SUPPLEMENTAL FILE

Supplemental Materials and Methods

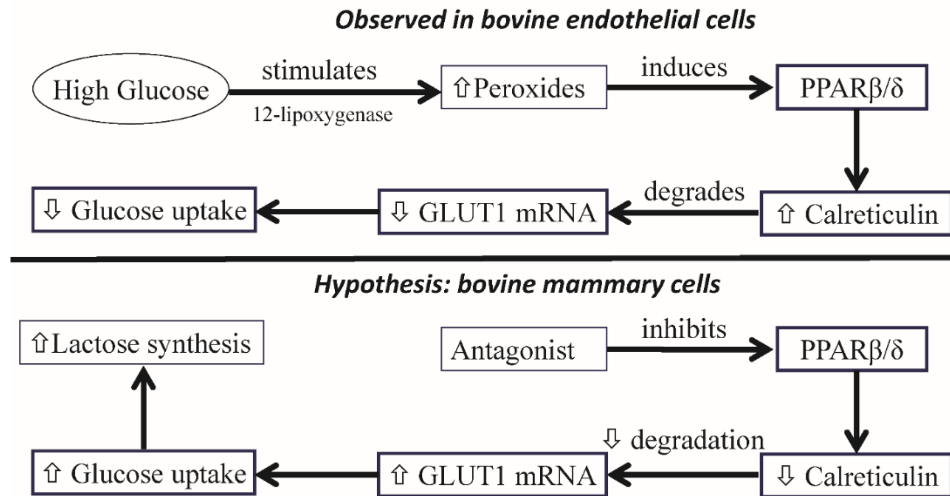
Cell culture: Cells were allowed to grow in 75-cm^2 flasks (Greiner Bio-one GmbH, Firckenhausen, Germany) in high-glucose Dulbecco modified Eagle's medium (DMEM) with sodium pyruvate (Cat# 25-500N; Genesee Scientific, San Diego, CA) containing, 10% fetal bovine serum (**FBS**; Cat# 1500-500; Seradigm, Radnor, PA), penicillin/streptomycin (10 mL/L, Cat# 97063-708; Amresco, Solon, OH), and Fungizone® Antimycotic (3 µL/mL, Cat# 15290-018; Life Technologies, Grand Island, NY). Cultures were maintained in a water-jacketed incubator (Cascade Scientifics, Portland, OR) with 5% CO_2 in air at 37°C. Culture medium was changed every 48 h and cells were sub-cultured to 70 to 80% confluence (approximately every 3 to 4 d) by rinsing once with 10 mL of PBS buffer (cat#25-508P, Genesee Scientific), adding 3 mL of 0.25% trypsin (cat#25-510, Genesee, Scientific), and incubated at 37°C for 6 to 10 min (i.e., until evidence of cell detachment). Trypsin activity was inhibited by addition of 6 mL of fresh culture media.

Suppl. Table 1. Gene symbol, description, accession numbers, primers and amplicon size of genes measured

