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Transcriptomics Comparisons of Mac-T cells Versus Mammary Tissue during Late Pregnancy and Peak Lactation

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Abstract

Epithelial cell cultures, including immortalized cells, have been used extensively to help answer basic questions of mammary gland physiology under normal or diseased conditions. The usefulness of using cell culture depends on the degree of identify compared to the *in vivo* mammary epithelium. Our objective was to compare the transcriptome by means of microarray and qPCR of immortalized bovine mammary epithelial cells (Mac-T) cultivated in plastic (i.e., 2 dimensional system) vs. mammary tissue obtained during late-pregnancy (-30 DIM) and peak lactation (+60 DIM). Functional analysis of microarray data was performed using enrichment analysis tools and the Dynamic Impact Approach (DIA). Overall, between 37% (vs. +60 DIM) and 44% (vs. -30 DIM) of the measured annotated genes were deemed as differentially expressed (DEG) between Mac-T cells and mammary tissue. These data, together with the bioinformatics results of the genes non-differentially expressed, indicated a larger overall similarity between Mac-T cells and lactating than non-lactating mammary tissue. However, Mac-T cells had a lower expression measured by qPCR of genes involved in milk synthesis compared to mammary tissue. The functional analysis of DEG further supported the poor lactation phenotype of Mac-T cells compared with mammary tissue. The bioinformatics analyses of DEG suggested that Mac-T cells cultivated on plastic had greater reliance on glucose, amino acids, and fatty acids for production of energy, greater cell-to-cell interactions, and more prone to react to inflammatory mediators relative to mammary tissue. Despites these differences, data suggest that Mac-T cells might be adequate for studying milk protein regulation. For studies of milk fat regulation Mac-T cells might be not as sensitive as the mammary tissue, particularly for its lower expression of PPARG and lower induction of PPARγ- and *LXR*-related pathways. Overall, this study revealed a marked difference in the transcriptome signatures between mammary tissue and Mac-T cells cultivated on plastic.

Keywords: Mac-T cells; Bovine Mammary Tissue; Transcriptomics

Introduction

Cell culture systems have been useful to help answer basic questions of mammary gland biology in normal and diseased states [1]. Several immortalized cell lines have been established from primary mammary epithelial cells. The primary cells or tissue explants are likely more representative of the *in vivo* system due to the temporal proximity to the original tissues/cells having, likely, more similar molecular milieu (e.g. more similar epigenome and transcriptome); however, it is cumbersome to consistently obtain tissue from non-laboratory species under controlled conditions (e.g. age of animal, stage of glandular development and parity). Therefore, despites the limitation in modeling the *in vivo* system the use of established cell lines can be helpful in the study of epithelial mammary gland biology.

Several ruminant mammary epithelial cell lines (five bovine, two ovine and one caprine) have been established and well-characterized [2-9]. Bovine cell lines BMEC+H (Bovine Mammary Epithelial Cells of the Hormone-adapted) [4], HH2A (spontaneously immortalized bovine mammary epithelial cell line) [10], and ET-C (epithelial and myoepithelial-like characteristics) [11] do not express lactation-specific proteins. Other bovine mammary epithelial cell lines such as BME-UV [5] express a low level of α-lactalbumin and αs1-casein, whereas Mac-T (Mammary Alveolar Cells) have a variable expression level of milk proteins [2,12,13]. Compared with other immortalized bovine mammary epithelial cells, the Mac-T cells can express and secrete α- and β-caseins [2]. These cells can be passed to >350 without sign of senescence [2]. The Mac-T cells, as ET-C and BME-UV cells, were established by stable integration of the Simian Virus large T–antigen (SV-40 large T-antigen) gene to induce immortalization. The SV40LTA is known to bind retinoblastoma protein and p53, thereby potentially modulating both the cell cycle and apoptotic pathways leading to an immortal phenotype. While this is useful in establishing and maintaining the cell line it is not clear how this modification affects the mammary epithelial-specific phenotype.

To date the Mac-T cells have been widely used in order to provide preliminary data for the effect of growth factors or hormones [12,14-16], fatty acids [17], amino acids [18,19], and bacterial cell wall components such as LTA and LPS [20]. However, the development of Mac-T cells was performed using a mixture of primary cells from slaughtered cows [2] without considering or reporting the stage of lactation of the cows. Therefore, it remains to be determined how reliable the Mac-T cells are for providing preliminary *in vitro* data relevant to mammary epithelial cells in general; more specifically, we aimed to determine how reliable the Mac-T cells cultured in lactogenic media on 2 dimensional plastic plates would be as a model to study *in vivo* mammary tissue biology.

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To that aim, we performed direct transcriptomics comparison between Mac-T cells and non-lactating (4 wk prior to parturition; -30 day in milk (DIM)) or lactating mammary tissue (+60 DIM) followed by a thorough bioinformatics analysis.

Materials and Methods

Samples

The total RNA from Mac-T cells was from 4 replicates of the control group maintained in lactogenic medium from a previous experiment [17]. The total RNA from mammary tissue of 3 multiparous Holstein cows at 1 month prior parturition (-30 DIM) and at peak lactation (+60 DIM) was from a previous experiment [21].

Microarray analysis

A specific bovine 13K oligo array previously described [22] was used. This platform is publicly accessible at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (GSE4304). The synthesis of cDNA, the protocol for microarray analysis, and data quality assessment were as previously described [23]. Microarrays were run in a dye-swap design as previously described [22] with the cDNA obtained from the Mac-T cells as reference. Few additional modifications compared with previous methods were introduced: prior to hybridization labeled cDNA of Mac-T cells was pooled for each dye and the same amount of labeled cDNA from mammary and Mac-T was added to two separate tubes prior to vacuum-drying in the dark in order to prepare the specimens for co-hybridization. The microarray data presented in this manuscript have been deposited at NCBI's Gene Expression Omnibus [24] and are accessible through GEO Series accession number *GSE46476*.

Verification of microarray data using quantitative real-time PCR (qPCR)

The results form microarray were validated performing qPCR for a selected panel of 32 genes considered important for the mammary gland functioning, e.g. growth and proliferation, transport, metabolic path ways, and regulatory elements (see primer pairs and amplicon sequencing results in Tables S1 and S2 in File S1 in supplementary online material). Methods for primer pair design and validation and qPCR were as previously described [21].

Essential for reliability of qPCR data is normalization using appropriate internal control genes (ICGs). Microarray data were mined to identify suitable ICGs as previously described [25]. A panel of more than 32 genes with high stability in expression between mammary and Mac-T (ratio ≈ 1) plus previously tested ICGs [26] were evaluated for co-regulation using Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). Among those *VPS4A, SLC26A11, ABHD11, GAPDHS, ZBTB2, SLC41A3, RECQL5, COL23A1, SH3BGR, MRPL39, MTG1, RPS15 and UXT* did not have known co-regulation. A qPCR analysis using all samples was performed for these 13 genes. The raw qPCR results from tested genes were used to select the most stable ICGs as well as the number of ICGs which should be used for accurate normalization using the geNorm software [27]. The results from geNorm suggested that *MRPL39, UXT, and MTG1* were the most reliable ICGs and the geometric mean of the three was used for qPCR normalization.

