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A review: Development of bovine mammary glandular structure in immuno-deficient mice and expression profiling of morphogenesis related genes

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Abstract
Raising Indian population leads to increases the future demand of protein for consumption. Especially milk protein demand growing annually, there has been increased activity in the research field of dairy production. But other side frequent outbreaks of diseases, vaccine failures and drug resistances developed against diseases in dairy animals which are the barriers to the meet out of future protein demand. So, want to increase the research activities in the field of drug discovery and development is very important now. But there has been no increasing activity in the new drug research due to practical difficulties in use live cattle for laboratory researches and preclinical studies. It is very easy when using laboratory animals like rodents for new drug development as well as the production of milk related research, as compared with live cattle. There is more attention need to develop a bovine mammary gland structure in laboratory mouse for bovine mammary gland related studies. In India, still we were lagging to develop such a mouse model. Therefore, in this review our prime objective of discussion is how to developing bovine mammary gland in mouse model.

Keywords: Bovine mammary glandular structure, immuno-deficient mice, morphogenesis

Introduction
The bovine mammary gland is a complex organ, made up of various cell types that work together for milk synthesis. Cell culture is n method which was successfully used to isolate bovine mammary epithelial cells. Collagenase dissociation was used successfully during isolation and culture of bovine epithelial cells *in vitro*. Mammary epithelial cell cultures have been widely used over the years as models to understand the physiological function of mammary gland. When using cell cultures/lines, it is inherently difficult to distinguish between primary mitogens and secondary regulators of mammary gland function/development. In these review discussing the isolated cells were not immortal, thus, additional work was required to develop a bovine mammary epithelial cell line and also explore the expression of genes involved in mammary gland morphogenesis, proliferation and differentiation of bovine mammary epithelial cells.

Bovine mammary cell transplantation
Generally, farm animals such as cattle and horses are difficult to handle for experiments. The mouse, on the other hand, is the most commonly used mammalian research model for laboratory-scale experiments. Hence, it would be beneficial to develop a mouse model of bovine mammary alveolar ducts for laboratory scale-studies. Here, we aimed to generate the bovine mammary gland ductal structure from *in vitro* bovine mammary epithelial cell culture or cell lines transplanted into nude mice dorsal tissue. The primary mammary duct invades the mammary fat pad at E17, and formation of a small, branched ductal tree begins at this time and develops its shape [1]. A previous study had established a clonal cell line from primary bovine mammary alveolar cells (MAC-T) [2]. Prolactin was used to induce the Bovine mammary epithelial cell differentiation. This differentiation, immortality, and a population doubling takes time of approximately 17 h. These cells may be very useful for dairy protein synthesis studies.

Morphogenesis of Bovine mammary gland

Transforming growth factor β (TGF-β) superfamily members play a key role in regulating mammary epithelium proliferation and are important mediators of mammary gland morphogenesis, development, and differentiation [3, 4, 5]. The TGF-β superfamily contains more than 30 members including TGF-β, bone morphogenetic proteins, growth and differentiation factors, activins, and inhibins. Activins in particular are vital for embryonic development and morphogenesis of most organs, including the mammary glands. Activins are dimers comprising 2 β subunits (βA or βB); therefore, 3 possible activins exist: A (βA/βA), B (βB/βB), and AB (βA/βB) [6]. They bind initially to their type II receptor, ActRIIA or ActRIIB, which leads to the recruitment, phosphorylation, and subsequent activation of the type I receptor, ActRIB. Upon activation, ActRIB binds and then phosphorylates a subset of the cytoplasmic signaling proteins of the Smad family (Smad 2, Smad 3, and Smad 4), forming a complex that translocates toward the nucleus and modulates gene expression [7, 8]. Whereas follistatin (FS) is an activin-binding single-chain glycoprotein that exists in 3 isoforms that differ in cell surface proteoglycans binding properties [9, 10, 11].