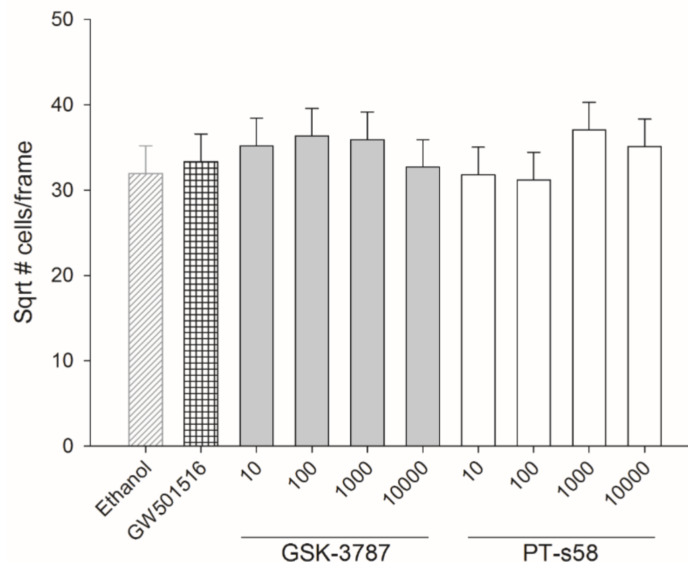
symbol	Name	NCBI Accession	Primers (3' →5')[1]	Size[2]	Ref
B2M	Beta-2-Microglobulin	NM173893.3	F - TCCAGCGTCCTCCAAAGATT R - CCCATACACATAGCAGTTCAGGTAA	65	(Kadegowda *et al.*, 2009)
CALR	Calreticulin	NM_174000.2	F - TTTGACAACTTCCTCATCACCAA R - TTGCTTTTCTGCTGCCTTTGTAA	90	This paper
EIF3K	Eukaryotic Initiation Factor 3K	NM_001034489.2	F - CCAGGCCCACCAAGAAGAA R - TTATACCTTCCAGGAGGTCCATGT	125	(Kadegowda *et al.*, 2009)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_001012668.1	F - TTGTCTCCTGCGACTTCAACA R - TCGTACCAGGAAATGAGCTTGAC	103	(Kadegowda *et al.*, 2009)
HK1	Hexokinase 1	NM_001012668.1	F - GCAGGTGAATCACGAGCAGAA R - TGATCAAAAAGCTGGCTTCCA	102	(Hosseini *et al.*, 2013)
LALBA	Lactalbumin	NM_174378.2	F - GAATTAACTACTGGTTGGCCCATAA R - CAGAAAGAGGACAGAAGCAGCAA	110	(Hosseini *et al.*, 2013)
MTG1	Mitochondrial Ribosome Associated GTPase 1	NM_001025327.2	F - GATCTGAAGGAGCAGCAGAAAATT R - GTTGGGATGACCTGCTTGACA	110	(Kadegowda *et al.*, 2009)
PDK4	Pyruvate Dehydrogenase Kinase 4	NM_001101883.1	F - ATGTTCCATCTCACCTTCACCAT R - AACTGTGGCCCTCATTGCAT	65	(Akbar *et al.*, 2013)
PPARD	Peroxisome Proliferator Activated Receptor Delta	NM_001083636.1	F - TGTGGCAGCCTCAATATGGA R - GACGGAAGAAGCCCTTGCA	100	(Moyes *et al.*, 2014)
RPS15A	Ribosomal Protein S15a	NM_001037443.2	F - TGCCGAAAAGAGAGGCAAAC R - TTCGCCAATGTAACCATGCTT	100	This paper
SLC2A1	Solute Carrier Family 2 Member 1	NM_174602.2	F - CCCCCAGAAGGTGATTGAAG R - GAACCAATCATGCCTCCCAC	135	(Bionaz & Loor, 2011)
SLC2A8	Solute Carrier Family 2 Member 8	NM_174602.2	F - CCCCCAGAAGGTGATTGAAG R - GAACCAATCATGCCTCCCAC	135	(Bionaz & Loor, 2011)
UXT	Ubiquitously Expressed Prefoldin Like Chaperone	NM_001037471.2	F - CAGCTGGCCAAATACCTTCAA R - GTGTCTGGGACCACTGTGTCAA	125	(Kadegowda *et al.*, 2009)