Statistical analysis

The GSDAT files were uploaded into Gene Spring GX7 (Agilent, USA), normalized using Lowess, and the statistical package included in the software was used to provide a list of similar (SEG) and differentially expressed genes (DEG) using ANOVA. Due to the microarray experimental design a direct comparison between the microarray signal from mammary tissue and Mac-T cells was performed using a Benjamini-Hochberg false discovery rate (FDR). The SEG and DEG between mammary tissue and Mac-T were determined by using an FDR cut-off ≥ 0.20 and 0.05, respectively.

The qPCR data were log2 transformed prior statistical analysis. The data were analyzed using a Generalized Linear Model (GLM) using SAS (v. 9.3) with cell/tissue type (Mac-T cells, mammary tissue at -30 DIM, and mammary tissue at +60 DIM) as main effect. Significance was declared at $P<0.05$.

Data mining

Data were mined by means of IPA (analysis performed the 12/15/2010) and Database for Annotation and Visualization and Integrated Discovery (DAVID; performed the 12/10/2010) v6.7 [28]. All the data from oligos on the array were uploaded into IPA with associated annotations plus expression ratios and FDR. Each annotated gene was mapped to its corresponding gene object in the IPA Knowledge Base and the whole annotated bovine microarray was used as background. Genes from the dataset that met the FDR ≤ 0.05 or FDR>0.20 and were associated with biological functions in the IPA Knowledge Base were used for the analysis. The functions and pathways in IPA were considered significantly enriched and discussed at a Benjamini-Hochberg FDR ≤ 0.05. The interpretation of IPA analysis was performed as previously described [23]. For DAVID analysis the lists of DEG and SEG with associated bovine Entrez gene ID were up-loaded into the system and the whole annotated microarray was used as background. The DAVID default annotation databases plus chromosome and UP-tissue were downloaded as Functional Annotation Chart and Functional Annotation Cluster with an Easy score ≤ 0.01.

For analysis of the KEGG pathways of DEG between mammary tissue and Mac-T cells we also used the novel Dynamic Impact Approach (DIA) [29]. The analysis of DEG was as previously described [29]. In order to increase reliability of the DIA results only KEGG pathways that had: 1) ≥ 4 bovine genes mapped to the pathway and 2) annotated genes in microarray that covered ≥ 30% of all bovine genes mapped in the pathway by the bovine genome were used for analysis.

Results

The complete dataset with expression ratio, p-value adjusted by FDR between each comparison, and a complete list of genes verified by qPCR is available in File S2.

Expression of several selected genes by qPCR

In Figure 1 are reported the results of genes verified with qPCR (plus CCND2 and PPARG, not present on the microarray) clustered based on main functions related to mammary biology. Overall >70% of genes measured with qPCR had a result deemed similar (considering also the statistical analysis) to microarray data (File S2 and Figure 1). Among the verified genes it was clear that Mac-T cells had a more pronounced expression of components of cell cycle, carbohydrate and lipid catabolism, and energy production compared with mammary gland (Figure 1). The mammary gland compared with Mac-T cells had a greater expression of genes involved in transport, lipid anabolism (particularly milk fat synthesis), caseins, and lactose synthesis. Among genes involved in regulatory functions mammary tissue vs. Mac-T

cells had greater expression of *PPARG, PRLR, and STAT5B*, while expression of AKT1 and *SREBF1* was similar or slightly greater in Mac-T cells compared with mammary tissue (Figure 1).

Number of similarly expressed genes (SEG) and differentially expressed genes (DEG) in the microarray data

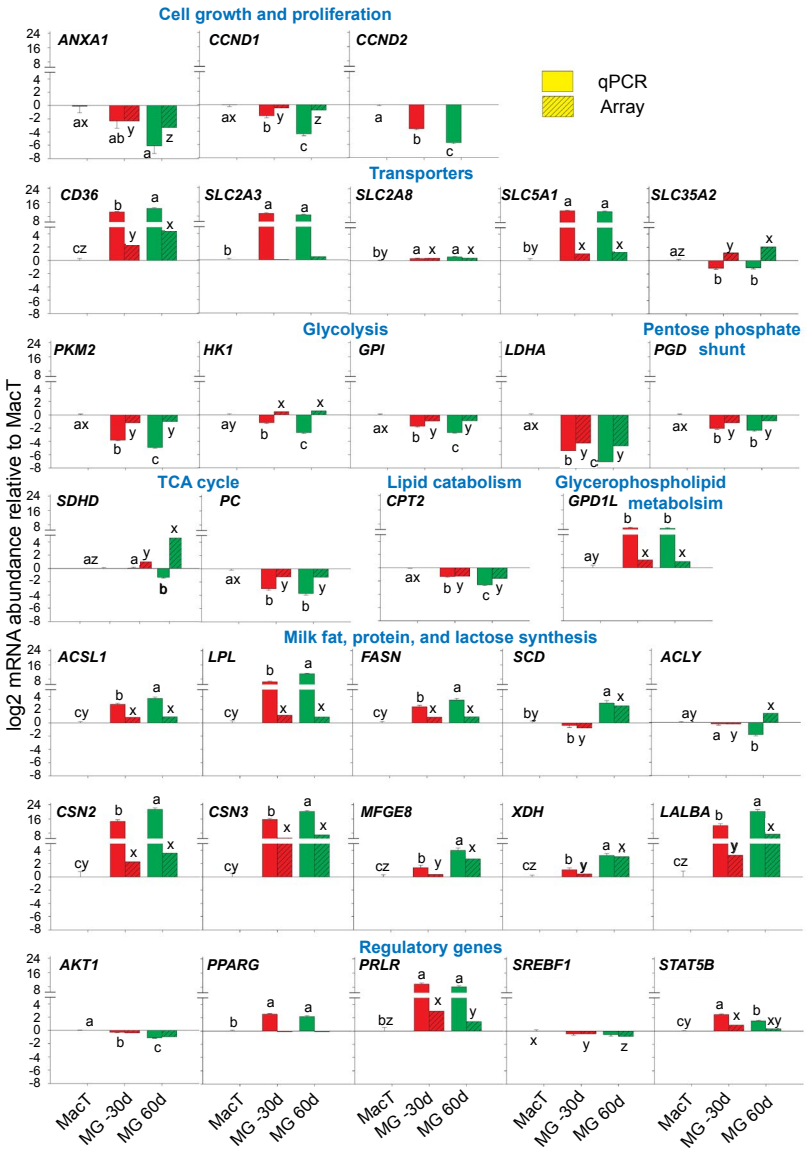
Overall there were ≥37% of SEG between mammary tissue and Mac-T cells at an FDR ≥ 0.20, and >53% at an FDR ≥ 0.05 (Table 1). We observed >46% overall DEG in mammary tissue vs. Mac-T cells (FDR<0.05). When comparing mammary tissue at -30 DIM and +60 DIM vs. Mac-T cells separately, the highest number of DEG was observed at -30 (ca. 44%) compared with +60 DIM (ca. 37%). The transcriptome of the Mac-T cells was more similar (FDR>0.20) to the transcriptome of the lactating (i.e., +60 DIM; >45%) compared to the non-lactating (i.e., -30 DIM) mammary tissue.