In mice, activins and inhibins play a pivotal role in mammary gland organogenesis. Actually, βB subunit knockout mice do not produce milk. Instead, they exhibit impaired ductal branching and epithelium differentiation during gestation, and at parturition alveolar lumina fail to expand because of the absence of secreted milk [12]. Moreover, activin-related proteins have been detected during non-gestational and lactational mammary gland development in mice, with a growth regulatory role in lactation being suggested [13] (Jeruss *et al.*, 2003). Similarly, in the bovine species, *in vitro* experiments have demonstrated that TGF-β treatment has an inhibitory effect on bovine epithelial cell growth [14]. Based on the βB subunit knockout mouse model and previous activin expressional studies, along with the fact that TGF-β members are associated with normal development, differentiation, and function of the mammary gland, we hypothesize that activins, inhibins, and their related proteins are expressed in the bovine mammary gland [15]. Therefore, in this study exploring the expression profile of the morphogenesis related genes of βA, βB, and α-inhibin subunits, and follistatin and ActRIB and ActRIIA receptors may help to access the important role in the developing mammary gland during different stages of pregnancy in the BME cell transplanted Nude Mice.

Materials and Methods

Animals and Sample Collection

All tissue samples used in the present study were collected from Mammary tissue from cross breed or desi heifers (Bos indicus; n = 20) was collected, frozen in liquid nitrogen and stored at −80 °C for real-time PCR analysis.

Materials

The basal growth media was DMEM/F12 containing 10% fetal bovine serum (FBS) (Invitrogen). Induction media, which could promote the synthesis of milk protein and fat, was the growth media containing 5 μg/mL bovine insulin, 5 μg/mL bovine Holo-transferrin, 5 μg/mL progesterone, 10^{-7} mol/L hydrocortisone, 10 ng/mL bovine epithelial growth factor and 5 μg/mL bovine estradiol (Sigma-Aldrich, cat. #I4434, T1283, P8783, H0888, E4127, E2758, respectively). The storage media prepared freshly was composed of 90% fetal bovine serum and 10% DMSO. A solution of 0.25% trypsin-0.02% EDTA solution (Sigma-Aldrich) used for cell digestion was prepared and stored at −20 °C until use.

Tissue Isolation

Bovine mammary tissue was obtained from a three-year-old mid-lactation dairy cow. Fresh tissue was placed in sterilized tubes containing ice-cold D-Hank's (balanced salt solution) and immediately transported to the laboratory. Tissue of ca. 1 cm^3 was washed with D-Hank's solution for several times until the solution was pellucid and without milk. Tissue was then cut into 0.5~1 mm^3 cubes and washed again with D-Hank's solution until clean. These smaller pieces of tissue were transferred with sterile tips onto empty plastic cell culture dishes (Corning, U.S.A) coated with collagen. Care was taken to ensure that tissue was kept wet. Culture dishes were incubated at 38 °C and 5% CO_2 and were monitored closely every 30 min. If the adjacent area surrounding the tissue was dry, several drops of basal media were added ensuring that the tissue would not float and separate from the bottom of the culture dish.

After 4 h, 0.5 mL basal media were added to every culture dish and 1 mL basal media were added after 12 h. The basal media was replaced with fresh media every 48 h until cells were visibly spread across the bottom of the culture dish. Cells were detached with 0.25% trpysin-0.02% EDTA and transferred to new culture dishes, which were used to remove fibroblasts. Subsequently, the pure mammary epithelial cells were isolated after 3 passages.

Growth Characteristics of Epithelial Cells

Growth curves and doubling time were determined by seeding 5×10^4 cells/well in 12-well flat-bottom culture plates (Corning 3513, U.S.A) containing induction media. Cell number and viability were determined each day in triplicate wells between 7 to 11 d post-seeding via trypan blue exclusion. Morphology of cultured cells was routinely evaluated with an inverted microscope with phase contrast (Olympus IX71, Japan), and photomicrographs were taken.