[1]Underline is the exon-exon junction

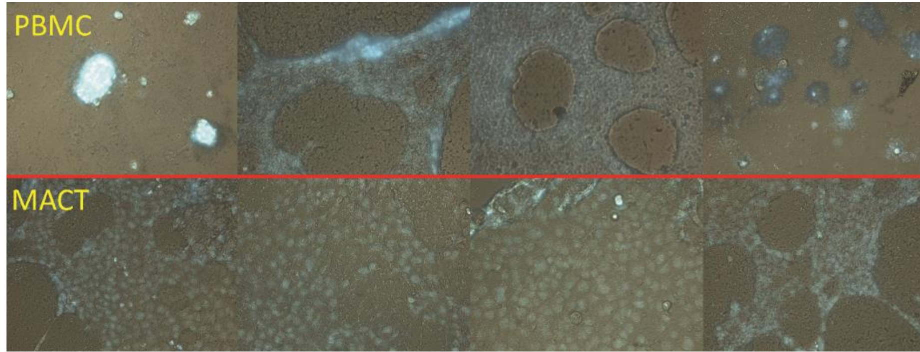
[2]Size of the amplicon in base pair



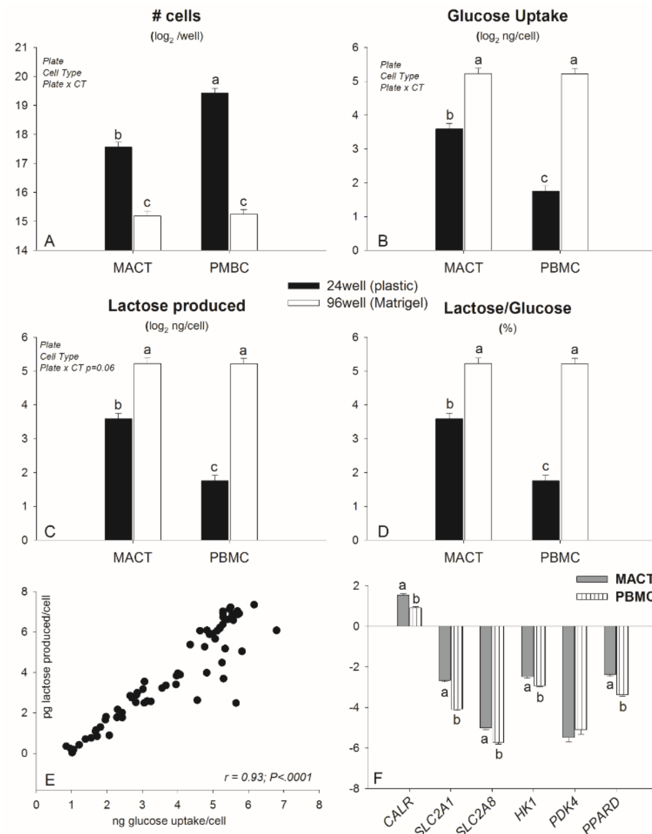
Suppl. Figure 1. ***Hypothetical model.*** In bovine endothelial cells it was observed that activation of PPARβ/δ by peroxides as product of excess glucose increased expression of PPARβ/δ down-stream target calreticulin that, in turn, degrades the transcript of glucose transporter 1 (GLUT1) (Riahi *et al.*, 2010). As a consequence, there is less uptake of glucose by the cells preventing toxicity. Based on the above observation, we hypothesized that an inhibition of PPARβ/δ would decrease the expression of calreticulin and, as a consequence, the amount of GLUT1 would increase in cells stimulating glucose uptake and lactose synthesis.



Suppl. Figure 2. Number of cells/frame counted using NucBlue™ Live ReadyProbes™ Reagent staining in combination with CellProfiler. The treatments were as described for Figure 2A. There was not effect of the treatment (P=0.92).



Suppl. Figure 3. PBMC tended to form tri-dimensional structures that can resemble alveoli, but this events were overall rare. We did not observe any tri-dimensional structure formation with MACT. Cells were stained with NucBlue (Life Technologies) and fluorescent image was overlaid with bright field at 20× magnification.



Suppl. Figure 4. ***Differences in cell number/well, glucose uptake, lactose synthesis, and transcription of glucose-related genes between MACT and PBMC and between plastic or Matrigel culture.*** Reported are the differences between MACT and PBMC cells either cultivated in plastic or Matrigel on the uptake of glucose and production of lactose (A-D). The correlation between glucose uptake and lactose produced is also reported (E). The difference in expression of the target genes between MACT and PBMC cells measured in the present work is also reported (F). Letters denote difference with a $P \leq 0.05$.

