New Research Strategies in Lactation Biology

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Summary

Different approaches have been used to study milk related traits in farm animals, reaching from statistical dissection of phenotypic variation to the search of candidate genes with major phenotypic effects. The aim of this study was to develop a new research tool devoted to *in vitro* studies of physiological pathways responsible for mammary gland development, lactation, remodeling and immune response, supported by a user friendly map based bioinformatics tool for integration of different types of data. We established goat mammary gland derived primary epithelial cell line with predominantly epithelial morphology, responsive to lactogenic hormones and exhibiting regeneration potential in heterologous mouse system. The response of primary epithelial cells to pathogenic bacterium *Mycoplasma agalactiae* was studied using RNA sequencing approach and 1553 differentially expressed genes were detected 24 h post infection. The majority of differentially expressed genes belonged to cell cycle regulating genes, pro-inflammatory cytokines, chemokines and genes involved in lipid metabolism. Bioinformatic analysis of 359 putative target sites for mammary gland expressed miRNAs revealed polymorphic miRNA target sites for bta-miR-199b, -199a-5p, and -361 in the *IL1B* gene and for -miR-126 in the *CYP11B1* gene. Graphical integration of different types of data to DairyVis platform allowed identification of genomic regions with higher number of potential functional elements that deserve further experimental analysis. The newly developed MEC line and integration of bioinformatics tools into DairyVis database represent a promising methodological support for further research in the field of lactation biology.

Key words

lactation, mammary gland biology, QTL, RNAi, RNA sequencing, stem cell, visualisation of genomic data

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Received: May 31, 2011 | Accepted: August 4, 2011

ACKNOWLEDGEMENTS
We thank the Slovenian Research Agency (J4- 3621, P4-0220 and US-SLO bilateral grant) for co-financing this study.

Introduction

Mammary gland has evolved through approximately 200 million years (Lemay et al., 2009) to become a highly specialized organ in mammals, devoted to the production of milk which is in the first period of life the only nutrition for the newborn. In the majority of mammalian species the milk plays the role of passive immunization of the newborn through the high level of immunoglobulins being present in milk at the beginning of lactation. In some species, i.e. kangaroo, females can even produce milk of different composition in different compartments of the mammary gland in order to meet nutritional needs of two offspring of different age. Lactation is embedded into the cycle of pregnancy associated proliferation, differentiation and involution of the mammary gland that occurs several times during the reproductive life of the female in all mammalian species. Dramatic tissue remodeling events, characteristic for mammary gland development, growth, lactation and involution make mammary gland one of the most dynamic organs in mammals. In addition, the lactation capacity in some domestic mammalian species that are widely used for commercial milk production (cow, sheep, goat, lama) exceeds several times the nutritional needs of the offspring. Therefore, the mammary gland is considered as a potent bioreactor for production of valuable biologically active proteins in animal biotechnology. The ability of mammary gland to regenerate after massive involution following the previous lactation suggested the existence of undifferentiated cells representing the cell pool responsible for renewal of the mammary gland, which was experimentally confirmed by transplantation of mammary explants into the cleared mouse mammary fat pads.

Genetic analyses of cDNAs coding lactoproteins revealed high number of mutations present in the majority of lactoprotein genes. A number of polymorphisms within the coding regions of lactoprotein genes were associated with technological properties of milk and differences in milk composition. Probably the most well known effect of lactoprotein variants on technological properties of milk is the positive effect of CSN3 allele B on micelle size, coagulation time, curd firmness and cheese yield (Buchberger and Dovč, 2000). The analysis of lactoprotein gene expression in heterozygous animals revealed quantitative differences in expression between alleles, called differential allelic expression (Van Eenennaam and Medrano, 1991a, b). In the case of *CSN3* gene, the higher expression of the B allele at protein as well as at RNA level could not be explained with allele specific polymorphisms in the *CSN3* gene proximal promoter (Debeljak et al., 2000). The allele specific mutations in the 3'-UTR of the *CSN3* gene suggested the possibility that RNA interference (RNAi) could be involved in the regulation of *CSN3* gene expression (Debeljak et al., 2005).