Functional analysis of SEG and DEG

Enrichment analysis

SEG between Mac-T cells and mammary tissue:

Enriched biological functions in IPA: No functions were significantly enriched with a Benjamini-Hochberg (B-H) FDR<0.05. Among the many functions enriched with a more liberal Exact Fisher test P<0.05 and using the "effect on function" analysis in IPA (Table S3 in File S1), we deemed interesting for our comparison those associated with lipid metabolism, carbohydrate metabolism, energy metabolism, and cell morphology. Within lipid metabolism were vitamin A and beta-estradiol. Apparently, vitamin A was more enriched in SEG at -30 DIM (Table S4) and beta-estradiol in SEG at +60 DIM (Table S5). In addition, the SEG between Mac-T cells and mammary tissue at -30



a,b,c denote differences with p<0.05 in qPCR data and x,y,z denote differences with p<0.05 in microarray data.

Figure 1: qPCR analysis of selected transcripts and comparison with microarray data. Transcripts are grouped based on main functions. Several of selected transcripts were present and differentially expressed between Mac-T and mammary tissue in the microarray data (File S2), others such as *PPARG* and *CCND2* were run with qPCR but were no present in the microarray data.

DIM were significantly enriched by functions related to induction of progesterone response (Table S4 in File S1).

The genes coding for proteins involved in increasing D-glucose import and ATP production were highly enriched in overall SEG (Table S3 in File S1), whereas genes involved in ATP production and AMP biosynthesis were significantly enriched among SEG at -30 DIM (Table S4). Other enriched lipid-related functions within SEG between Mac-T cells and mammary tissue at -30 DIM were modification and esterification of fatty acids (Tables S4 in File S1). Genes involved in cell morphology and cell-to-cell signaling, including cytoskeleton modification, vesicle formation, and tight junctions were enriched in SEG between Mac-T cells and mammary tissue at +60 DIM (Tables 3S and 5S in File S1).

Enriched pathways in IPA: Among overall SEG the most enriched pathways uncovered by IPA was 'Fatty Acid Elongation in Mitochondria' (File 4S in File S1). The 'Synaptic Long Term Depression', 'Keratan Sulfate Biosynthesis', and 'CD27 Signaling in Lymphocytes' were the most enriched pathways in SEG between Mac-T cells and mammary tissue at +60 DIM (File 4S in File S1). 'Taurine and Hypotaurine Metabolism' was the most enriched in genes with similar expression between Mac-T cells and mammary tissue at -30 DIM (File 4S in File S1).

Enriched biological functions and pathways in DAVID: Except 'SH3 domain' (SRC Homology 3 Domain) in SEG between Mac-T cells and mammary tissue at +60 DIM, no biological terms were significantly enriched with FDR ≤ 0.05 among SEG between Mac-T cells and mammary (Files S5 and S6).

DEG more expressed in Mac-T cells vs. mammary tissue

Enriched biological functions in IPA: Functions related to cellular migration and movement, cell death, cell growth and proliferation, cell

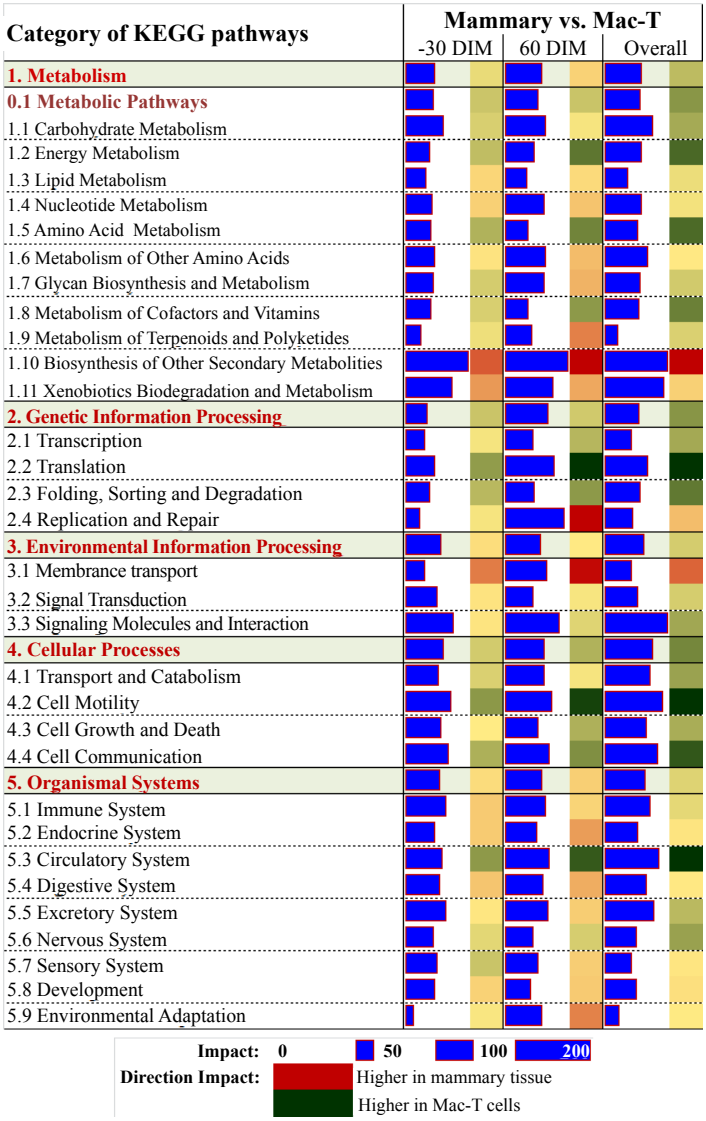


Figure 2: Overall calculated flux and impact of KEGG pathways by DIA of DEG between mammary tissue and Mac-T cells. Shown are the main categories of KEGG pathways (e.g., 'Metabolism', 'Genetic Information Processing') with related sub-categories. For each category and sub-category are reported the impact (horizontal bar; larger the bar larger the impact) and the direction of the impact (red more induced in mammary tissue vs. Mac-T cells and green more induced in Mac-T cells vs. mammary tissue).

morphology and development (including epithelial cells), cell-to-cell signaling and interaction (particularly cell adhesion and cytoskeleton organization), protein synthesis and modification, and amino acid transport were the most enriched (B-H FDR<0.05) in IPA for DEG with greater expression in Mac-T cells vs. mammary tissue (Tables S6 and S7 in File S1). All these functions, with exception of cell death, were more induced in Mac-T cells vs. mammary tissue. Interestingly, protein synthesis was the most enriched function in DEG with greater expression in Mac-T cells vs. mammary tissue at +60 DIM and apparently more induced in Mac-T cells.

