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Characterization of mammary epithelial cell line HC11 using the NIA 15k gene array reveals potential regulators of the undifferentiated and differentiated phenotypes

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ABSTRACT

Differentiation of undifferentiated mammary epithelial stem and/or progenitor cells results in the production of luminal-ductal and myoepithelial cells in the young animal and upon pregnancy, the production of luminal alveolar cells. A few key regulators of differentiation have been identified, though it is not known yet how these proteins function together to achieve their well-orchestrated products. In an effort to identify regulators of early differentiation, we screened the NIA 15k gene array of 15,247 developmentally expressed genes using mouse mammary epithelial HC11 cells as a model of differentiation. We have confirmed a number of genes preferentially expressed in the undifferentiated cells (*Lgals1*, *Ran*, *Jam-A* and *Bmpr1a*) and in those induced to undergo differentiation (*Id1*, *Nfkbiz*, *Trib1*, *Rps21*, *Ier3*). Using antibodies to the proteins encoded by *Lgals1*, and *Jam-A*, we confirmed that their proteins levels were higher in the undifferentiated cells. Although the amounts of bone morphogenetic protein receptor-1A (BMPR1A) protein were present at all stages, we found the activity of its downstream signal transduction pathway, as measured by the presence of phosphorylated-SMAD1, -SMAD5, and -SMAD8, is elevated in undifferentiated cells and decreases in fully differentiated cells. This evidence supports that the BMPR1A pathway functions primarily in undifferentiated mammary epithelial cells. We have identified a number of genes, of known and unknown function, that are candidates for the maintenance of the undifferentiated phenotype and for early regulators of mammary alveolar cell differentiation.

1. Introduction

The mammary gland is composed of different cell lineages including myoepithelial and luminal epithelial cells; the latter is comprised of ductal, and alveolar epithelial cells. In the adult mammary gland, development is highly regulated by hormones, growth factors and cytokines, as well as by the information supplied by the environment of the fat pad. One important aspect of mammary gland development is the appearance of a new cell fate, alveolar cells, that occurs during pregnancy. In early pregnancy, these luminal cells of the alveoli proliferate then subsequently become further polarized and able to produce milk protein, such as β-casein. Following weaning, most of the new alveolar cells are removed by apoptosis during involution. This cyclical nature of the gland led to the hypothesis that a mammary stem cell population exists, which is able to self-renew cell types persistently within the gland (Gudjonsson et al., 2002; Welm et al., 2002; Alvi et al., 2003; Smith and Boulanger, 2003; Shackleton et al., 2006; Stingl et al., 2006; Smalley and Ashworth, 2003; Sleeman et al., 2006; Jonker et al., 2005; Stingl et al., 1998). In general, stem cells will self-renew to produce new stem cells and asymmetrically divide to produce fate-restricted progenitor cells that proliferate and then differentiate. Therefore, part of the investigation of stem cell biology includes the elucidation of common mechanisms controlling the stem cell functions such as self-renewal and differentiation.

The HC11 cell line, a prolactin (PRL) responsive clone of the COMMA-1D mouse (Balb/c) mammary epithelial cell line, has been widely used as a suitable model to study mammary epithelial cell proliferation, signal transduction and differentiation

Abbreviations: BMPs, bone morphogenetic proteins; BMPR1A, bone morphogenetic protein receptor 1A; Dex, dexamethasone; EGF, epidermal growth factor; DIP, dexamethason, insulin, prolactin; Ins, insulin; JAM, junction adhesion molecule; NIA, National Institute on Aging; NFκB, nuclear factor-kappa B; PRL, prolactin; RT, reverse-transcriptase; TGF-β, transforming growth factor-β

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in vitro. It is one of the few normal mouse mammary epithelial lines that are capable of differentiation in culture. It has long been known that mammary epithelial cell differentiation requires interaction of the epithelial cells with extracellular matrix in order for the cells to respond to lactogenic hormones (Katz and Streuli, 2007), and HC11 produce their own extracellular matrix proteins, such as laminin (Chammas et al., 1994). With a controlled hormonal regime, the cells in the undifferentiated stage will become competent to respond to lactogenic hormones (prolactin, insulin and dexamethasone) and will differentiate to produce milk protein such as β-casein (Ball et al., 1988). This biochemical differentiation is accompanied by a morphological change on a two-dimensional plate (Desrivieres et al., 2003). The controlled production of milk protein indicates that HC11 cells likely contain an alveolar progenitor cell population that differentiates to form a milk protein secretory cell.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, are known to play a crucial role in regulating differentiation of epithelial cells. BMPs control proliferation, specification and differentiation in different epithelial tissues (Abe, 2006; Owens et al., 2008; Patel et al., 2006; Zaghloul et al., 2005). Although BMPs were identified by their ectopic bone formation activity (Urist, 1965), many studies now indicate that members of the BMP subfamily play critical roles during organogenesis. Some BMP-related ligands are known to be involved in the generation of specific neuronal cell types (Shah et al., 1996; Lee et al., 1998), and there is also evidence that many act in a redundant manner (Dudley and Robertson, 1997; Solloway et al., 1998; Zhao et al., 1998). BMPs exert their effects by activating a complex of type I and II receptors.