A nice example how promoter mutations can affect expression of lactoprotein genes is the G to C transversion in the promoter region of the *BLG* gene at position -430 within the AP-2 binding site. This mutation was proposed to be associated with the reduced binding efficiency of AP-2 transcription factor (Lum et al., 1997) and consequently with reduction of BLG synthesis that was demonstrated *in vitro* (Folch et al., 1999) and *in vivo* (Kuss et al., 2003) where G to C transversion within the AP-2 binding site was associated with lower expression of *BLG* gene and consequently higher casein number in German HF and Simmental populations.

In addition to the genetic variation within coding and regulatory regions of lactoprotein genes, there is an additional mechanism contributing to the variability of lactoproteins in the milk: the alternative inclusion of particular exons into the mRNA transcripts at different lactoprotein loci results in a number of different transcripts from the same locus generating proteins of different length. The differential splicing of mRNA for CSN2 and CSN3 has been thoroughly studied in horse, where a universal splice regulatory element has been identified (Lenasi et al., 2006).

Due to the economic importance of lactation a number of studies have been performed in order to identify genomic regions affecting lactation traits. Large genomic sections designated as Quantitative Trait Loci (QTL), were identified on the majority of bovine chromosomes, however, interactions between environment and genotype, epistasis and genetic imprinting compromise robustness and reliability of QTL detection. Although a number of QTL, affecting milk production traits was identified in cattle, there are only a few reports demonstrating reliable QTL regions that could be confirmed in different breeds and/or in different commercial populations. The first reported successful positional cloning of a QTL in an outbred mammal revealed the missense mutation in the bovine *DGAT1* (acylCoA:diacylglycerol acylransferase 1) gene with the major effect on milk yield and composition (Grisart et al., 2002). The gene identification was based on bioinformatics, comparative mapping and functional analysis. The bovine *DGAT1* gene was cloned by means of positional candidate cloning targeting the 3 cM chromosomal region on the telomeric end of BTA14. The presence of the milk trait QTL in this chromosomal region was detected earlier (Dovč et al., 2006), but Grisart et al. (2002) detected the nonconservative K232A substitution in the *DGAT1* gene as a molecular cause for the QTL effect on BTA14. The second example for identified missense mutation as a cause of QTL effect in an outbred cattle population is the Y581S mutation in the bovine *ABCG2* gene at BTA6 (Cohen-Zinder et al., 2005).

The availability of fully sequenced genomes (The Bovine Genome Sequencing and Analysis Consortium, 2009) and further development of DNA sequencing techniques, mainly due to introduction of the next generation sequencing approaches, allowed acquisition of complex genomic data from a number of animals and promises to overcome traditional problems with interpretation of fragmented genomic data provided by other genomic techniques. Association studies using entire genomic sequence from animals with different phenotypic performance will allow identification of genomic regions with significant effect on important economic traits (Eck et al., 2009).

The aim of this study was to establish a new cell-based model for *in vitro* studies of gene expression in the mammary gland that will allow the identification of most important biological pathways responsible for mammary gland development, lactation, remodeling and immune response. In addition, our aim was to develop a new bioinformatics tool that would enable a map based, holistic approach to mammary gland research, allowing integration of different types of data.

Material and methods

Primary culture of mammary epithelial cells

Mammary tissue was obtained from a seven month-old juvenile Saanen goat. The udder was aseptically removed after slaughter, rinsed in 70% ethanol, cut in small pieces and washed in Hank's Buffered Salt Solution (HBSS) containing penicillin (200 µg/ml), streptomycin (200 µg/ml), gentamicin (200 µg/ml), ampicillin (200 µg/ml) and amphotericin B (10 µg/ml). Pieces were transferred into HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered HBSS containing collagenase (Biochrom AG) and hyaluronidase (Sigma), both at concentration of 400 U/ml. Degradation was performed at 37°C under soft shaking. Released cells were collected in three fractions, after 60, 120 and 180 min. of degradation. Cells were filtered through 40 µm mesh, centrifuged and resuspended in 90% FBS and 10% DMSO and frozen in liquid nitrogen. Aliquots were plated on plastic dishes in the RPMI 1640 medium (Sigma) with insulin (1 µg/ml), hydrocortisone (1 µg/ml) and prolactin (1 µg/ml). Cells were grown at 37°C in 5% CO_2 and 100% humidity.

