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MicroRNA expression profiling of porcine mammary epithelial cells after challenge with *Escherichia coli* in vitro

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Abstract

Background: Coliform mastitis is a symptom of postpartum dysgalactia syndrome (PDS), a multifactorial infectious disease of sows. Our previous study showed gene expression profile change after bacterial challenge of porcine mammary epithelial cells (PMECs). These mRNA expression changes may be regulated through microRNAs (miRNAs) which play critical roles in biological processes. Therefore, miRNA expression profile was investigated in PMECs.

Results: PMECs were isolated from three lactating sows and challenged with heat-inactivated potential mastitis-causing pathogen *Escherichia coli* (*E. coli*) for 3 h and 24 h, in vitro. At 3 h post-challenge with *E. coli*, target gene prediction identified a critical role of miRNAs in regulation of host immune responses and homeostasis of PMECs mediated by affecting pathways including cytokine binding (miR-202, miR-3277, miR-4903); IL-10/PPAR signaling (miR-3277, miR-4317, miR-548); and NF-κB/TNFR2 signaling (miR-202, miR-2262, miR-885-3p). Target genes of miRNAs in PMECs at 24 h were significantly enriched in pathways associated with interferon signaling (miR-210, miR-23a, miR-1736) and protein ubiquitination (miR-125, miR-128, miR-1280).

Conclusions: This study provides first large-scale miRNA expression profiles and their predicted target genes in PMECs after contact with a potential mastitis-causing *E. coli* strain. Both, highly conserved miRNAs known from other species as well as novel miRNAs were identified in PMECs, representing candidate predictive biomarkers for PDS. Time-dependent pathogen clearance suggests an important role of PMECs in inflammatory response of the first cellular barrier of the porcine mammary gland.

Keywords: Coliform mastitis, Immune response, Mammary epithelial cells, microRNA, Target genes

Background

Coliform mastitis (CM) represents one of the most important cardinal symptoms of postpartum dysgalactia syndrome (PDS) in sows [1]. This multifactorial infectious disease has high economic relevance for the pig industry, because both postpartum sows and their piglets can be critically affected by mastitis and lactation failure [2]. Husbandry and management conditions, pathogen contamination, and genetic predisposition are suspected etiological factors that contribute to PDS [3]. Gram-negative pathogens such as *Escherichia coli* (*E. coli*) are most commonly isolated from milk of PDS-positive sows, but are found in non-affected sows as well [4, 5]. Therefore, it is

unclear which determinants result in development of subclinical or clinical signs of infection within 12–48 h postpartum, or otherwise preserve clinical health.

Lipopolysaccharide (LPS) endotoxin is a pathogenic factor of *E. coli* and can induce acute and severe inflammation in sows and other animal species [6]. Our previous study showed that *E. coli* induce a fast and strong inflammatory response in porcine mammary epithelial cells (PMECs) [7]. In these cells, bacterial challenge strongly upregulates mRNA expression of genes encoding cytokines, chemokines, and cell adhesion molecules. Furthermore, these changes suggest rapid activation of other immune-competent cells mediating pathogen clearance and host homeostasis through interleukin 1 beta (IL1B) and tumor necrosis factor (TNF) expression [7]. Therefore, cellular as well as molecular factors may

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be highly relevant to animals' susceptibility to intramammary infection and mastitis.

MiRNAs are small non-coding RNA molecules (~22 nucleotides long) and are highly conserved across higher eukaryotic species and can modulate host immune responses [8] as well as are involved in a variety of signaling pathways and biological processes, such as development, cell proliferation, cell differentiation, cell death, metabolism, and disease [9].

In epithelial cells, miRNAs can modulate production and release of cytokines/chemokines and expression of adhesion and costimulatory molecules [10]. According to different microbe-associated molecular patterns (MAMPs) from bacteria, innate and adaptive host immune responses are activated through stimulation of pattern recognition receptors (PRRs) [11]. In bovine mammary epithelial cells, *E. coli* affect expression of miR-184, miR-24-3p, miR-148, miR-486, and let-7a-5p, which target genes involved in developmental/cellular processes, biological regulation, cell growth, and death [12]. Furthermore, miR-142-5p, miR-223, miR-2898, miR-16, and miR-181a are potential markers for mastitis in cattle [12–16].

In contrast, the function of miRNAs in innate and adaptive immunity of porcine mammary glands has not yet been described. Therefore, our study focused on miRNA expression changes in PMECs induced by a potential mastitis-causing *E. coli* strain isolated from milk of PDS-positive sows. We characterized miRNA expression differences in PMECs at 3 h and 24 h after challenge with heat-inactivated *E. coli* to investigate and to compare early and late phase of immune response since specific kinetics and extents of global changes in the transcriptome of bovine mammary epithelial cells were found in a study with comparable experimental design [17]. The response of PMECs was determined by comparing miRNA expression profiles between pathogen-challenged and unchallenged control groups. Together with our previous mRNA expression profiles, miRNA expression data were then integrated based on pairwise correlations and computational target predictions to identify potentially affected signaling pathways. Finally, we performed selective analysis of the most and strongest affected biological processes, molecular functions, and Kyoto Encyclopedia of Genes and Genomes (KEGG)/ canonical pathways to investigate the role of miRNAs in pathogen clearance of PMECs.
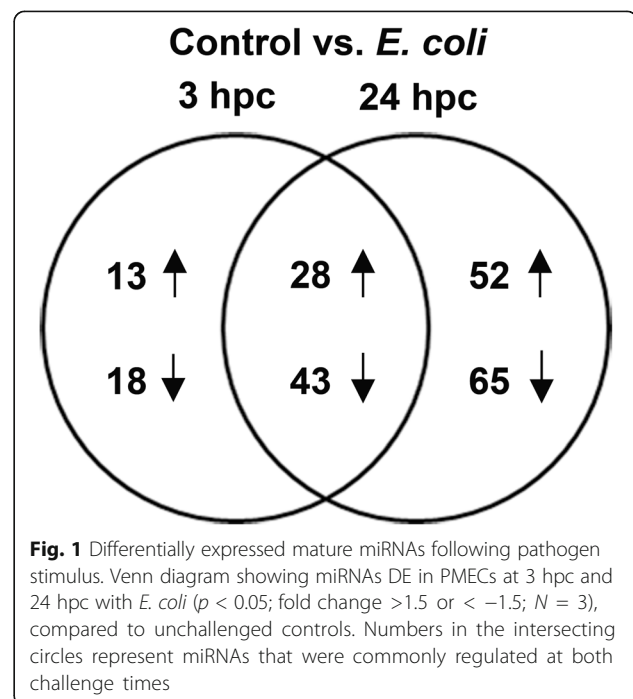
Results
Effects of *E. coli* challenge on miRNA expression in PMECs
MiRNA expression profiling was performed at 3 h post-challenge (hpc) and 24 hpc of PMECs with *E. coli* in comparison to unchallenged control cells using Affymetrix GeneChip miRNA 3.0 arrays. After quality control

and filtering of raw data, 3277 probesets corresponding to 1332 mature miRNA sequences were further analyzed. Top10 most highly expressed miRNAs (normalized log2 signal intensity >13) in unchallenged PMECs are miR-23a, miR-24, miR-24a, miR-24-3p, miR-27a, miR-31, miR-31b, miR-221, miR-222, and miR-222a. Compared with other published results in the same cell type, some miRNAs which are highly expressed in pig, were also found as highly expressed in cattle and mice, e.g. miR-26a, miR-205, miR-221, and miR-222 [12, 18, 19]. Furthermore, miR-21, miR-22-3p, miR-27b, miR-27a-3p, miR-31, miR-92a, miR-182, and miR-191 are highly expressed in porcine as well as bovine mammary epithelial cells [12, 18].