FDR cut-off	Higher expression	Day in milk					
		-30	% array	+60	% array	Combined	% array
≥ 0.05		5,771	56.0	6,495	63.0	5,525	53.6
≥ 0.20		3,811	37.0	4,698	45.6	3,839	37.2
<0.05		4,537	44.0	3,813	37.0	4,783	46.4
	Mammary	2,184	21.2	1,564	15.2	2,106	20.4
	Mac-T	2,353	22.8	2,249	21.8	2,677	26.0

Table 1: Number of transcripts similarly (FDR ≥ 0.05 and FDR ≥ 0.20) and differentially (FDR<0.05) expressed in mammary tissue at -30 and +60 DIM (or analysis when those were combined) vs. Mac-T cells. The number of genes was determined using the 10,308annotated transcripts on the microarray that passed all the criteria to select reliable data (see Materials and Methods).

Pathway	Main category	p-val*	FDR*	Ratio	Genes
DEG with greater expression in Mac-T cells vs. mammary tissue overall					
Integrin Signaling	Cell-to-cell interaction	8.7	6.5	0.17	34
Actin Cytoskeleton Signaling		5.5	3.8	0.12	27
Ephrin Receptor Signaling		3.9	2.3	0.11	22
Regul. of Actin-based Motility by Rho		3.4	2.1	0.14	13
14-3-3-mediated Signaling	Signal transduction	3.6	2.1	0.14	16
Leukocyte Extravasation Signaling	Immune-related	3.5	2.1	0.11	21
Clathrin-mediated Endocytosis	Endocytosis	3.3	2.0	0.11	19
Oxidative Phosphorylation	Energy metabolism	8.2	6.2	0.17	26
Mitochondrial Dysfunction		6.5	4.7	0.13	23
Ubiquinone Biosynthesis		3.5	2.1	0.10	10
Gly, Ser and Thr Metabolism	Amino acid metabolism	3.2	2.0	0.06	9
Glycolysis/Gluconeogenesis	Glucose metabolism	3.2	2.0	0.08	12
DEG with greater expression in Mac-T cells vs. mammary tissue at -30 DIM					
Integrin Signaling	Cell-to-cell interaction	8.4	6.1	0.22	43
Ephrin Receptor Signaling		5.0	3.4	0.17	32
Actin Cytoskeleton Signaling		3.7	2.3	0.14	31
Tight Junction Signaling		3.6	2.3	0.16	26
Axonal Guidance Signaling		3.3	2.0	0.11	44
14-3-3-mediated Signaling	Signal transduction	4.6	3.1	0.20	23
fMLP Signaling in Neutrophils	Immune-related	3.6	2.3	0.17	21
Leukocyte Extravasation Signaling		3.2	2.0	0.14	27
PI3K/AKT Signaling	Cell cycle/protein synthesis	5.3	3.7	0.20	27
Oxidative Phosphorylation	Energy metabolism	7.7	5.7	0.20	32
Mitochondrial Dysfunction		6.3	4.5	0.17	29
Ubiquinone Biosynthesis		3.7	2.3	0.12	13
Methane Metabolism		3.2	2.0	0.08	5
Phenylalanine Metabolism	Amino acid metabolism	3.3	2.0	0.08	9
DEG with greater expression in Mac-T cells vs. mammary tissue at +60 DIM					
Integrin Signaling	Cell-to-cell interaction	6.7	4.9	0.21	41
Actin Cytoskeleton Signaling		5.2	3.5	0.16	36
Ephrin Receptor Signaling		3.8	2.4	0.15	30
Reg. of Actin-based Motility by Rho		3.6	2.3	0.20	18
Cell Cycle: G1/S Checkpoint Reg.	Proliferation	3.3	2.1	0.22	13
Wnt/β-catenin Signaling	Development/ differentiation	4.6	3.1	0.18	30
TGF-β Signaling		3.4	2.1	0.20	17
ERK/MAPK Signaling		3.3	2.1	0.16	30
Oxidative Phosphorylation	Energy metabolism	12.8	10.5	0.26	41
Mitochondrial Dysfunction		7.4	5.4	0.19	32
Ubiquinone Biosynthesis		4.8	3.2	0.14	15
Hyp. Signal. Cardiovascular System	Other	3.9	2.5	0.24	17

Table 2: Most enriched canonical pathways (-log10Benjamini-Hochberg FDR corrected P-value >2) by DEG in various comparisons using Ingenuity Pathways Analysis. Reported are the name of the pathway, the main general category of the pathway, the -log10 Fisher's- exact test P-value of the enrichment (p-value), the -log10 of the Benjamini-Hochberg FDR corrected P-value (FDR), the ratio of DEG/total number of genes in the pathway, and number of DEG in the pathway (Genes).

Enriched pathways in IPA: The DEG with greater expression in Mac-T cells vs. mammary tissue (both overall, at -30, and + 60 DIM) were significantly enriched by pathways related to energy production in mitochondria (e.g. 'Oxidative Phosphorylation', 'Ubiquinone Biosynthesis'), cell-to-cell interaction, amino acid and glucose metabolism (e.g. 'Glycine, Serine and Threonine Metabolism', 'Glycolysis/Gluconeogenesis'), cell proliferation (e.g. 'p53 signaling'), and protein synthesis (e.g. 'Aminoacyl-tRNA Biosynthesis') (File S3 and Table 2).

Enriched biological functions and pathways in DAVID: Similarly to IPA, the most enriched functions and pathways in DAVID among DEG with greater expression in Mac-T cells vs. mammary tissue were related to protein synthesis, energy production, and cell-to-cell interaction (particularly cell adhesion, extra-cellular matrix, and cytoskeleton organization; Table 3 and details in Files S5 and S6). The analysis also uncovered that DEG with greater expression in Mac-T cells vs. mammary tissue were highly enriched by genes located on the chromosome 29 (Table 3 and File S5).

DEG more expressed in mammary tissue vs. Mac-T cells:

Enriched biological functions in IPA: There were no functions significantly enriched with a B-H FDR<0.05 in DEG with greater expression in mammary tissue compared with Mac-T cells (Table S8 in File S1). The use of a more liberal Exact Fisher's test P<0.05 indicated that functions related to carbohydrate and lipid metabolism were the most enriched and more induced in mammary vs. Mac-T (Table S8 in File S1). The analysis indicated a greater triacylglycerol (TAG), phospholipid, and ganglioside synthesis in mammary tissue vs. Mac-T cells (Table S8 in File S1). Also transport of constituents such as TAG, fatty acids, amino acids, and calcium, and cell death were apparently more induced in mammary tissue vs. Mac-T cells (Table S8 in File S1).