Karyotyping Analysis of Epithelial Cells

The cells from three periods (primary, purified, and resuscitated cells) were examined via changes in karyotyping analysis as described by Seabright. Exponentially-growing cells were incubated with colchicine (0.2 μg/mL) for 2–2.5 h. Cells were trypsynized with 0.25% trypsin and treated with warm hypotonic KCl solution (0.075 mol/mL) for 30 min at 37 °C. The solution was centrifuged at 1200 × g for 10 min and the cells were harvested. Cells were then fixed with ice-cold methanol and acetic acid mixture (volume 3:1) 3×, commencing after 30 min and subsequently twice at 15 min intervals. Each time, cells were centrifuged at 1200 × g for 10 min prior to harvesting of cells. Cells were suspended with 0.5 mL fixed solution and spotted onto ice-cold glass slides. The sample slides were allowed to dry at room temperature and stored at −20 °C until use. All the slides were stained with Giemsa solution (1.0 g giemsa, glycerin 66 mL, and methanol 66 mL) 1 mL/slide for 10 min, washed with distilled water and dried at room temperature. Chromosomes were visualized and detected with a phase-contrast microscope (Olympus IX71) and analyzed with the soft Video TesT Karyo3.1 (Nature Gene Corp., USA).

Bovine mammary epithelial cell line

Bovine mammary epithelial (BME) cell line is cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, DM, USA) containing 25 mM glucose, supplemented with 10% fetal bovine serum (FBS; Welgene, Daejeon, South Korea), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco 15140-122). The cells were grown in a humidified 5% CO2 atmosphere at 37o C.

Bovine mammary cell transplantation to Nude Mice

Seven-week-old female BALB/C nude mice were purchase from Jackson lab, USA and animals were housed in an environmentally controlled room with IVC System (temperature: 23±2o C, relative humidity: 50±10%, programmed ventilation, and 12:12 h light-dark cycle) prior to experiment. All of the animal experiments will be approved by the Institutional Animal Ethical Committee (IACE), IIIM, Jammu. BME cells were suspended in BD Matrigel ™ diluted 1:1 (v/v) in Hank's Balanced Salt Solution (HBSS, Gibco). Cell suspension in Matrigel was injected into the 8 weeks male BALB/C nude mice dorsal using syringe for transplantation. After 6 weeks, small portion of the transplanted tissue was dissected from BALB/C nude mice and fixed in Bouin solution for immune-staining. For analyzing of bovine mammary gland protein expression, to perform a mammalian gland biopsy from fourteen-month calf and fixed in Bouin solution.

Immunocytochemistry

The BME cells were seeded in 6-well plate at a density of $1X10^5$ cells/well and cell were fixed for 10 min with 4% paraformaldehyde, permeabilized for 10 min with 0.2% Triton X-100, and then incubation for 1 hour with 3% BSA in PBS to block nonspecific antibody binding. Cells were incubated with a 1:200 dilution of anti-cytokeratin 18 (CK-18), anti-cytokeratin 14 (CK-14) or anti Prolactin for 1 hour. The cell were then rinsed with PBS and incubated with anti-mouse Alexa 568 and anti-rabbit Alexa 488 antibody (both 1:500; Invitrogen, Carlsbad, CA, USA) for 1 hour. Samples were rinsed in PBS for 15 min and incubated on DAPI for 10 min.

Immunohistochemistry

Tissues were rinsed PBS, fixed in Bouin solution for 5 hours at 22 ºC, and then dehydrated through an alcohol gradient consisting of 1 hour's incubations in 25%, 50%, 70%, 83%, 90%, and 100% ethanol. The dehydrated tissue was cleared in xylene, infiltrated with melted paraffin for 2 hours at 65 ºC, and embedded in paraffin blocks. Tissues were sectioned at 4 µm thickness using rotary microtome (Leica), and the sections were placed on glass slides. Tissue sections were deparaffinized in xylene, rehydrated, and equilibrated in water. Antigen un-masking was performed by boiling sections in 10 mM sodium citrate buffer for 10 minutes. Nonspecific binding was blocked using 3% BSA in PBS for 30 minutes at 22 ºC. samples were incubated overnight at 4 ºC with 1:200 dilutions of primary antibodies. Primary antibodies included the following: cytokeratin14 (ab7800), cytokeratin 18 (ab668) from Abcam (Cambridge, UK) and Prolactin (SC-7805) from Santa Cruz Biotechnology. After several washes, tissue sections.