Statistical analysis identified 143 significantly differentially expressed (DE) probesets ($p < 0.05$; fold change >1.5 or < −1.5) corresponding to 102 mature miRNA sequences (41 up- and 61 downregulated) at 3 hpc with *E. coli* when compared with unchallenged controls (Fig. 1; Additional file 1). At 24 hpc with *E. coli*, 299 significantly DE probesets corresponding to 188 mature miRNA sequences (80 up- and 108 downregulated) were identified as compared to unchallenged controls (Fig. 1; Additional file 1). Approximately 70% of significantly DE miRNAs at 3 hpc with *E. coli* (71 mature miRNA sequences; 28 up- and 43 downregulated) also differed at 24 hpc with *E. coli* (Fig. 1; Additional file 1). In addition, 31 mature miRNA sequences (13 up- and 18 downregulated) were significantly DE only at 3 hpc with *E. coli*. In contrast, 117 mature miRNA sequences (52 up- and 65 downregulated) were significantly DE only at 24 hpc


Fig. 1 Differentially expressed mature miRNAs following pathogen stimulus. Venn diagram showing miRNAs DE in PMECs at 3 hpc and 24 hpc with *E. coli* ($p < 0.05$; fold change >1.5 or < −1.5; $N = 3$), compared to unchallenged controls. Numbers in the intersecting circles represent miRNAs that were commonly regulated at both challenge times

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with *E. coli* (Fig. 1; Additional file 1). Some of these miR-NAs are not yet annotated in *Sus scrofa*. As concern to this point, we compared these miRNA sequences with *Sus scrofa* miRNA in miRBase (http://www.mirbase.org) and alignment with pig genome (*Sus scrofa* 10.2 download from NCBI: http://www.ncbi.nlm.nih.gov/ on 1.9.2015) and added in Additional file 1 in the column of pig chromosome and position. On the basis of the top 100 significantly DE miRNAs after pathogen challenge, a heat map was generated to identify distinguishable miRNA expression profiles between short-term (3 hpc) and long-term (24 hpc) challenge with *E. coli* compared to unchallenged controls (0 hpc) (Fig. 2). Hierarchical clustering analysis determined similar patterns in miRNA expression profiles of both pathogen-challenged groups compared to unchallenged control group (Fig. 2).

Prediction of miRNA targets identifies biological processes and molecular functions that differ at 3 hpc and 24 hpc with *E. coli*

Pairwise correlation coefficient analysis was performed to evaluate associations of expression levels between 442 significantly DE miRNA probesets and 1345 significantly DE mRNA probesets (previously identified by Jaeger et al. [7]). In total, 36,610 miRNA–mRNA pairs were significantly negatively correlated ($p < 0.05$) and in silico predicted as functionally linked. TargetScan and RNAhybrid software were used to further filter predicted miRNA–mRNA pairs by setting the threshold for microRNA:target duplex energy to −25 kcal/mol. Finally, we identified 135 miRNA–mRNA pairs corresponding to 34 mature miRNA sequences and 71 genes at 3 hpc with *E. coli* (Table 1; Additional file 2). At 24 hpc with *E. coli*, 531 miRNA–mRNA pairs corresponding to 67 mature miRNA sequences and 214 genes were filtered (Table 2; Additional file 3).

Gene ontology (GO) analysis identified biological processes and molecular functions predicted for target genes of DE miRNAs. Predicted target genes of up- and downregulated miRNAs in PMECs at 3 hpc with *E. coli* were significantly associated with negative regulation of biosynthetic processes, metabolic processes, and transcription (*ARID5B*, *BCOR*, *BMP2*, *CDC6*, *GLI3*, *HELLS*, *ID4*, *ITGAV*, *PPARG*, and *RNF2*); lipid storage (*ITGAV*, *NFKBIA*, and *PPARG*); cell differentiation (*GLI3*, *ID4*, *ITGAV*, *JAG1*, *NFKBIA*, and *PPARG*); and regeneration (*JAK2*, *LIFR*, *PLAUR*, and *PPARG*) (Fig. 3a; Additional file 4). Most genes were predicted targets of predominantly miR-101, miR-125a-3p, miR-202, miR-2262, miR-29a, miR-29b, miR-30b-star, and miR-3277 (Additional file 4).
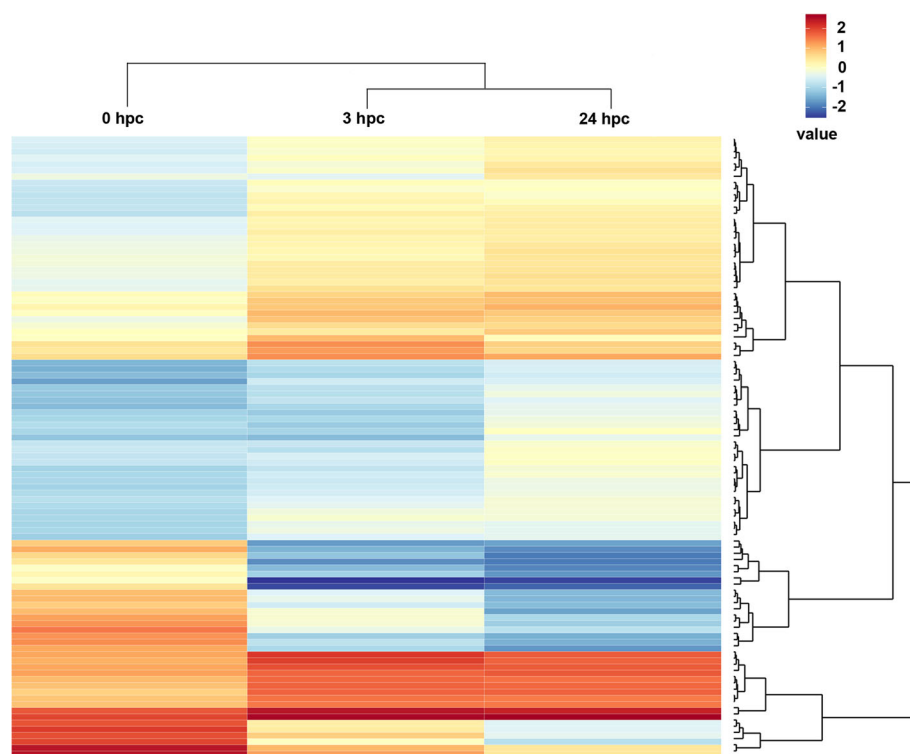
Fig. 2 Heat map and hierarchical clustering analysis of the top 100 DE miRNA probesets. Heat map based on processed array signals (Lsmean values log2-transformed; $p < 0.05$; fold change >1.5 or < −1.5) from PMECs at 3 hpc and 24 hpc with *E. coli*, compared to unchallenged controls (0 hpc). Red indicates higher relative expression and blue indicates lower relative expression. Hierarchical clustering dendrogram (ward.D2 method) reveals a distinguishable miRNA expression profile among samples