Among DEG with greater expression in mammary tissue at -30

Category	Term	Count	B-H*
DEG with greater expression in Mac-T cells vs. mammary tissue at -30 DIM			
Chromosome	29	66	<0.001
GOterm_BP_FAT	GO:0006412~translation	56	0.002
GOterm_CC_FAT	GO:0042470~melanosome	21	0.002
GOterm_CC_FAT	GO:0070469~respiratory chain	21	0.009
GOterm_CC_FAT	GO:0005739~mitochondrion	111	0.015
KEGG_pathway	bta00190:Oxidative phosphorylation	33	0.002
KEGG_pathway	bta04512:ECM-receptor interaction	20	0.016
KEGG_pathway	bta04510:Focal adhesion	38	0.016
KEGG_pathway	bta03010:Ribosome	22	0.022
KEGG_pathway	bta04810:Regulation of actin cytoskeleton	35	0.031
UP_tissue	Ileum	187	<0.001
UP_tissue	Heart	28	0.034
DEG with greater expression in Mac-T cells vs. mammary tissue at +60 DIM			
Chromosome	29	60	0.002
Chromosome	3	111	0.002
Chromosome	18	91	0.015
Chromosome	23	57	0.020
Chromosome	5	90	0.021
Chromosome	2	76	0.041
GOterm_BP_FAT	GO:0006412~translation	95	<0.001
GOterm_BP_FAT	GO:0006091~gener. precur. metab. and energy	50	<0.001
GOterm_BP_FAT	GO:0022900~electron transport chain	27	0.010
GOterm_BP_FAT	GO:0006414~translational elongation	9	0.017
GOterm_CC_FAT	GO:0005840~ribosome	73	<0.001
GOterm_CC_FAT	GO:0030529~ribonucleoprotein complex	95	<0.001
GOterm_CC_FAT	GO:0022627~cytosolic small ribosomal subunit	10	<0.001
GOterm_CC_FAT	GO:0070469~respiratory chain	24	<0.001
GOterm_CC_FAT	GO:0005743~mitochondrial inner membrane	52	0.003
GOterm_CC_FAT	GO:0042470~melanosome	19	0.013
KEGG_pathway	bta03010:Ribosome	60	<0.001
KEGG_pathway	bta00190:Oxidative phosphorylation	38	<0.001
UP_tissue	Ileum	229	<0.001
UP_tissue	Heart	37	<0.001
UP_tissue	Lymphoid epithelium	20	<0.001
DEG with greater expression in mammary tissue at +60 DIMvs. Mac-T cells			
GOterm_CC_FAT	GO:0005576~extracellular region	96	0.01
UP_tissue	Mammary gland	16	0.02

*Benjamini-Hochberg false discovery rate corrected P-value.

Table 3: Results from DAVID analysis for terms enriched with a Benjamini-Hochberg FDR<0.05. Additional results are available in Files S5 and S6. Category denotes the main group of terms in DAVID (BP=biological process, CC=cellular component, KEGG and UP Tissue=Uniprot Tissue). The count denotes the number of genes among the DEG in the specific term. No significant terms were observed for the DEG with greater expression in mammary tissue at -30 DIM vs. Mac-T cells and genes similarly expressed between mammary tissue and Mac-T with FDR>0.20.

DIM vs. Mac-T cells we observed enrichment (Exact Fisher's test P<0.05) of genes coding for proteins involved in lipid metabolism, particularly TAG synthesis, immune system activity and inflammation, cell death, transport of folic acid and mobilization of calcium (Table S10 in File S1). All those functions were more induced in mammary tissue vs. Mac-T cells.

In DEG with greater expression in mammary tissue at +60 DIM vs. Mac-T cells we observed enrichment (Exact Fisher's test P<0.05) of genes coding for proteins involved in tissue development, cellular growth and proliferation, immune cell trafficking, and inflammatory response (Table S11 in File S1). The data also indicated a significant enrichment of protein trafficking and lipid, carbohydrate, amino acid, nucleic acid, and calcium metabolism but the significance of the enrichment was lower compared with the above functions. In all cases the terms were overall more induced in mammary tissue compared with Mac-T cells.

Enriched pathways in IPA: As for the biological functions, no pathways were significantly enriched with a B-H FDR<0.05 by DEG with greater expression in mammary tissue vs. Mac-T cells. Considering a more liberal Exact Fisher's test P<0.05, the most enriched pathways were related to protein metabolism (e.g. 'Glutathione metabolism'), immune system (e.g. 'Antigen presentation pathway'), and regulation of lipid metabolism (e.g. '*LXR*/RXR signaling'), particularly for the DEG with greater expression in mammary tissue at +60 DIMvs. Mac-T cells (File S3).

Enriched biological functions and pathways in DAVID: Similarly to IPA also in DAVID very few terms were significantly enriched in DEG with greater expression in mammary tissue vs. Mac-T cells (Files S5 and S6). Among the few significant enriched terms were the gene ontology terms related to extracellular matrix and mammary gland as UP-tissue (Files S5 and S6 and Table 3).

Dynamic Impact Approach analysis of KEGG pathways and Chromosome

Impact on main KEGG pathway categories: The overall view of the KEGG categories of pathways (Figure 2) evidenced an overall greater induction of pathways in Mac-T cells compared with mammary tissue with few exceptions such as pathways related to the biosynthesis of secondary metabolites, xenobiotics degradation, and membrane transport that were more induced in mammary tissue vs. Mac-T cells. The metabolic pathways were overall more induced in Mac-T cells, particularly in comparison with mammary tissue at -30 DIM. The 'Genetic Information Processing' was evidently more induce in Mac-T cells vs. mammary tissue, with a very strong impact and larger induction of the 'Translation' while the 'Replication and Repair' was more induced in mammary tissue (Figure 2). The Mac-T cells also had an apparent greater activation compared with mammary tissue of pathways related to 'Cellular Processes', particularly evident for the 'Cell Motility' and 'Cell Communication' (Figure 2). Several pathways related to 'Organismal Systems' were more induced in mammary tissue vs. Mac-T cells such as the ones related to the 'Endocrine System', 'Digestive System', and "Environmental Adaptation', particularly when considering mammary tissue at +60 DIM (Figure 2).

Integrated view of KEGG pathways using DIA: The DIA analysis of pathways indicated that the overall metabolism was greater in Mac-T cells compared with mammary tissue (Files S7 and S8). Most of the pathways related to carbohydrate metabolism were more induced in Mac-T vs. mammary tissue, particularly at +60 DIM. The Mac-T cells had a greater induction of pathways related to the use of glucose for energy purpose (e.g. 'Glycolysis/Gluconeogenesis', 'Pyruvate metabolism', 'Pentose and glucuronate interconversions'; File S8) while the mammary tissue had a more induced "Galactose metabolism' and 'TCA cycle' compared with Mac-T cells.

The lipid catabolism was overall more induced in Mac-T cells compared with mammary (e.g. 'Fatty acid metabolism' and 'Synthesis and degradation of ketone bodies') while mammary tissue (especially at +60 DIM) vs. Mac-T cells had a greater induction of lipid anabolism, particularly for synthesis of fatty acids and TAG (e.g. 'Glycerolipid metabolism', and 'Biosynthesis of unsaturated fatty acids') but not for sterols (Files S7 and S8).

With exception of 'Taurine and hypotaurine' and 'Glutathione metabolism' that were more induced in mammary tissue, the metabolism of amino acids was more pronounced in Mac-T cells compared with mammary tissue, chiefly the catabolism of Cys and Met and the biosynthesis of Phe, Tyr, and Trp (Files S7 and S8).

The synthesis of several glycans was more induced in mammary tissue vs. Mac-T cells, especially the synthesis of O-mannosyl, keratan sulfate glycosaminoglycan, glycosylphosphatidylinositol-anchor, and lacto and neolacto series of glycosphingolipids (Files S7 and S8). As for the above pathways this difference with Mac-T cells was more pronounced in lactating (i.e., at +60 DIM) compared with non-lactating mammary tissue.