Infection of mammary epithelial cells

Primary goat epithelial cell culture was grown until 80% confluence on a thin layer of Geltrex (Gibco). Buoyant culture of *Mycoplasma agalactiae* was diluted in Frey's medium and each plate with mammary epithelial cells was infected with about 1×10^9 bacterial cells and incubated in triplicates for 3, 12 and 24 hours. After incubation, the total RNA was isolated using TRI-reagent (Ambion) and mRNeasy extraction kit (Qiagen).

RNA sequencing

RNA sequencing was performed on Illumina Gene Analyzer IIx. Samples were prepared following the Illumina mRNA sequencing protocol using Illumina kit. Each of the 12 samples was sequenced in total of five lanes in three different single read flow-cells. Obtained sequences, 50 base pairs in length were mapped onto bovine NCBI-Refseq (ftp://ftp.ncbi.nih.gov/refseq/Bos_taurus/) using Burrows-Wheeler Alligner - BWA (http://bio-bwa.sourceforge.net/). Differentially expressed genes were identified using edgeR software package (http://bioconductor.org/packages/2.6/bioc/html/edgeR.html). The strongest regulated genes during the infection were integrated into functional networks using Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/). Functional annotation of differentially expressed genes was performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp).

Search for miRNA loci involved in lactation

Putative target sites for mammary gland expressed miRNAs in candidate genes were obtained using Sanger's mir-Base Targets (http://microrna.sanger.ac.uk/). Ensembl transcript identifiers for candidate genes were matched to the list of identifiers with putative miRNA target sites for miRNAs experimentally confirmed in the mammary gland. Polymorphisms in bovine miRNA targets were obtained from the Patrocles database (http://www.patrocles.org/).

Xenotransplantation

For xenotransplantation collagen gels were made as previously described (Eirew et al., 2008). Each gel contained $1.6\text{x}10^5$ 10T1/2 fibroblasts previously treated with 2 µg/ml mitomycin C and $7.5\text{x}10^4$ caprine primary mammary cells. The gels were kept on ice until surgically implanted. A two cm anterior-to-posterior cut was made through the skin of recipient NOD-SCID mouse along a median line followed by a smaller incision in the abdominal wall directly above the kidney position. Collagen gels were implanted under the capsule and the abdominal wall was sutured. A slow release pellet containing 2 mg β-estradiol and 4 mg progesterone (both Sigma-Aldrich) in silicone (MED-4011, NuSil Technology) was placed subcutaneously before finally suturing the skin. The gels were removed after 4-6 weeks, embedded in paraffin and processed for immunofluorescence and hematoxylin/eosine (H&E) staining.

Visual integration of data

DairyVis consists of several tightly integrated software components. The core of the system is a MySql database (http://www.mysql.com) which is used as storage for the genetic features. Database is populated via custom made Python scripts. They are used to load a list of genetic features and for periodical update of the DairyVis database. The DairyVis Flash based graphical interface has been developed using the latest Flex 4 technology using the Adobe R Flash R BuilderTM 4 development environment.

Results

Mammary epithelial cell model

The study of host-pathogen interactions in farm animals is limited by the relatively high costs of animal experiments and animal welfare issues. Therefore, the development of cell models, which can give an impression about the regulatory mechanisms at cellular level and can also be extrapolated to the whole animal, opens new possibilities for the study of these interactions. Our goat mammary gland derived primary cell culture shows morphological properties of two main cell types in the udder: epithelial (luminal and myoepithelial) and fibroblasts. Both cell types are present in the primary culture (Figure 1, A), however, clonal selection and specific culture conditions can lead to enriched epithelial phenotype (Figure 1, B) with typical morphology and even detectable level of beta casein expression. Furthermore, in our infection experiment with *Mycoplasma agalactiae* the epithelial cells expressed several immune response related genes and demonstrated their suitability for *in vitro* challenging experiments.