RNA isolation and cDNA synthesis

Total RNA was extracted from mammary tissue by the acid guanidinium thiocyanatephenol-chloroform method (Trizol Reagent, Invitrogen, Carlsbad, CA) in a proportion of 1 mL/100 mg of tissue. Concentration of RNA was determined by measuring absorbance at 260 nm. The DNA contamination was removed by the use of DNase I, amplification grade (Promega), and the SuperScriptTM III First-Strand Synthesis Super Mix Kit (Promega) was used according to manufacturer's instructions to reverse transcribe 3 µg of total RNA. Integrity of RNA was evaluated by gel electrophoresis under denaturing conditions; intact bands were visualized by ethidium bromide staining.

Primer Design

All PCR primers were designed with the Real Time PCR Primer Design program (DNA Star) based on sequences obtained from the National Center for Biotechnology Information database[16] Table.1. Primers were designed to span exon region whenever possible in order to avoid any accidental amplification of genomic DNA.

Table 1: Oligonucleotide sequences used for real time PCR amplification

S. No	Gene	Oligonucleotide Sequence 5′→3′	Bp	GenBank
1	βA subunit2	GGAATCAGCACAGCCAGGAA TCCTGTTGGCCTTGGGAACT	117	NM_174363
2	βB subunit2	CAGCTTCGCCGAGACAGATG CTGGCCTGCACCACAAACAG	98	NM_176852
3	α-Inhibin	CACGTATGTGTTCCAGCCAT GTCTGTCCAGTCCTGTGTGG	86	NM_174094
4	Follistatin	TTTCTGTCCAGGCAGCTCTA GTCACTCCATCATTCCCACA	126	L21716
5	ActRIB	GCATCAAGAAGACCCTCTCC AGAGGTAGGCCTCCATCGTA	137	AY185302
6	ActRIIA	AAAGTTTGAGGCTGGCAAGT GCATCCCTTTGGAAGTTGAT	108	U43208
7	β-Actin	AGCAGATGTGGATCAGCAAG TAACAGTCCGCTTAGAAGCA	82	AY141970

Real-Time PCR

Real-time PCR was performed using (Applied Biosystems, Bio-rad, or Roche) in a final volume of 25 µL and all samples were run in duplicate. Also, 1.7 µL of cDNA was added to 2 µL of each primer plus 17 µL of SYBR Green Master Mix (Applied Biosystems) and completed with 4.3 µL of RNase-free water. Control reactions were performed omitting either the reverse transcriptase enzyme or template RNA to test for contamination with genomic DNA or nonspecific amplification. The PCR runs were programmed as follows: stage 1, 1 cycle of 52 °C for 2 min; stage 2, 1 cycle of 95 °C for 10 min; stage 3, 40 cycles of 95 °C for 15 s and 50 °C for 1 min. Expression levels of β-actin were used as endogenous controls within each sample [17] given that target and housekeeping genes had similar slopes. Positive controls consisted of cDNA from bovine ovary that is known to

express activin-related proteins.

Statistical analysis

Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method, where CT is cycle threshold. Statistical Analysis for real-time PCR analysis, the ΔCT values obtained for different stages of mammary gland development were normally distributed and therefore expressed as means ± standard deviation and compared using 1-way ANOVA followed by Student-Newman-Keuls post hoc test for multiple comparisons. The relative mRNA concentrations are summarized as fold increase over the non-pregnant group [18].

Conclusion

It is concluded that using mammary gland transplanted mice models will be more reliable and accurate for the new drug development for mastitis as well as the production of milk related research, as compared with live cattle. There is more attention need to develop a bovine mammary gland structure in laboratory mouse for bovine mammary gland related studies. In India, still we were lagging to develop such a mouse model and further research studied required this kind of reliable transplant animal model for accurate drug targeting strategies.

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