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Table 1 Top 30 significantly DE miRNAs and their predicted target genes from microarray analysis in PMECs at 3 hpc with *E. coli* compared with unchallenged controls

3 hpc with *E. coli*		
Upregulated miRNA	Sequence (5′ - > 3′)	Downregulated target genes
miR-29b	UAGCACCAUUUGAAAUCAGUGUU	BCOR, KLHDC7A
miR-140	CAGUGGUUUUACCCUAUGGUAG	RAB40B
miR-210	UUGUGCGUGCGACAGCGACUUC	RAB40B
miR-101	UACAGUACUGUGAUAACUGAAG	EML5, HELLS, RBM12
miR-29a	UAGCACCAUUUGAAAUCGGUU	CDC6, CYP26A1, DNAJA1, EML5, RNF2, TIMM44
miR-24-star	GUGCCUACUGAGCUGAUAUCAGU	USP46
miR-30b-star	CUGGGAGGUGGAUGUUUACUUC	BCOR
miR-B6	AAGUGCCCGACGCGGGGAACGUG	ENC1
let-7 g-star	CUGUACAGGCCACUGCCUUGC	DUSP6
miR-125a-3p	ACAGGUGAGGUUCUUGGGAGCC	ARID5B, ID4, ITGB8, KIAA0182, NUAK1, PHF17, RAB40B, TOX, USP2, VGLL3, WDR35
Downregulated miRNA	Sequence (5′ - > 3′)	Upregulated target genes
miR-371	ACUCAAAAAAUGGCGGCACUUU	SGSM1
miR-548u	CAAAGACUGCAAUUACUUUUGCG	NFKBIA
miR-5128	CAAUUGGGGCUGGCGAGAUGGCU	SGSM1
miR-8-5p	CAUCUUACCGGGCAGCAUUAGA	ARHGAP28, HIVEP2, IFNAR2
miR-259-star	CCACCGAUUUGGCAUGGGAUUGAC	SGSM1
miR-3277	UGGGCAUGUUUCUGAAAUUCGAU	ARHGAP28, IFNAR2, IL1A, PPARG
miR-4903	UACCCCGGUAGGCAUAGGUUG	GLRX, IL1RAP, MAP3K8, PLAUR
miR-202	AGAGGCAUAGGGCAUGGGAAAA	ARHGAP28, CHL1, GLI3, HIVEP2, IFNAR2, JAG1, LIFR, MAP3K8, OSBPL3, P2RX4, RFFL, RMND5A, RNF19B, TEP1, TNFAIP3, TPM2, VPS37C, YPEL2
miR-5100	UCGAAUCCCAGCGGUGCCUCU	OBFC1
miR-2287	CUGGGACUGGUGGCAGCACUU	CLDN1, ITGAV, RNF19B
miR3949	UGAUGUUGAGGCAAAAAUGUAG	CCDC152, RBM39, RFX1
miR-885-3p	AGGCAGCGGGGUGUAGUGGAUA	BIRC3, DCUN1D3, HIVEP2, IL10RB, MAP3K8, TNFAIP3
miR-2518	UCGAGCAGCGGGUCGAUCCGAGC	CYP1A1, JAG1

Table 1 Top 30 significantly DE miRNAs and their predicted target genes from microarray analysis in PMECs at 3 hpc with *E. coli* compared with unchallenged controls *(Continued)*

miR-3042	GAGGGCAGAUUAUUUCUGAUAC	JAG1, LPIN1, YPEL2
miR-4031-5p	CCCAAAGUGUCGGCGCAUAU	SGSM1, TNFAIP3
miR-4317	ACAUUGCCAGGGGAGUUU	NFKBIA
miR-216b	AAAUCUCUGCAGGCAAAUGUGA	ETV5, PDLIM5
miR-890	UACUUGGAAAGGCAUCAGUUG	EHF, FCHSD2, IL10RB, PLXND1, SGSM1, SLC4A7, TAP1
miR394b-star	AGGUGGACAUAUUGCCAACA	ITGAV, JAG1
miR-648	AAGUGUGCAGGGCACUGAU	HIVEP2, IFNAR2, JAG1, SGSM1

MiRNAs with $p < 0.05$ and fold change >1.5 or < −1.5 are listed and sorted by fold change in descending order. TargetScan-predicted miRNA-mRNA pairs showing negative correlation in expression profile and energy-value of at least −25 kcal/mol

Molecular functions influenced by predicted target genes of up- and downregulated miRNAs in PMECs at 3 hpc with *E. coli* included cytokine receptor activity and cytokine binding (*IFNAR2*, *IL10RB*, *IL1RAP*, and *LIFR*); transcription repressor activity (*ARID5B*, *BCOR*, *BMP2*, *ID4*, *PPARG*, and *RNF2*); chromatin binding (*CDC6*, *GLI3*, *HELLS*, and *RNF2*); and heat shock protein binding (*BCOR*, *DNAJA1*, and *NFKBIA*) (Fig. 3b; Additional file 4). Most genes were predicted targets of predominantly miR-29a, miR-202, and miR-3277 (Additional file 4).

miRNAs that were up- and downregulated in PMECs at 24 hpc with *E. coli* were negatively correlated with target genes that were also involved in negative regulation of metabolic processes, transcription, gene expression, biosynthetic processes, and regulation of transcription from RNA polymerase II promoter (e. g., *ATF7IP*, *BCOR*, *BMP2*, *HEXIM1*, *ID3*, *MDM4*, *PPARG*, *RBM15*, *RNF2*, *SP100*, *TLE1*, and *TRIM27*) (Fig. 4a; Additional file 4). Most genes were predicted targets of predominantly miR-10a, miR-1934-star, miR-23a-star, miR-263a-star, miR-289, miR-29a, miR-30b-star, miR-3216, miR-3277, miR-34c, miR-363, miR-4592, miR-660, miR-92b, miR-M9-3p, miR-rL1−34-5p, and miR894 (Additional file 4).