Metabolic pathways related to cofactors and vitamins were overall more induced in Mac-T cells compared with mammary tissue, with a very large induction of 'One carbon pool of folate' in Mac-T cells (File S7). The pathways related to the biosynthesis of secondary metabolism and the degradation of xenobiotics were more induced in mammary vs. Mac-T cells, particularly concerning the 'Caffeine metabolism' and the 'Drug metabolism - cytochrome P450' (Files S7 and S8). The large induction of 'Caffeine metabolism' in mammary tissue vs. Mac-T was due to the larger expression of xanthine dehydrogenase (*XDH,* see File S2).

Among non-metabolic related pathways the DIA analysis uncovered a greater induction in Mac-T cells vs. mammary tissue of pathways related to transcription and, with an even greater induction, translation which was more pronounced in Mac-T cells vs. mammary tissue at +60 DIM (Files S7 and S8). All pathways related to DNA replication and repairs were more induced in mammary tissue vs. Mac-T cells (File S8). In accord with this observation was the greater induction of cell cycle in mammary tissue vs. Mac-T cells, particularly considering the non-lactating mammary tissue confirmed also by a greater induction in Mac-T of the 'p53 signaling pathway'(File S7).

The membrane transport involving ABC transporters was greater in mammary tissue vs. Mac-T cells (File S7) while cell-to-cell interaction and signaling was overall more pronounced in Mac-T cells vs. mammary tissue with few exceptions (File S7). Among these pathways 'Calcium signaling' and 'Jak-STAT signaling' were more induced in mammary tissue vs. Mac-T cells. The KEGG pathway analysis by the DIA also indicated that Mac-T cells vs. mammary tissue were characterized by having larger cell adhesion involving chiefly extracellular matrix interaction, focal adhesion, adherens junctions, and cytoskeleton regulation (File S7).

The overall greater induction in mammary tissue vs. Mac-T cells of the 'Organismal Systems' categories of pathways was mostly due to several pathways related to the innate immune system (File S7 and S8). The mammary tissue at +60 DIMvs. Mac-T cells had a larger induction

of 'Complement and coagulation cascades', pathways related to the differentiation of immune-cells (i.e., 'Hematopoietic cell lineage'), sensitivity to bacteria and virus DNA (i.e., 'Cytosolic DNA-sensing pathway') and, particularly, response to antigens (i.e., 'Intestinal immune network for *IgA* production'), but a less induced 'Antigen processing and presentation', pathways related to the migration of leukocytes (i.e., 'Leukocyte trans endothelial migration'), and 'NOD-like receptor signaling pathway' (Files S7 and S8). The non-lactating mammary tissue had instead a more induced 'Antigen processing and presentation' compared with Mac-T cells (File S7 and S8).

Among endocrine-related pathways the 'PPAR signaling', the 'Renin-angiotensin system', and, with a minor impact, the 'GnRH signaling', were more induced in mammary tissue (chiefly at +60 DIM) vs. Mac-T cells (File S7). The pathways related to excretory system appeared to be more induced in mammary tissue vs. Mac-T cells, particularly for calcium and water reabsorption (Files S7 and S8).

For other pathways with likely less biological relevance in the present experiment (e.g. 'Human Disease' category of pathways), it is interesting to point out that the pathway 'Bacterial invasion of epithelial cells' was more induced in Mac-T cells compared with mammary tissue (Files S7 and S8).

In summary the overall analysis of KEGG pathways using the DIA indicated that:

-Mac-T cells compared with mammary tissue had a greater catabolism in order to produce energy, a greater protein synthesis and degradation capacity, a greater induction of cell-to-cell interaction heavily involving the cytoskeleton, and greater response to bacterial invasion.

-Mammary tissue compared with Mac-T cells was characterized by having a greater anabolism particularly involving glucose and lipid, production of secondary metabolites, degradation of xenobiotic, DNA replication and cell cycle, innate immune capacity, and signaling pathways known to be important for milk fat and protein synthesis.

Most impacted chromosomes uncovered by DIA: Overall, the *BTA6* was the chromosome with the greatest impact due to DEG between mammary tissue and Mac-T cells with an obvious greater induction of transcription in mammary tissue (File S7). This was mostly due to the greater expression of casein genes in mammary tissue vs. Mac-T cells, but not exclusively; few genes, several involved in transport, such as SLC39A8 (Zn transporter) and *SLC34A2* (phosphate transporter), are also located in the *BTA6* and had greater expression in lactating mammary tissue vs. Mac-T cells (File S2 and Figure S1). Among the most impacted chromosomes was *BTA14* in which transcription overall was more induced in mammary tissue vs. Mac-T cells (File S7). Several other chromosomes, including *BTA29* and *BTA23*, were among the most impacted by the DEG between mammary tissue and Mac-T cells and transcription was overall more induced in the latter (File S7 and Figure S2). Several of the chromosomes with greater impact in the DIA analysis were also significantly enriched in the DAVID analysis (Table 3).

Discussion

Immortalized mammary epithelial cell lines can be useful to conduct *in vitro* studies to obtain data pertinent to the bovine mammary gland. The immortalized Mac-T cells have been extensively used to invest*IgA*te the effect of fatty acids [12,14-16], growth factors or hormones [17], and amino acids [18,19], to study the milk fat [17] and protein synthesis [30], and inflammatory response [20,31]. The usefulness of the data obtained using immortalized cell lines is related to the similarity with mammary tissue. In order to evaluate this similarity, in the present study we performed a comparison at the transcriptomics level between Mac-T cells cultivated in a 2 dimensional (2D) *in vitro* system and mammary tissue during the end of pregnancy and peak lactation.

The use of a 2D culture system in the present study is an important point considering that the 3 dimensional (3D) structure has a strong effect on determining the identity, therefore function, of cells [32]. This appears to be even more important for mammary epithelial cells that are strongly polarized. It has been clearly shown that mammary epithelial cells, including the ones from bovine, cultured on 2D systems have an impaired expression of the main milk protein genes compared with cells cultivated in collagen, where they can form the 3D structure [33]. The importance of a 3D culture for a full differentiation of Mac-T cells was already demonstrated in the first paper describing the Mac-T cells [2].

A direct comparison between mammary explants and primary cells from the same animals indicated a greater quantity and secretion of β-casein in explants vs. expanding primary cells in response to somatotropin and insulin like growth factor I (IGF-I); however, the difference decreased when cells reached confluence [34]. The authors concluded that "primary cell cultures are comparable to explant cultures when used to study mechanisms of DNA and milk protein synthesis and secretion". The mammary explants stem compared with isolated cells preserves mammary tissue composition, the 3D structure, and the extracellular matrix; all factors essential to obtain a fully functional lactating mammary epithelial [35]. In another study Mac-T cells had a very similar response compared with primary mammary epithelial cells isolated from prepubertal heifers when treated with IGF-I or inflammation-related proteins such as IL-6, IL-1b, MCP-1, and PAI-1 [36]. Similarly, the transcriptomics response to growth hormone in Mac-T cells, bovine mammary tissue explants, and primary mammary epithelial cells isolated from milk was similar [37]. In a recent study, a similar effect of amino acids and a greater effect of insulin on mTOR signaling was observed in Mac-T cells compared with mammary tissue explants [19]. All the above studies provided evidence that Mac-T cells are an adequate system to study bovine mammary *in vitro*; however, a large transcriptomics analysis might provide a more comprehensive comparison.