Stem cells are undifferentiated, division competent cells that are present virtually in all differentiated tissues and represent a cell pool from which specific cell types composing that particular tissue can develop. The biological potential of stem cells includes self renewal, i.e.: the ability to regenerate new stem cells, as well as the potential to generate all different cell types present in the particular tissue. Regeneration and/or replacement of different cell types that compose mammary gland is possible because of the presence of the mammary stem cells (MaSCs). The presence of MaSCs, which can give rise to both mammary gland specific cell types (epithelial and myoepithelial cells) has been shown in mammary glands of several species. Our primary goat epithelial cell line was used for xenotransplantations in NOD-SCID mice in order to demonstrate regeneration potential of this cell fraction and to demonstrate indirectly the presence of MaSCs.

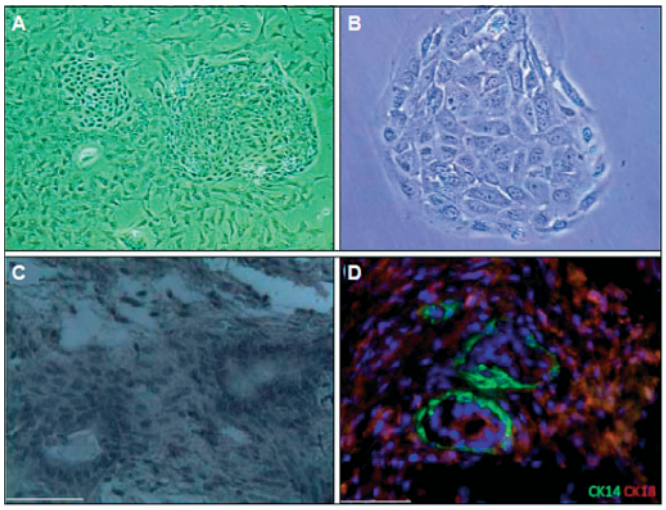

Figure 1. Morphology and proliferative potential of primary mammary gland cells. Epithelial cells build islands surrounded by fibroblast cells (A). Morphology of epithelial cell colony resembles alveolar structures in the mammary gland (B). Haematoxylin/eosin staining of xenograft sections (C) and expression of cytokeratins 14 and 18 in xenografts (D). The xenotransplanted gels were removed after 4 weeks (C) or 6 weeks (D). Scale bars: 50 µm.

After 4-6 weeks, the xenografts were removed from the murine kidney capsule and analysed immunohistochemically. The Haematoxylin/eosin (H&E) stained sections show round, slightly elongated regenerated structures (Figure 1, C and D). In the immunostained xenograft sections (Figure 1, D) the spatial distribution of markers CK14 and CK18 is similar to that seen in the caprine mammary tissue, where CK 14–positive myoepithelial cells are lining the CK18$^+$ luminal cells. As in the mammary gland, transplanted epithelial fraction of goat mammary epithelial cells formed a polarized bilayered epithelium enclosing a lumen (Figure 1, C and D).

mRNA sequencing

In our study, the newly developed goat primary mammary epithelial cells (MECs) and *Mycoplasma agalactiae,* strain PG2 as infectious agent, were used for infection experiment. The applied Illumina mRNA sequencing strategy revealed 853 differentially expressed genes (DEG) 3 h post infection, 112 DEG 12 h post infection, and 1553 DEG 24 h post infection. The low number of DEG 12 h post infection is a bit surprising, but it most likely reflects early down regulation of genes involved in immediate immune response as well as slow up regulation of genes involved in the response. The majority of differentially expressed genes belonged to cell cycle regulating genes, chemokines, pro-inflammatory cytokines and genes involved in lipid metabolism. Infection of the cell culture affected numerous cell processes and using the DAVID program package we discovered that 326, 108 and 457 biological pathways were affected 3, 12 and 24 h post infection, respectively. Among them were also processes that are involved in induction of innate immune response effector-genes. Using IPA software we integrated DEG into metabolic pathways. An example of metabolic pathway related to immune response 24 h post infection is shown in Figure 2.