Molecular functions influenced by predicted target genes of up- and downregulated miRNAs in PMECs at 24 hpc with *E. coli* included transcription repressor activity (e. g., *ARID5B*, *ATF7IP*, *ATXN1*, *BCLAF1*, *BMP2*, *CREBZF*, *HEXIM1*, *PPARG*, *SP100*, and *TLE1*); binding of nucleotides, ribonucleotides, purine ribonucleotides, purine nucleotides, adenyl ribonucleotides, and ATP (e. g., *ABCA7*, *AKAP9*, *CAMKK2*, *CHD7*, *CLK1*, *FGFR2*, *FGFR3*, *IGF1R*, *TOP1*, and *UBE2R2*); RNA binding (e. g., *ATXN1*, *DDX18*, *DGCR8*, *EIF4E*, *LARP4*, *NOL6*, *OBFC2A*, *RBM41*, *RBM5*, and *RPUSD4*); chromatin binding (*CDC6*,

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Table 2 Top 30 significantly DE miRNAs and their predicted target genes from microarray analysis in PMECs at 24 hpc with *E. coli* compared with unchallenged controls

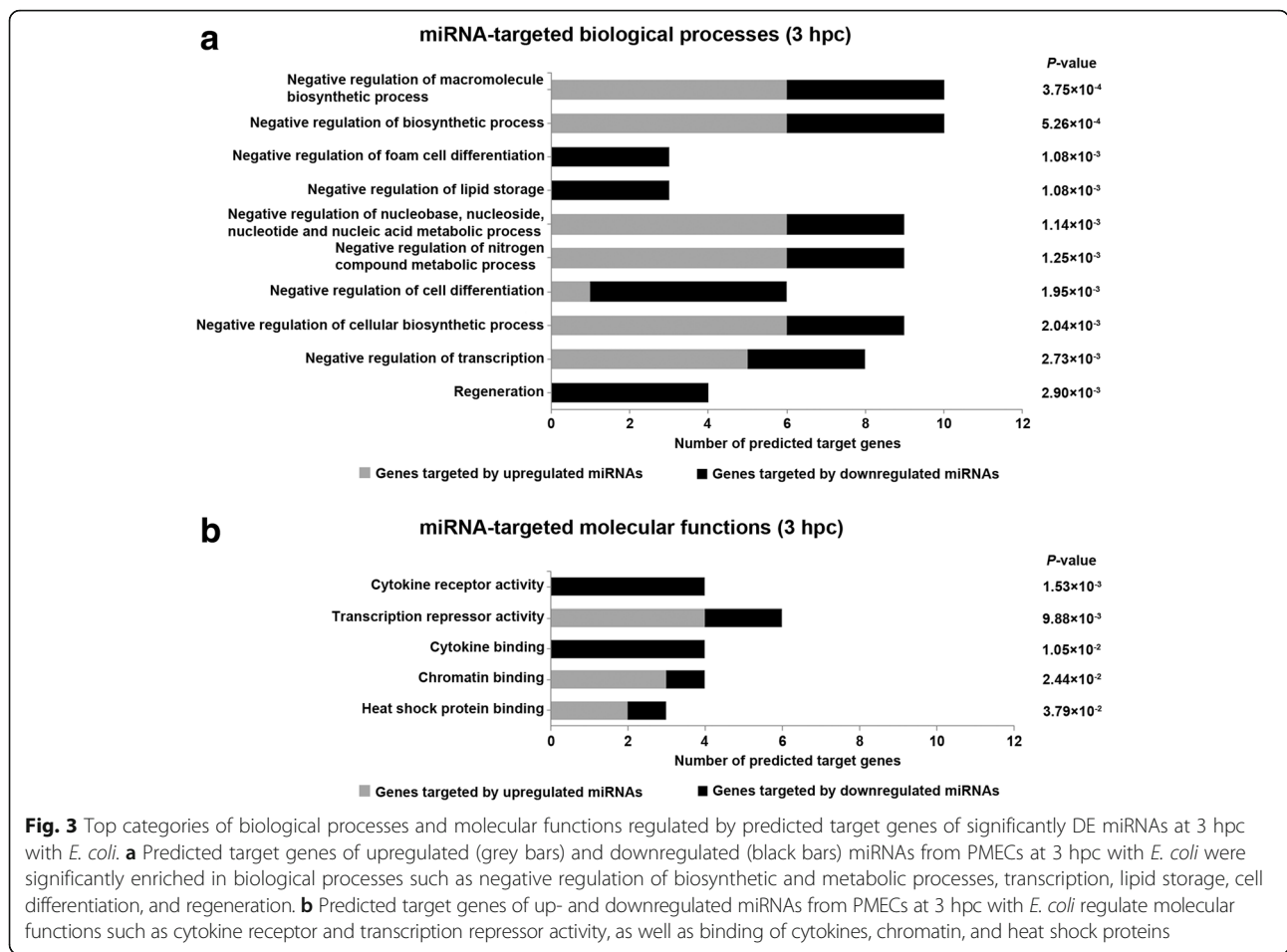| 24 hpc with *E. coli*		
Upregulated miRNA	Sequence (5′ -> 3′)	Downregulated target genes
miR-505	CGUCAACACUUGCUGGUUUCCU	ABCA7, DDX18, DDX52, EIF4E, GRPEL1, LARP4, MSL1, PLEKHH1, SDC1, SERTAD1, SOCS4, TATDN2, WDR3, YTHDF2, ZBED4
miR-29a	UAGCACCAUUUGAAAUCGGUU	CDC6, CYP26A1, DNAJA1, EML5, RNF2, TIMM44
miR-101	UACAGUACUGUGAUAACUGAAG	CHD7, EML5, HELLS, LARS, MID1, RBM12
miR-24-star	GUGCCUACUGAGCUGAUAUCAGU	USP46
miR-289	UAAAUAUUUAAGUGGAGCCUGCGACU	KIAA1430, RNF2, TMCC3
miR-125-star	ACGGGUUAGGUUCUUGGGAGC	GIT2, LARS, MSL1, PAFAH1B1, RBM12, SDC1, TMEM79, UBE2R2, ULK3, USP46
miR-484	UCAGGCUCAGUCCCCUCCCGAU	FGFR3
miR-22-star	AGUUCUUCAGUGGCAAGCUUUA	ARGLU1
miR-660	UACCCAUUGCAUAUCGGAGCUG	ARMC8, BCLAF1, CAMKK2, CPT1A, DDX42, MSL1, NOL6, NRARP, PLEKHH1, PPP1R8, PPP6C, PSMD12, PSMF1, PYGO2, RRP12, SDC1, TRIM27, ZFX, ZNF280D
miR-B6	AAGUGCCCGACGCGGGGAACGUG	ENC1
miR-363	AAUUGCACGGUAUCCAUCUGUA	ABCA7, ADPRH, ATXN1, CAMKK2, DGCR8, FAM53C, GABPB1, GATAD2A, HERPUD2, PAFAH1B1, PLEKHH1, POP4, PPRC1, RBM15, S1PR2, SYK, TATDN2, TFAP2C, TRIM35, TXLNA
miR-128	UCACAGUGAACCGGUCUCUUUU	ADPRH, AQP3, GABPB1, KCNJ14, NDNL2, PLEKHH1, RRP12, SDC1, SNX6, TCEB3, UBE2R2, ULK3, ZFAND2A, ZFX, ZNF764
miR-10a	UACCCUGUAGAUCCGAAUUUGUG	ADPRHL2, EML5, FARSA, FEN1, IQCC, KLHL18, LMNB2, MFSD9, SFN, SLC16A3, SLC39A6, TATDN2, TLE1, TMCC3, TUBB2B, TXLNA, UBAP2L, ZNF367