Similarities between Mac-T and mammary tissue

Overall our data indicated a more similar transcriptome in Mac-T cells induced to lactation and lactating mammary tissue. The DIA analysis of SEG also supported a closer functional similarity between Mac-T cells and lactating than non-lactating mammary tissue. Due to the very large number of SEG compared to the analysis of DEG, it is not surprising that no biological terms were significantly enriched at a B-H<0.05 [29,32]. However, this might also indicate that Mac-T cells are not "specialized" compared with the mammary tissue either at -30 or +60 DIM.

Differences between Mac-T cells and mammary tissue

The functional analysis of DEG compared with the analysis of SEG has to be considered more important for our objectives. Overall, the large number of DEG between Mac-T cells and mammary tissue (Table 1) raises doubts about the reliability of the former to study the latter.

Are Mac-T cells a good model for studying milk synthesis?

The qPCR analysis of selected genes (Figure 1) clearly indicated that the Mac-T cells cultivated in a 2D system do not closely resemble the lactating mammary tissue, particularly for the tasks that the mammary tissue does best: synthetizing milk. The expression of all measured transporters of fatty acids, amino acids, and glucose, the main milk proteins, and genes involved in milk fat synthesis had greater expression in mammary tissue compared with Mac-T cells (Figure 1). For most of those genes the greater expression in mammary tissue vs. Mac-T cells was observed even at -30 DIM. This is not novel, particularly for expression of caseins [33]. Even though it has been reported that Mac-T cells can express and synthetize caseins [13,38] the amount produced is often below the limit of detection [2]; certainly not at the level of mammary tissue. Therefore, contrary to previous reports [19,34], the qPCR data in the present study indicated that the usefulness of Mac-T cells for studying milk protein synthesis, particularly if expression of milk proteins is considered, is limited.

The Mac-T cells also cannot be considered a good model for studying milk fat and lactose synthesis. This was evident both by using qPCR (Figure 1) and by the functional analysis of microarray data, particularly with the DIA analysis (File S7). These differences appear to be not only related to production of milk protein, fat, and lactose, but also other milk synthesis-related functions. This is supported by the large differences between Mac-T cells and mammary tissue observed in the present study for pathways previously shown to be highly activated during lactation such as glutathione metabolism, GPI-anchor biosynthesis, TCA cycle, and caffeine metabolism (Files S3 and S7) [39].

Interesting also is the marked translational capacity in Mac-T cells vs. mammary tissue. It was previously observed the paradoxical phenomenon of a strong decrease in expression of ribosomes from pregnancy to lactation both in bovine and mouse mammary tissue [33,39], despite an overall increase in protein synthesis. We have interpreted such findings as a way for the mammary gland to prioritize the translation of transcripts coding for proteins for milk production, i.e., a way for the mammary tissue to specialize [33,39]. This observation seems to be not only related to mammary tissue but also other tissues such as adipose [40]. Therefore, if we accept this hypothesis, the data in the present experiment further indicate that the Mac-T cells were less "specialized" than mammary tissue, particularly for milk synthesis.

Are Mac-T cells a good model for studying milk fat and protein regulation?

Despite the large differences observed for the genes involved in milk synthesis, the Mac-T cells have been often used successfully to study regulation of milk protein [12,14,18] and milk fat [17,41,42] synthesis.

Milk protein synthesis regulation: All the studies involving regulation of milk protein synthesis where Mac-T cells were compared with mammary explants suggested a very similar response between both systems [18,19,37]. In mammals the main pathway involved in the regulation of translation is the insulin-mTOR pathway. This also seems to be the case for the bovine mammary tissue [33]. Our data indicated that both mTOR and insulin signaling were slightly more induced in Mac-T cells vs. mammary at +60 DIM, indicating that Mac-T cells might be more sensitive to regulation by insulin. This also has been shown by recent data [19]. It has to be considered that the Mac-T cells in the present study were cultured in a supra physiological dose of insulin [17].

The regulation of casein expression is known to be under control of the Jak-STAT signaling. This has been clearly established in rodents and bovine [43,44]; however, for the latter this remains somewhat controversial [33]. Recently from transcriptomics data it was observed a large induction of the *Jak-STAT* signalling from pregnancy to lactation [39]. In the present study the DIA analysis suggested the Jak-STAT signalling was overall more induced in mammary tissue compared with Mac-T cells (File S7). This might partly explain the greater expression of milk protein genes in the former (Figure 1). In summary, it appears that Mac-T cells cultivated in 2D might be an adequate model to study regulation of protein synthesis, particularly for the insulin-mTOR signaling.

Milk fat synthesis regulation: Three main transcription factors have been reported to control milk fat synthesis: SREBP1 [45], LXRα [46,47], and PPARγ [17,48]. Our data, both from qPCR and microarray, indicated a marked degree of similarity between Mac-T cells and mammary tissue for the *SREBF1* expression (Figure 1). Unfortunately our microarray did not contain probes for co-factors of SREBP1 such as *SCAP* and *INSIG1* (however, expression of *INSIG2* was not different between Mac-T cells and mammary at +60 DIM, see File S2). Similarly, we only had data for the LXRβ (or *NR1H2*) for which the expression was greater in Mac-T cells vs. mammary tissue (File S2). Even though only with an Exact Fisher's test <0.05, functional analysis in IPA indicated that LXR/RXR activation was the most enriched pathway in genes with greater expression in mammary at +60 DIM vs. Mac-T cells (File S3). This might indicate an overall greater sensitivity of the mammary tissue compared to Mac-T cells to activation of LXR.

The *PPARG* expression was ca. 4-fold greater in mammary compared with Mac-T cells (Figure 1). In addition, the 'PPAR signaling' was among the most induced pathways in lactating mammary tissue compared with Mac-T cells (File S7). If the observations for LXR and, particularly, PPARγ, hold true, the findings in Mac-T cells about activation of LXR and PPARγ might be underestimating the response of the mammary tissue *in vivo*. In summary, it appears that Mac-T cells are a good model to study transcription regulation of milk fat synthesis, particularly for SREBP1.

Metabolic differences between Mac-T cells and mammary tissue

The results of the enrichment and DIA analyses of pathways indicated that the overall metabolism was greater in Mac-T cells compared with mammary tissue. The functional analyses indicated an overall greater utilization of glucose, amino acids, and fatty acids for energy production (particularly in mitochondria) in Mac-T cells compared with mammary tissue. The marked utilization of those molecules for energy production might be partly due to a reduction in anabolism associated with low expression of milk-related genes (Figure 1) and/or the higher availability of glucose from the medium (>4 g/L in the medium vs. <1 g/L in blood), despite the lower expression of glucose transporters (Figure 1, File S2). However, the apparent greater glucose utilization for energy production in Mac-T cells in 2D, therefore deprived of ECM, compared with mammary tissue, that contains the ECM, seems to contradict with results of a previous study [49]. In that study it was observed detachment of human mammary epithelial cells from ECM negatively affected ATP production mostly due to a decrease in glucose import into cells; therefore, glucose availability and ATP production [49]. Therefore, the suggested greater use of glucose as energy source by Mac-T cells as a result of the greater expression of the genes involved in production of energy might be a reaction of the cells

to an inner lower capacity, due to absence of ECM, for the utilization of energy compared with mammary tissue.