The obtained data allow insights into the dynamics of the immune response signaling and opens possibilities to identify promising candidate genes, which could be beneficial for development of new therapeutic methods and marker assisted selection towards enhanced mastitis resistance (Ogorevc et al., submitted).

Identification of miRNA targets in mammary gland expressed genes

To elucidate the regulatory role of miRNA in the mammary gland, we performed search for miRNA target sites within mammary gland expressed genes. We found 359 putative target sites for mammary gland expressed miRNAs in the candidate genes, including polymorphic miRNA targets. Among them we iden-

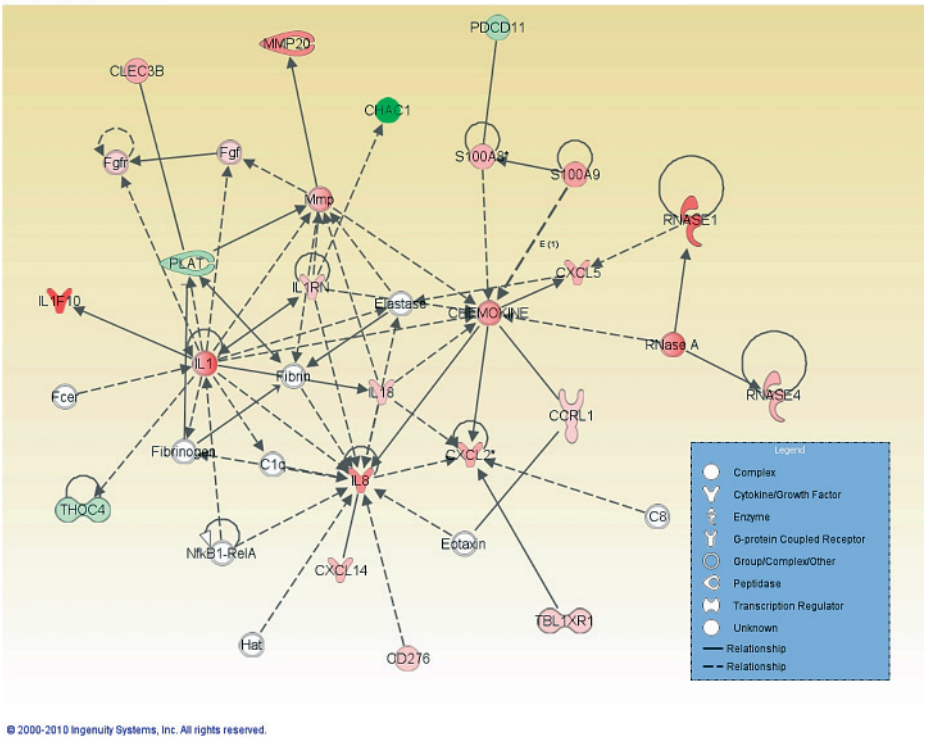

Figure 2. Metabolic pathway "Cell movement, development and hematologic functions, immune system signalling" 24 h post infection (False discovery rate, FDR ≤ 0,05). The intensity of grey color reflects the magnitude of change in gene expression.

Polymorphism		Ancestral allele				Derived allele(s)				
SNPId	Alleles	Octamer	Expr	Source	miRNA	Octamer	Expr	Source	miRNA	Gene Id
rs41255722	C/G					ACACTGGA	n/a	L X	miR-199a-5p miR-199b	IL1B_BOVIN
rs41255717	A/G	TCTGATAA	n/a	L	miR-361					IL1B_BOVIN

Figure 3.
Polymorphic miRNA targets in the bovine *IL1B* gene.

tified using the Patrocles database polymorphic miRNA target sites for bta-miR-199b, -199a-5p, and -361 in the *IL1B* gene and for -miR-126 in the *CYP11B1* gene. Interestingly, the expression of -miR-199b, -199a-5p, and -126 in the bovine mammary gland has already been experimentally confirmed (Figure 3).