Table 2 Top 30 significantly DE miRNAs and their predicted target genes from microarray analysis in PMECs at 24 hpc with *E. coli* compared with unchallenged controls *(Continued)*

miR-92b	AAUUGCACUAGUCCCGGCCUGC	CPT1A, GPRC5A, HEXIM1, LIN37, PHF23, PKP1, ULK3, YWHAG
miR-92b	UAUUGCACUCGUCCCGGCCUCC	EML5
Downregulated miRNA	Sequence (5′ -> 3′)	Upregulated target genes
miR-371	ACUCAAAAAAUGGCGGCACUUU	SGSM1
miR-4423-3p	AUAGGCACCAAAAAGCAACAA	RASAL2
miR5019	UGUUGGGAAAGAAAAACUCUU	RASAL2
miR-3128	UCUGGCAAGUAAAAAACUCUCAU	SLC2A12
miR5021	UGAGAAGAAGAAGAAGAAAA	CIRBP
miR-M9-3p	AAACUCCGAGGGCAGGAAAAAG	ARHGAP12, ARHGAP28, BIRC3, CEP68, FCHSD2, GRK5, ISG15, LPIN1, MDM4, PCDH7, PDLIM5, SLC2A12, TCTN2, YPEL2
miR3949	UGAUGUUGAGGCAAAAAUGUAG	CCDC152, RBM39, RFX1,
miR-B8-5p	CGCGGGCAAAAAAUCCAAUGGC	BIRC3, MAP3K8, PLXND1, RNF19B
miR-2326	CCCCCCUUCCUCUGGAAAAA	ARHGAP28, CEP68, CHI3L1, CXCL2, FNBP1, LPIN1, PCDH7, PLXND1, PPAP2B, RFFL, RHOQ, SCUBE3, SLC4A7, TPM2
miR-5100	UCGAAUCCCAGCGGUGCCUCU	OBFC1
miR-259-star	CCACCGAUUUGGCAUGGGAUUGAC	SGSM1
miR-2779	AUAUCCGGCUCGAAGGACCA	CLDN1, PLXND1
miR-4592	CCAGCGGCGGUGCCGUGAUGGCGA	CLDN1, CEP68, CXCL2, ITGB8, MDM4, RHOQ
miR-202	AGAGGCAUAGAGCAUGGGAAAA	ARHGAP28, BIRC3, CHL1, GLI3, LIFR, MAP3K8, P2RX4, PLN, RASAL2, RFFL, RMND5A, RNF19B, SGSM1, TEP1, TNFAIP3, TPM2, VPS37C
miR-3277	UGGGCAUGUUUCUGAAAUUCGAU	ARHGAP28, IFNAR2, IL1A, PPARG

MiRNAs with $p < 0.05$ and fold change >1.5 or < -1.5 are listed and sorted by fold change in descending order. TargetScan-predicted miRNA-mRNA pairs showing negative correlation in expression profile and energy-value of at least −25 kcal/mol

CHD7, GLI3, HELLS, RNF2, SF3B1, TLE1, and *TOP1*); and protein domain specific binding (*BMP2, CLDN1, ELMO1, FUT8, ID3, LUC7L, RHOQ, RIPK1, SFN, SP100, SYK,* and *YWHAG*) (Fig. 4b; Additional file 4). Most genes were predicted targets of predominantly miR-10a, miR-1280, miR-1934-star, miR-23a-star, miR-263a-star, miR-363, miR-660, miR-92b, and miR894 (Additional file 4).

The number of target genes predicted for DE miRNAs and involved in the regulation of biological processes or

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Fig. 3 Top categories of biological processes and molecular functions regulated by predicted target genes of significantly DE miRNAs at 3 hpc with *E. coli*. **a** Predicted target genes of upregulated (grey bars) and downregulated (black bars) miRNAs from PMECs at 3 hpc with *E. coli* were significantly enriched in biological processes such as negative regulation of biosynthetic and metabolic processes, transcription, lipid storage, cell differentiation, and regeneration. **b** Predicted target genes of up- and downregulated miRNAs from PMECs at 3 hpc with *E. coli* regulate molecular functions such as cytokine receptor and transcription repressor activity, as well as binding of cytokines, chromatin, and heat shock proteins