# cells	Lactose	% lactose	*HK1*	*LALBA*	*PDK4*	*PPARD*	*SLC2A8*	*CALR*	*SLC2A1*	
-0.48 0.002	0.99 <.0001	-0.12 0.490	0.27 0.086	0.14 0.566	-0.17 0.306	0.58 <.0001	0.52 0.001	0.48 0.002	0.27 0.086	Glucoseuptake
	-0.56 0.000	-0.39 0.017	-0.56 0.000	-0.33 0.163	-0.02 0.883	-0.65 <.0001	-0.59 <.0001	-0.63 <.0001	-0.71 <.0001	# cells
		0.03 0.846	0.36 0.024	0.05 0.851	-0.18 0.286	0.63 <.0001	0.58 0.000	0.57 0.000	0.41 0.010	Lactose
			0.38 0.019	-0.41 0.079	-0.13 0.437	0.18 0.285	0.22 0.180	0.41 0.010	0.44 0.005	Lactose/Glucose
				-0.04 0.876	0.01 0.951	0.72 <.0001	0.64 <.0001	0.76 <.0001	0.70 <.0001	*HK1*
					-0.07 0.756	0.55 0.011	0.46 0.039	0.31 0.179	0.14 0.552	*LALBA*
						-0.08 0.629	-0.20 0.212	-0.20 0.212	-0.10 0.550	*PDK4*
							0.92 <.0001	0.90 <.0001	0.76 <.0001	*PPARD*
								0.90 <.0001	0.72 <.0001	*SLC2A8*
									0.79 <.0001	*CALR*

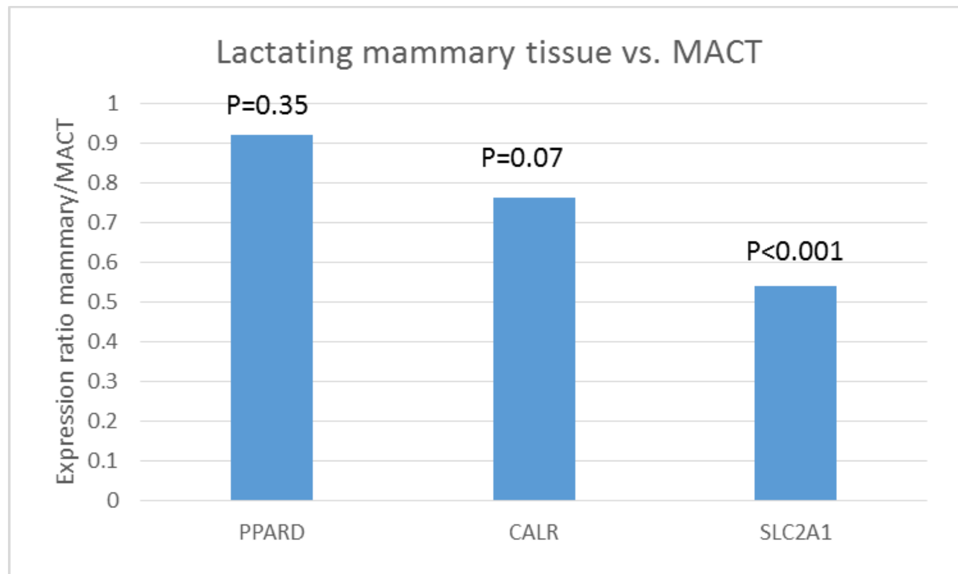
Suppl. Figure 5. Correlation analysis between number of cells, glucose uptake/cell, lactose produced/cell, % of glucose used for lactose synthesis (i.e., % lactose), and expression of glucose metabolism-related genes in MACT and PBMC cultivated in 24-well plastic dishes. Reported are in each cell on the top the Pearson product-moment correlation coefficient (r) and on the bottom the significance of the correlation. Correlation with a P<0.01 are highlighted in red.

The lactose production was highly positively correlated with glucose import (r = 0.99; P < 0.0001). When the analysis was performed including the cells cultivated in Matrigel, the correlation was still very positive (r=0.93; P<0.0001; Figure 5E), confirming prior data (Lin *et al.*, 2016) and corroborating our underline assumption that higher glucose uptake would increase lactose synthesis. Surprisingly, expression of *LALBA* was not correlated (r=0.05; P=0.85) with lactose synthesis, contrasting with prior reviewed data (Bionaz *et al.*, 2012); however, the expression was very low in MACT cells and determined to be undetectable in PBMC (Figure 3). Among the measured transcripts, the highest correlation with lactose production/cell and glucose import/cell was detected for *PPARD* (r=0.58; P< 0.001), followed by *SLC2A8* (r=0.52; P< 0.01), and *CALR* (r=0.48; P<0.002). A lower correlation (r=0.27; P ≤0.09) was detected for lactose with *SLC2A1*. However, this was the only transcript with a significant positive correlation with the %lactose produced/glucose uptake. A high positive correlation (r=0.76; P< 0.001) was detected for *PPARD* with the transcripts coding for the two measured glucose transporters and *CALR*. Our data also indicated a large positive correlation (r=0.79; P< 0.001) between the transcript abundance of *CALR* and the transcript abundance of *SLC2A1*.