Differences in cell-to-cell communication

The Mac-T cells also had a more pronounced cell-to-cell interactions and cell movement, particularly involving cytoskeleton and ECM compared with mammary tissue. This might be a consequence of the effect of the *in vitro* system on the epithelial cells. The ECM, together with the 3D formation of the alveolus and the establishment of the polarity, is essential for the formation of a functional mammary epithelium, primarily via integrins and cytoskeleton [50-52]. The ECM, through integrins, might affect the expression of milk-related genes via epigenetic mechanisms [53]. Therefore, the large differences observed between Mac-T cells and mammary tissue in the present experiment might be partly explained by the lack of ECM and 3D structure [54].

It is interesting in this regard the differences in the type of glycosaminoglycan biosynthesis observed (File S7). Intriguingly, the data indicated a more induced formation of heparin sulfate in Mac-T cells, considered to be absent in undifferentiated mammary epithelial cells [54], and a more induced formation of chondroitin sulfate in mammary tissue, which is highly abundant in undifferentiated mammary cells (File S7) [54]. This observation, together with the greater induction in Mac-T cells vs. mammary tissue of the cell-to-cell interaction, might indicate a reaction of the cultured cells to a lack of functional structure. This reaction might be aimed to recover the original alveolus structure. This idea is supported by the observed high capacity of the mammary epithelial cells, even the immortalized, to easily form lobule-like structure when cultured in laminin and/or collagen [2,55]. It will be interesting, from this point of view, to compare the transcriptome between Mac-T cells cultured in 3D and bovine mammary tissue.

Membrane transport and signaling

The membrane transporters, particularly the ABC transporters, and pathways of excretory systems appeared to be more induced in mammary tissue vs. Mac-T cells, particularly for calcium and water reabsorption (Files S7 and S8). The expression of the ABC transporters is mainly controlled by LXR/RXR signaling to regulate cholesterol homeostasis [56]. Among the ABC transporters the expression of ABCG2 dramatically increased from pregnancy to lactation in bovine mammary [21] and a single-point mutation strongly affected milk yield [57]. This gene was >4-fold greater in expression in mammary tissue at +60 DIM vs. Mac-T cells (File S2).

Extensive calcium loads occur in lactating mammary gland to support the requirements of milk calcium. Besides the provision for producing milk, the calcium in mammary gland also plays pivotal roles as a signaling molecule for regulation of proliferation, differentiation, and apoptosis [58].

Do Mac-T cells respond to inflammation as mammary epithelial cells *in vivo*?

Mac-T cells have been used to study the *in vitro* response of mammary epithelial cells to bacteria, their cell wall components [20], and activated neutrophils [59,60]. Our analysis indicated that potential inflammatory response was not similar between mammary tissue and Mac-T cells. Most of the functions and pathways uncovered to be more induced in mammary tissue (especially the lactating one) vs. Mac-T cells were immune cells-rather than epithelial cells-related (e.g. 'Intestinal immune network for *IgA* production'). This might be partly due to the presence of a relatively important number of immune cells in mammary tissue that tends to increase during lactation, particularly for macrophages [39,61].

Not considering the presence of immune cells, our data indicate a potentially greater response of Mac-T cells to bacteria and viruses. This was suggested by the superior induction in Mac-T cells of 'NOD-like receptor signaling' pathway (Files S7 and S8), one of the most activated pathways during mastitis induction through intramammary infusion of Escherichia coli or Staphylococcus uberis [61], and 'Leukocyte trans endothelial migration' pathway (Table 2 and Files S7 and S8).

It is interesting in this regard the observation made from transcriptomics analysis of the bovine mammary tissue from pregnancy to end of subsequent lactation [39]. Overall functional analysis of the data clearly indicated that the lactating vs. non –lactating mammary tissue had an overall decrease of sensitivity to bacteria invasion due to evident inhibition of the antigen presentation pathway but had put a large effort to keep a prepared immune system during lactation. This strong reduction of the antigen presentation was likely pertinent to epithelial cells [61]. Therefore, this indicates that *in vivo* epithelial cells are likely having an overall low responsiveness to bacteria invasion.

Generally, our data indicate that the Mac-T cells have a potentially stronger response to inflammatory challenges, particularly using bacteria, compared with the lactating epithelia cells *in vivo*. In addition, the data indicated a stronger capacity of the Mac-T cells compared with mammary tissue to recruit and to allow invasion of immune cells; however, this might be also a consequence of an inexistent 3D structure and a strong cell-to-cell interactions and cytoskeleton activity for the Mac-T cells that can be considered independent from any inflammatory response.

Conclusions

Results suggest that the transcriptome between Mac-T cells and mammary tissue differs substantially, but with a slightly greater degree of similarity of the former with lactating compared to non-lactating mammary tissue as also suggested by the analysis of SEG. But this overall similarity was not associated with the lactation phenotype. In fact, when functional analysis of DEG was performed the Mac-T cells appeared to be more different than lactating compared with non-lactating mammary tissue, particularly for lactation-specific functions/pathways. This was concluded by the greater catabolism of glucose and lipid for production of energy in Mac-T cells, rather than anabolism for production of major milk constituents.

Concerning milk constituents, the data clearly indicated that Mac-T cells cultured in a 2D plastic system differ fundamentally from lactating mammary tissue because of an impaired ability to synthesize lactose, not adequate milk fat synthesis and secretion (including the control of it through PPAR signaling), a compromised expression of major milk proteins and control of milk protein synthesis (e.g. lower Jak-STAT signaling, very large translational machinery), and mechanisms concerning uptake and metabolism of calcium. Furthermore, several pathways and chromosomes previously found to be important during lactation (e.g. GPI- anchor biosynthesis, *BTA6*, *BTA14*) were also the ones with greater differences between lactating mammary tissue and Mac-T cells.

Our data also indicated that the Mac-T cells do not substantially differ from mammary tissue with regards to the regulation of milk protein synthesis by the insulin-mTOR pathway. With the exception

of SREBP1, our analysis indicated that results from studies of milk fat synthesis regulation using Mac-T cells are likely underestimating the *in vivo* mammary response. Lastly, the Mac-T cells appeared to be more immune responsive than lactating mammary tissue; therefore, likely overestimating the *in vivo* response.

The present study suffers from the limitation of having Mac-T cells cultivated in a 2D plastic system. As previously demonstrated, the Mac-T cells can be a better model for *in vivo* mammary tissue if cultivated in a 3D system. In this regard, it would be interesting to perform the same study presented here on Mac-T cells cultivated in a 3D system.

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