molecular functions in PMECs is about two- to tenfold higher at 24 hpc than at 3 hpc with *E. coli*, respectively.
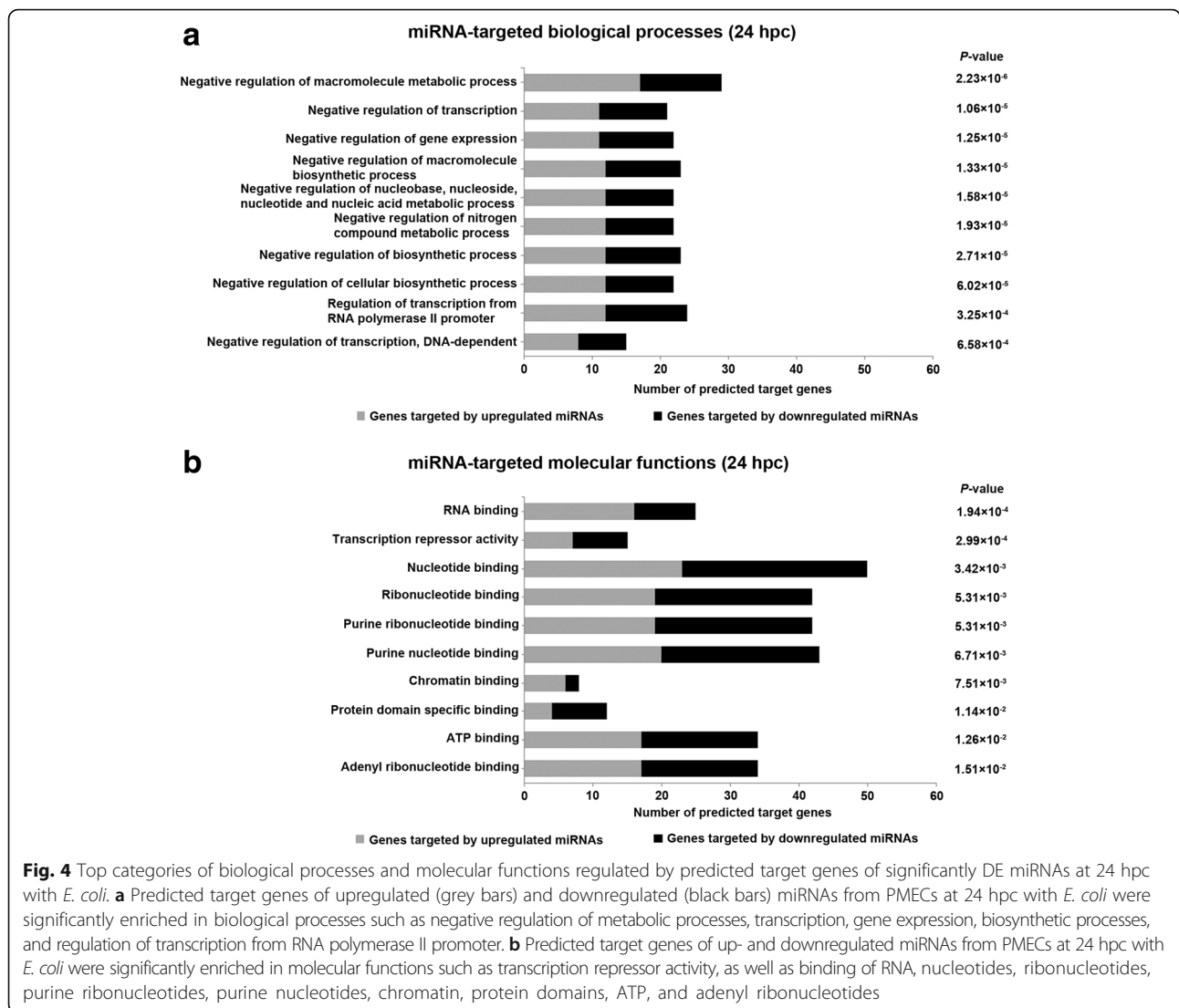
Enriched KEGG pathways and canonical pathways of predicted gene targets of DE miRNAs differ at 3 hpc and 24 hpc

In KEGG analysis, target genes of mostly downregulated miRNAs in PMECs at 3 hpc and 24 hpc with *E. coli* were significantly enriched in pathways in cancer (e. g., *BIRC3, BMP2, GLI3, ITGAV, NFKBIA*, and *PPARG*) and apoptosis (e. g., *BIRC3, IL1A*, and *NFKBIA*) (Fig. 5a). miR-3277, miR-548u, and miR-885-3p were highly predicted to play important roles in regulating both KEGG pathways (Additional file 5).

At 3 hpc with *E. coli*, target genes of downregulated miRNAs were significantly enriched in cytokine–cytokine receptor interactions (*BMP2, IFNAR2, IL10RB, IL1A, IL1RAP*, and *LIFR*); nucleotide-binding oligomerization domain (NOD)-like receptor signaling pathways (*BIRC3, NFKBIA*, and *TNFAIP3*); and Janus kinase/signal transducers and activators of transcription (Jak-STAT) signaling pathways (*IL10RB, IFNAR2, JAK2*, and *LIFR*) (Fig. 5a). miR-202, miR-2262, and miR-885-3p were

highly predicted to play important roles in regulating expression of genes that are involved in the three named KEGG pathways. Furthermore, most genes regulating cytokine signaling and Jak-STAT signaling were highly predicted to be targets of miR-3277, miR-648, miR-8-5p, and miR-890 (Additional file 5). Target genes of predominantly downregulated miRNAs in PMECs at 3 hpc with *E. coli* were significantly enriched in canonical pathways such as IL-10 signaling (*IL1A, IL10RB, IL1RAP*, and *NFKBIA*); NF-κB signaling (*BMP2, IL1A, MAP3K8, NFKBIA*, and *TNFAIP3*); peroxisome proliferator-activated receptor (PPAR) signaling (*IL1A, IL1RAP, NFKBIA*, and *PPARG*); tumor necrosis factor receptor 2 (TNFR2) signaling (*BIRC3, NFKBIA*, and *TNFAIP3*); and the role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis (*BIRC3, BMP2, IL1A, IL1RAP*, and *NFKBIA*) (Fig. 5b). miR-3277, miR-4317, miR-4903, miR-548u, and miR-885-3p were highly predicted to be important for regulation of most of the named canonical pathways (Additional file 5).

Target genes of predominantly downregulated miRNAs in PMECs at 24 hpc with *E. coli* were significantly enriched in canonical pathways such as interferon signaling (*MX1, ISG15, STAT1*, and *TAP1*); protein

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Fig. 4 Top categories of biological processes and molecular functions regulated by predicted target genes of significantly DE miRNAs at 24 hpc with *E. coli*. **a** Predicted target genes of upregulated (grey bars) and downregulated (black bars) miRNAs from PMECs at 24 hpc with *E. coli* were significantly enriched in biological processes such as negative regulation of metabolic processes, transcription, gene expression, biosynthetic processes, and regulation of transcription from RNA polymerase II promoter. **b** Predicted target genes of up- and downregulated miRNAs from PMECs at 24 hpc with *E. coli* were significantly enriched in molecular functions such as transcription repressor activity, as well as binding of RNA, nucleotides, ribonucleotides, purine ribonucleotides, purine nucleotides, chromatin, protein domains, ATP, and adenyl ribonucleotides

ubiquitination (*PSMB9*, *BIRC3*, *DNAJA1*, *DNAJC11*, *PSMD12*, *TAP1*, *UBE2R2*, *USP28*, and *USP46*); glioma invasiveness signaling (*DIRAS3*, *FNBP1*, *ITGAV*, and RHOQ); myc-mediated apoptosis signaling (*FAS*, *IGF1R*, *SFN*, and *YWHAG*); and Gq signaling (*DIRAS3*, *FNBP1*, *GNA14*, *HRH1*, *NFKBIA*, and *RHOQ*) (Fig. 5b). miR-210, miR-23a-star, and miR-324-5p were highly predicted to play important roles in regulating most of the named canonical pathways (Additional file 5).

Validation of selected miRNA expression by real time quantitative PCR

Seven miRNAs (miR-210, miR-423-5p, miR-17-3p, miR-193a-5p, miR-320, miR-339-5p, and miR-362) that were DE in miRNA microarray analyses were selected for validation by real time quantitative PCR (RT-qPCR). MiRNA expression analyzed by RT-qPCR was in good accordance with microarray data, as shown by similar

fold changes (FC) (Additional file 6). This suggested that our microarray data were reliable, although we used pools of samples for microarrays compared to individual samples for qPCR.