Limitations of the present study

Our experiment presents several limitations that could have compromised a full demonstration of the original hypothesis:

1) We were able to detect a significant and somewhat consistent inhibition of PPARβ/δ by 1000 nM of GSK-3787 using a gene reporter assay; however, the inhibition of PPARβ/δ did not consistently translate in a down-regulation of *PDK4*, as should have been expected. This might indicates a less than effective dose or effect of the used antagonist. However, in the present paper we assumed that GSK-3787 is a specific inhibitor of PPARβ/δ without affecting the other PPAR isotypes. It is not possible to date to demonstrate that this antagonist is PPARβ/δ-specific in bovine using the gene reporter assay because the reporter assay used cannot distinguish between the three PPAR isotypes and a better method should be developed, as previously proposed (Bionaz *et al.*, 2015).
2) The use of immortalized cells such as MACT can be a limitation. Prior data clearly indicated that the MACT cells are not a good model to study bovine mammary biology (Hosseini *et al.*, 2013). However, the expression of PPARD is not different compared to bovine mammary tissue but the expression of CARL and SLC2A1 is lower, especially for the latter (**Suppl. Figure 6**) (Hosseini *et al.*, 2013) This was the main reason in the present study for the use of PBMC, another bovine mammary cells line. The PBMC cells are not immortalized and were previously partly characterized and indicated as good model to study bovine mammary biology (Hu *et al.*, 2009). Our data indicated instead that the PBMC are likely less indicated in the study of mammary biology compared to MACT cells, especially if lactose synthesis is studied considering the undetectable expression of *LALBA*. Furthermore, the PBMC had lower expression compared to MACT cells of most of the genes involved in glucose metabolism, including *SLC2A1*. The PBMC cells also have a lower expression of *PPARD* compared to MACT cells that might have translated in a lower response to the PPARβ/δ modulators. Therefore, it is not possible to exclude that the lack of observed effects was due to the use of less than adequate cellular models.



Suppl. Figure 6. Ratio of mRNA abundance between lactating mammary tissue and MACT cells.

3) The lack of formation of alveoli-like structure was also a limitation of the present study. It is possible that the short time used in the present experiment (i.e., 1 week) was not long enough to allow the cells to fully organize in a tri-dimensional structure. Prior data showed a full formation of alveoli-like structure in bovine mammary cells when cultivated for 16 days in Matrigel; however, tri-dimensional structure was started to be observed at 6 day (Kozlowski *et al.*, 2009). The PBMC tended to form tri-dimensional structure, but they were far from having a defined morphology as previously observed (Kozlowski *et al.*, 2009). It is unclear the reason for the lack of tri-dimensional structure formation, especially in MACT cells, but this has likely impaired a large production of lactose and, therefore, the sensitivity of a possible response of lactose synthesis to the treatments. We did not measure the expression of milk-related genes in cells cultivated in Matrigel; however, the formation of tri-dimensional structures in PBMC compared to MACT in Matrigel might have increased the expression of milk-related genes. This is partly supported by the same lactose production between the two cells when cultivated in Matrigel whereas the PBMC produced less lactose compared to MACT when cultivated in plastic.
4) Another possible limitation of the study was the use of a medium containing high glucose. As previously discussed (Osorio & Bionaz, 2017) the high glucose media is likely providing a condition far from the physiological condition of the animals. However, as argued above, the use of high-glucose media in the present experiment was similar to the amount of glucose used for the experiment in bovine endothelial cells (Riahi *et al.*, 2010).

Therefore, in order to fully disprove our hypothesis further experiments should be performed. Prior to performing the experiment preliminary data should be generated to determine a more effective and consistent PPARβ/δ inhibition compared to the present study and physiological level of glucose in the media and primary bovine MEC that can form alveoli-like structure should be used.

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