Bioinformatics approach

Expression studies, genome and RNA sequencing, QTL mapping and association studies caused a flood of information during the last years that will even increase with the sequencing of single animal genomes and wide use of RNA sequencing for gene expression studies. These data are deposited in a number of public and commercial databases that makes their retrieval and use in bioinformatics studies difficult. Therefore we made an effort to integrate different types of data related to lactation and mammary gland health (Ogorevc et al., 2009) in a map based web application DairyVis (http://dairyvis.fri.uni-lj.si/DairyVis/DairyVis.html). The application integrates data from expression studies, animal models, QTL mapping, association studies, epigenetics and miRNA studies on a physical map, enabling holistic approach to identification of promising genomic regions with an impact on milk traits and udder health (Figure 4). This web application is user friendly and it is periodically updated.

Graphical integration of different types of data allows researchers to identify genomic regions with higher number of

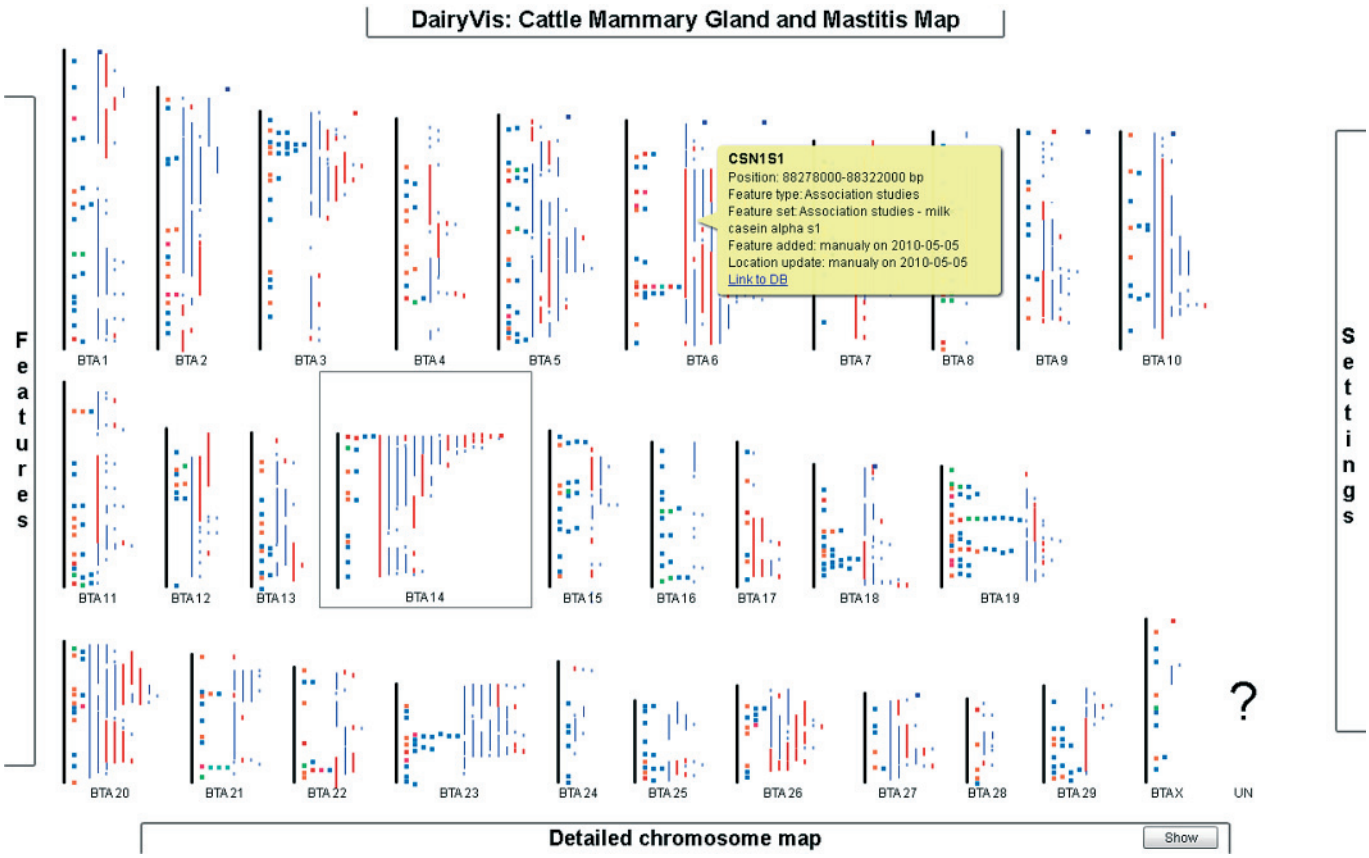

Figure 4. A snap shot from DairyVis web tool showing bovine chromosomes with locations of QTL regions, identified genes expressed in the mammary gland, informative SNPs and miRNA genes.

potential functional elements that deserve further experimental analysis. Furthermore, such integration enables identification of genes and regions that can then be integrated using other bioinformatics tools as predictive interaction databases (STRING, STICH, Scansite), pathway databases (KEGG, SPIKE) or databases for visualization of relationships among pathways (IPA, Cytoscape).

Discussion

In the near future, we can expect that the amount of genomic information related to lactation and gene expression in the mammary gland will grow exponentially and a very potent statistical and bioinformatics tools will be needed for integration and interpretation of different "-omics" data. The complex analysis and interpretation of experimental data will enable identification of the most important genomic variants that will significantly contribute to better understanding of the biological relevance of genetic factors affecting lactation and development of the mammary gland. In addition to the high number of interconnections between particular genes, will the holistic approach, trying to sum all genetic effects on these complex traits, remain the central strategy in lactation biology research. However, even the most sophisticated bioinformatics approaches require verification of genetic effects in experimental models either at cellular, organismic or even population level.

