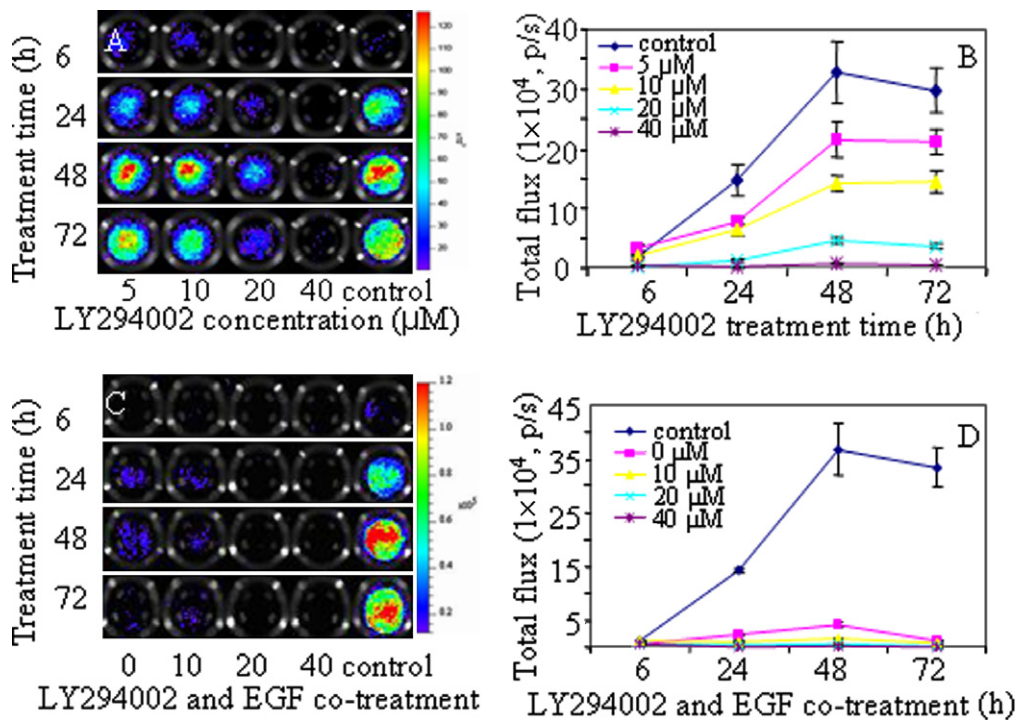


Fig. 5. Imaging of cell differentiation following inhibition of PI3K/Akt pathway using PI3K-specific inhibitor LY294002. LY294002 was added into DM at different concentrations before treatment. (A,B) Representative images and quantitative results showing dose-dependent inhibition of DIP-induced differentiation. (C,D) Cotreatment of cells using LY294002 and EGF showing complete arrest of DIP-induced differentiation. Control: DM alone. In other samples, EGF was added at the concentration of 1 ng/ml and LY294002 was added as indicated in panels C and D.

intensity was only approximately 40% of the controls at different treatment time points (24, 48, and 72 h). At the concentration higher than 20 μM, the light signal intensity was significantly

lower, indicating that DIP-induced differentiation was nearly completely arrested. When the cells were treated with LY294002 in the presence of EGF, the imaging results revealed

that the cell differentiation was completely inhibited even at very low concentrations of both EGF (1 ng/ml) and LY294002 (5 μ M/ml). EGF and LY294002 had an additive or synergistic effect on the inhibition of DIP-induced mammary epithelial cell differentiation. These results suggest that the PI3K/Akt pathway plays an important role in DIP-induced β -casein expression.