Discussion

The role of miRNAs in innate and adaptive immunity of the mammary gland is increasingly well described in different species, e. g. human, mice, and cattle. In pigs, the miRNA role regulating inflammatory defense mechanisms during microbial infection of the mammary gland has not yet been described. Therefore, we used the previously described PMEC model [7] to analyze specific pathogen defense mechanisms under standardized experimental conditions in vitro. To the best of our knowledge, this is the first study describing specific miRNAs and their predicted target genes in PMECs after contact with a potential mastitis-causing *E. coli* strain, indicating

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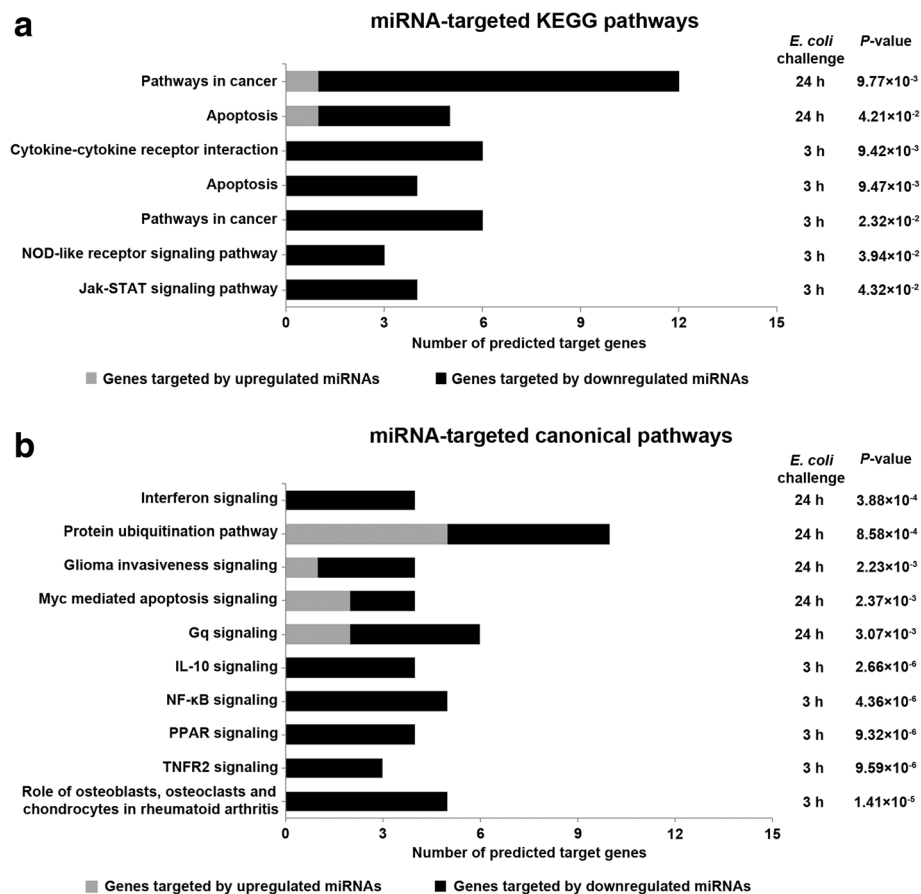
Fig. 5 Top KEGG pathways and canonical pathways regulated by predicted target genes of significantly DE miRNAs at 3 hpc and 24 hpc with *E. coli*. **a** Predicted target genes of predominantly downregulated (black bars), but also upregulated (grey bars), miRNAs from PMECs at 3 hpc and 24 hpc with *E. coli* were significantly enriched in KEGG pathways such as cytokine–cytokine receptor interactions, apoptosis, cancer pathways, NOD-like receptor signaling pathways, and Jak-STAT signaling pathways. **b** Predicted target genes of downregulated miRNAs were significantly enriched in canonical pathways such as IL10 signaling, NF-kB signaling, PPAR signaling, TNFR2 signaling, and the role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis in PMECs at 3 hpc with *E. coli*. Predicted target genes of up- and downregulated miRNAs were significantly enriched in canonical pathways such as interferon signaling, protein ubiquitination, glioma invasiveness signaling, myc-mediated apoptosis signaling, and Gq signaling in PMECs at 24 hpc with *E. coli*

the important roles of these cells in pathogen clearance within the first cellular barriers of the porcine mammary gland.

DE miRNAs that were identified in PMECs at 3 hpc and 24 hpc with *E. coli* were analyzed together with our previous mRNA expression profile and then integrated based on pairwise correlations and computational target predictions.

Pathogen challenge interfered with many host cellular processes, including modulating miRNA production, which influences host cell physiology and defense. As shown in this study, many miRNAs were significantly upregulated in PMECs at 3 hpc and 24 hpc with *E. coli*. Expression data showed that long incubation of PMECs with heat-inactivated *E. coli* resulted in more DE miRNAs than short incubation. Early response of PMECs at 3 hpc with *E. coli* was followed by a late, more intensive

host response at 24 hpc, as indicated by an approximately two-fold increase of significantly DE miRNAs. This is in accordance with our mRNA microarray analysis, which also identified a more intense host response after long-term challenge with *E. coli* [7].

Approximately 46% and 17% of identified mRNAs are predicted to be miRNA-regulated in PMECs at 3 hpc and 24 hpc with *E. coli*, respectively. Some of the identified DE miRNAs have not yet been described in the current literature. But based on our target prediction analysis and existing literature, most of the identified DE miRNAs likely play important roles in regulation of innate immunity and cellular homeostasis of PMECs. Among these, miR-101, miR-24-star, miR-30b-star, miR-210, miR-34c, and miR-17-3p were significantly DE in PMECs after *E. coli* challenge and are also affected during host responses of human, mice, or cattle to LPS

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stimulus or *E. coli* infection [12, 20–24]. In addition, some DE miRNAs in our analysis play pivotal roles in regulation of host defenses against other bacterial infections (miR-23a-star, miR-125-star, and miR-128) [12, 25, 26]; viral infections (miR-29a, miR-505, and miR-423-5p) [27–29]; parasitization (miR-8-star) [30]; and inflammatory bowel disease (miR-10a and miR-5128) [31, 32]. Furthermore, miR-101, miR-29b, miR-10a, miR-17-3p, and miR-92b were DE expressed in PMECs after pathogen challenge and are important regulators in bovine (endo-) metritis [33]. In addition, miR-484, miR-660, miR-34c, miR-339, miR-193a-5p, and miR-362 have regulatory functions in breast cancer progression [34–39] and were also deregulated in PMECs after pathogen challenge. Likewise, some miRNAs that were DE in PMECs at 3 hpc and 24 hpc with *E. coli* are potential regulators of genes that were enriched in KEGG pathways, such as cancer-related or apoptosis pathways. Therefore, a deregulation of growth regulation or an alteration of cellular physiological processes such as cell proliferation, cell death and repair of genes in PMECs by *E. coli* infection is assumed, which might play a pivotal role in the genesis of PDS.