In order to better understand the molecular background of mammary gland renewal it is of crucial importance to identify the exact location of MaSCs and the mechanisms involved in their proliferation and differentiation. Undifferentiated cells with stem cell potential seem to be present in all parts of the mammary gland either in more primitive form, represented by MaSCs or as more determined stem/progenitor cells (Woodward et al., 2005). Our transplantation experiment indirectly shows that progenitor cells are present in the mammary primary cell culture and have renewal potential. Culturing MECs under conditions which stimulate formation of 3D structures leads to enrichment of undifferentiated cells and formation of mammospheres which contain $CD49f^{+}$, $K5^{+}$ and $CD10^{+}$ cells, whereas some of the cells also express luminal (ESA) and myoepithelial (K14) markers (Dontu et al., 2004). When grown on Matrigel, the single cell can differentiate into ductal and alveolar structures and when adding prolactin, the mammospheres form functional alveolar cells which secrete caseins. Recent results show that CD24 (heat stable antigen), CD29 (beta1 integrin) and CD49f (alpha 6 integrin) are very informative for identification of MaSC (Visvader and Lindeman, 2006).

RNA sequencing is increasingly being used for transcriptomic studies, due to its better dynamic range compared to microarrays and possibility to discover new transcripts that may give a useful information about expressed genomic sequences and additional information about splicing variants (Medrano et al., 2010; Wickramasinghe et al., 2011). However, the potential of bioinformatics analyses is still restricted due to incomplete annotation data and missing knowledge about complex interactions among genes (Lemay et al., 2009). In our case an additional problem was the necessity to use bovine genome for transcript mapping, although from the evolutionary point of view a very similar one to the caprine genome, which is still not available. RNA sequencing is also a very potent tool for discovery of new informative SNPs (Canovas et al., 2010).

In conclusion, our newly developed goat mammary epithelial cell model was successfully applied for studying transcriptional response of MECs to infection with *Mycoplasma agalactiae*. Due to the responsiveness to lactogenic hormones and their proven ability to develop alveolar structures in xenotransplantation experiments, we propose wider use of this cell model for functional genomics of the mammary gland. The positive response to our integrative holistic approach to mammary gland biology has initiated development of a novel visualization tool DairyVis, which is freely available on the web and will be periodically updated in order to meet expectations of the research community and to allow permanent integration of novel data.

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acs76_76