Type I receptors such as BMPR1A (also known as ALK3), BMPRIB (also known as ALK6), and ACTR-I (also known as ALK2) are responsible for the specific transduction of BMP signals into the cell (Kawabata et al., 1998). The expression patterns of BMPR1A and BMPR1B in the central nervous system (Dewulf et al., 1995; Zhang et al., 1998) suggest that they have distinct roles in the transduction of BMP signals. Upon binding of the ligand to type I receptors, a heteromeric complex is formed with type II receptors, which phosphorylate the type I receptors. Type I receptors in turn phosphorylate the DNA-binding proteins SMAD1, SMAD5, and SMAD8 (Wagner, 2007), each of which can then heteromerize with SMAD4 and translocate to the nucleus to regulate transcription of downstream target genes (Itoh et al., 2000).

In an effort to identify early regulators of mammary epithelial cell differentiation, and specifically regulators controlling the differentiation of alveolar progenitor cells, we performed expression-profiling analysis of HC11 cells at different stages of differentiation. We hypothesized that early regulators of differentiation could be identified by comparing the gene expression profile of undifferentiated cells to those that were induced to differentiate into a secretory cell.

2. Methods

2.1. Antibodies

Goat and rabbit-anti-BMPR1A antibodies were purchased from Santa Cruz (amino-terminus) and Abgent (Biolynx) (carboxyl-terminus), respectively. Goat-anti-JAM-A antibody (amino-terminus and carboxyl-terminus) and anti-β-casein (M-14, carboxyl-terminus) were obtained from Santa Cruz. Mouse-anti-GRB2 (amino acids 1–217) and anti-phospho-SMAD antibodies (phospho-SMAD1,5 Ser 463–465, phospho-SMAD8 Ser 426–428) were purchased from Cell Signaling Technology. Alexa donkey-anti-goat (Alexa 647) and donkey-anti-mouse (Alexa 488) fluorescent antibodies were purchased from Invitrogen (Molecular Probes), and secondary antibodies conjugated to horseradish peroxidase were obtained from Bio-Rad and Pierce.

2.2. Cell culture

Mouse mammary epithelial HC11 cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine and 100 units/ml penicillin-streptomycin. For cell maintenance, the medium was supplemented with 5 μg/ml insulin (Ins) and 0.01 μg/ml epidermal growth factor (EGF). RPMI 1640 medium was purchased from Hyclone, glutamine, penicillin, streptomycin and trypsin from Gibco-Invitrogen. Ins, dexamethasone (Dex) and prolactin (PRL) were purchased from Sigma, EGF from BD Biosciences-VWR.

2.3. Differentiation regime

HC11 cells were maintained in RPMI 1640 medium (with 5 μg/mL Ins, 0.01 μg/ml EGF). Cells that were used for RNA extraction and gene array validation were kept in growing media until 100% confluent, treated with competence medium (EGF) for 4 days then with DIP medium (1×10^{-7} M Dex, 5 μg/mL Ins, 5 μg/mL PRL, DIP-treated cells) for 1 h or for 4 days for full differentiation. Alternatively, to induce differentiation with a short protocol, confluent cells were treated with competence medium for 1 day, with pre-differentiation media (Ins, Dex) for 1 day, and 3 days in DIP medium.

2.4. Gene array sample preparation and hybridization

RNA was prepared from HC11 cells that were undifferentiated (80% confluent), competent or induced to differentiate for 1 h. RNA was extracted using Trizol and purified on RNA Easy columns. Incubation with the NIA 15k array and analysis was performed as per Florin et al (Florin et al., 2004). Essentially, reverse transcription was performed using an Omniscript RT Kit (Qiagen) and Cy3/Cy5-dUTPs (NEN Life Science Products, Cologne, Germany). Subsequently, Cy3- and Cy5-labelled cDNA samples were pooled, purified and concentrated using Microcon YM-30 PCR filter units (Millipore, Eschborn, Germany).

For microarray hybridization, pre-heated (50 °C) UltraHyb hybridization buffer (Ambion, Huntingdon, UK) was added to each cDNA-pool and applied to into the hybridization chamber of a GeneTAC automated slide processing system (Genomic Solutions, Ann Arbor, MI). Hybridisations were performed for 16 h at 50 °C with gentle agitation. Thereafter, the slides were washed in SSC/SDS solutions followed by final washing step in 70% ethanol and finally air-dried.

2.5. Data acquisition, processing and analysis

Microarrays were scanned and analyzed using a GenePix 4000A microarray scanner (Axon Instruments Inc., Union City, NJ) controlled by GenePix Pro 3.0 software (Axon Instruments Inc.). Individual data reports for all conducted experiments (including, e.g., median fluorescence intensities of all spots and local background) were exported and further analyzed using MS-Excel 2000 (Microsoft, Walldorf, Germany). All data sets were ranked according to spot homogeneity as assayed by the ratio of median and mean fluorescence intensities, spot intensity and the standard deviation of log ratios for replicate spots. Fluorescence