Discussion

Recent technological advances have made it feasible to conduct HTS of gene expression changes and small molecules based on visual phenotypes of individual cells or functional reporters using automated imaging [9,10]. Luciferase-based bioluminescent imaging is cost-effective and easy to use, with an extremely high signal-to-noise ratio relative to other imaging techniques. In the presence of oxygen, Mg^{2+} , and adenosine triphosphate (ATP), luciferase catalyzes the conversion of d-luciferin to oxyluciferin, which is accompanied by release of a photon. This article has described a simple and sensitive image-based method that is reproducible and permits real-time evaluation and visualization of the molecular events related to milk protein production. We tested the feasibility of this technique for imaging DIP-induced differentiation and underlying signal pathways. The hallmark of mammary gland differentiation is secretion of milk protein, including β -casein. Transcription of the β -casein gene is highly dependent on prolactin. Insulin and dexamethasone increase prolactin-stimulated β -casein mRNA transcription and stability. The close correlation between the image findings and β -casein promoter activity as measured following protein extraction indicates that this method was precise in quantification. The consistency of the image-based findings with the endogenous β -casein mRNA expression level suggests that findings in imaging indeed reflected the status of cell differentiation. DIP-induced differentiation involves JAK2/Stat5A, Raf-1/MEK/MAKP, and PI3K/Akt pathways [15–17]. On normal mammary epithelial cells, JAK2/Stat5A mediates growth-suppressive and differentiation-inducing effects, whereas the Raf-1/MEK/MAKP pathway plays a key role in cell proliferation and the PI3K/Akt pathway contributes to cell survival. In mammary gland carcinogenesis, JAK2/Stat5A signaling plays a dual role in breast cancer initiation and progression [19–21]. Aberrant activation of the Raf-1/MEK/MAKP pathway is related to development of various cancers, including breast cancer [22]. The PI3K/Akt pathway contributes to resistance of mammary gland tumors to chemotherapy [18]. We imaged the role of the three key pathways in DIP-induced cell differentiation. Inhibition of the JAK2/Stat5A and PI3K/Akt pathways using specific inhibitors and overactivation of Raf-1/MEK/MAKP using EGF all led to failure of differentiation, presenting significant low light signal in optical imaging. Close correlation or consistency for the findings between the image-based technique and conventional methods demonstrates that this technique is reliable.

Compared with conventional methods, the significant advantage of the image-based method is that it is simple, is rapid, and permits real-time visualization and evaluation of cell differentiation status. These features make the image-based technique ideal for laboratory automation. This method can be extended to robust HTS of natural or human-made small molecules for their effects on mammary differentiation, carcinogenesis, or drug development. Analysis using signal pathway-specific inhibitors demonstrated the potential as an HTS technique. HTS primarily aims to systematically test hundreds of small molecules to find candidates. Recently developed and more complex phenotypic assays, such as cell-based and whole-organism-based ones, precede HTS to validate new targets and characterize new lead com-

pounds in cellular contexts [23]. The cell-based phenotypic assay is an upcoming methodology for the investigation of cellular processes and their alteration by multiple chemical or genetic perturbations [24,25]. Cell-based screens use genetic engineering to narrow the field of targets in an attempt to reduce their complexity to that of a biochemical assay. Ding and coworkers developed a method using a neuronal-specific promoter luciferase construct in embryonic stem cells to screen compounds that can induce neuron differentiation [26]. This embryonic stem cell system allows compounds to induce complex phenotypic change, differentiation of cells into neurons, without prior knowledge of the mechanism underlying this transformation. Valley and coworkers described a two-step homogeneous bioluminescent assay for monoamine oxidase (MAO) and screened small molecules to find MAO inhibitors [27]. With the help of automated fluorescent microscopy, Li and coworkers identified some blockers of gap junction communication between cells by coculturing donor cells (preloaded with a membrane-impermeable dye) and acceptor cells and measuring junction-dependent transfer of fluorescence into acceptor cells [28]. In contrast to biochemical screening, these assays detect the responses within the context of intercellular structural and functional networks of normal and diseased cells, respectively. However, high variation of many cell-based assays and not yet fully satisfying solutions for quantitative image analysis are limitations of the technology. The method described in the current study used the characteristics of the HC11 cell system and highly sensitive bioluminescent imaging to visualize and quantify the mammary epithelial cell differentiation. To our knowledge, it is the first image-based system for signal pathway analysis, target validation, and compound screening related to mammary epithelial cell differentiation.

Acknowledgments

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