Staedel and Darfeuille [25] reported that recognition of intruding pathogens by PAMPs — such as membrane-associated TLRs and cytoplasmic NOD-like receptors — is essential for subsequent activation of innate and adaptive immunity, mostly stimulated by NF-κB signaling. Accordingly, we identified miR-202, miR-2262, miR-4031-5p, miR-4317, miR-548u, and miR-885-3p as potential regulators of target genes that are enriched in NOD-like receptor signaling pathways in PMECs, referring to recognition of *E. coli*. In contrast, the TLR signaling pathway was not identified as one of the top enriched canonical pathways in PMECs after pathogen challenge as discuss in our previously study [7]. It is possible that the preparation of heat kill particles somehow removed TLR agonists from the particles from pMECs but not from bMECs [40]. This is still lack of knowledge in this field of experiment. However, miR-101, miR-125-star, miR-8-star, and miR-29a were DE in PMECs after pathogen challenge and are involved in TLR signaling [20, 25, 30].

MiRNAs can interact with several signaling molecules, such as cytokines, chemokines, and transcription factors, and therefore influence various signaling pathways and cellular processes [25]. In our study, miR-202, miR-3277, miR-4903, miR-648, miR-8-5p, miR-885-3p, and miR-890 were identified to play critical roles in regulation of genes that control cytokine receptor activity and cytokine binding. Additionally, some miRNAs that were DE in PMECs after pathogen challenge are known to regulate genes that are involved in NF-κB signaling (miR-210 and miR-23a-star) [41, 42] or production of cytokines

IL-6, TNF-alpha, IL-1 beta, and IL-10 (miR-30b-star, miR-210, miR-17-3p, miR-23a-star, miR-24-star, miR-29b, miR-125-star, miR-1934-star, and miR-320) [21, 22, 24, 42–47]. Furthermore, our analysis identified predicted target genes of DE miRNAs in PMECs at 3 hpc with *E. coli* that are enriched in top canonical pathways such as IL-10 signaling, PPAR signaling, NF-κB signaling, TNFR2 signaling, or arthritis-associated roles. These signaling pathways are important for fast pathogen recognition and effective nonspecific removal of infectious agent as well as moderate the inflammatory response to limit tissue damage of the host. At 24 hpc of PMECs with *E. coli*, predicted target genes of DE miRNAs were enriched in top canonical pathways such as interferon signaling and protein ubiquitination, which were also significantly enriched in our previous mRNA analysis of pathogen-challenged PMECs [7]. In this late immune response phase these signaling pathways can increase host-defense by activation of immune cells and regulation of a variety of fundamental cellular processes.

Altogether, the number of target genes predicted for DE miRNAs and involved in regulation of biological processes or molecular functions in PMECs was two- to tenfold higher at 24 hpc than at 3 hpc with *E. coli*.

In addition to our target prediction analysis, current literature suggests that some DE miRNAs in PMECs also regulate genes involved in Jak-STAT signaling (miR-125-star, miR-289, miR-22-star, and miR-23a-star) [45, 48–50]; interferon signaling (miR-29a) [28]; MAPK signaling (miR-289, miR-324-5p, and miR-193a-5p) [48, 51, 52]; TGF signaling (miR-125-star, miR-324-5p, miR-193a-5p, and miR-505) [45, 51–53]; PTEN/Akt signaling (miR-92b) [54]; and Wnt signaling (miR324-5p and miR-193a-5p) [51, 52]. Interaction of signaling molecules that are involved in these pathways seems to be important to balance pro- and anti-inflammatory processes and to ensure immune homeostasis as well as host protection [55]. This is confirmed by our results, as some miRNAs that were DE in PMECs after pathogen challenge play crucial roles in innate and adaptive immune responses (miR-101, miR-125-star, miR-423-5p, miR-10a, miR-23a-star, miR-1934-star, miR-29a, miR-29b, miR-24-star, let-7 g-star, miR-320, miR-17-3p, miR-324-5p, and miR-8-star) [20, 25, 27, 31, 42, 46, 56–64] as well as negative feedback regulation of inflammation (miR-210 and miR-17-3p) [22, 62].

Our target prediction analysis revealed several DE miRNAs as potential regulators of genes that control biosynthetic, metabolic, and transcriptional processes in PMECs at 3 hpc as well as 24 hpc with *E. coli*. In addition, some DE miRNAs in our study are known to target genes involved in regulation of cell adhesion/cytoskeleton (miR-17-3p, miR-339, and miR-8-star) [62, 65, 66]; cell survival/DNA repair (miR-324-5p, miR-8-star, miR-890, miR-885-3p, and miR-363) [63, 65, 67–70];

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catabolism (miR-320 and miR-140) [44, 47, 70]; migration/invasion/tumorigenesis (miR-339, miR-193a-5p, miR-362, miR-125-star, miR-92b, miR-324-5p, miR-320, miR-202, miR-371, miR-125a-3p, and miR-140) [37–39, 45, 54, 63, 71–75]; cellular transport (miR-10a and miR-5100 [60, 76]; proliferation/differentiation/cell cycle/growth/development (miR-484, miR-34c, miR-339, miR-193a-5p, miR-362, miR-125-star, miR-92b, miR-24-star, miR-10a, miR-324-5p, miR-363, miR-320, miR-202, miR-371, miR-125a-3p, miR-140, miR-5100, miR-128, miR-216b, miR-4423-3p, and miR-320b) [34, 36–39, 45, 54, 58, 60, 63, 67, 71–80]; and apoptosis/ autophagy/ phagocytosis (miR-30b-star, miR-125-star, miR-324-5p, miR-505, miR-92b, miR-24-star, miR885-3p, miR-320, miR-128, miR-216b, miR-320b, miR-125a-3p, miR-363, and miR-423-5p) [43, 45, 51, 53, 54, 58, 69, 71, 77, 78, 80–84]. These results are consistent with our mRNA study, in which pathogen challenge of PMECs resulted in DE genes that regulate cellular growth, proliferation, development, cell death, and survival [7]. All these processes are physiologically significant for determining epithelial cell fate, ensuring epithelial cell functions, and fine-tuning epithelial immune responses against infection [85].

Conclusions

Combined with current literature, our data show that many functions of several miRNAs seem to be highly conserved across species. However, while many miRNAs can have similar functions, specific miRNAs also can have different functions in PMECs. We suppose the identified miRNAs have critical roles in pathogen recognition and initiation of local and systemic immune responses in PMECs. Altogether, our results strongly suggest that DE miRNAs in PMECs contribute to pathogenesis of porcine mastitis induced by *E. coli.* The detection of several known and novel miRNAs in context with predicted target genes in PMECs is another indicator for the immune competence of these cells and provides a strong impulse to further examine porcine mammary gland immune defense mechanisms.

Methods
Cell culture and pathogen challenge

PMECs are originated from our previous study [7]. Therefore, cell cultures were established from mammary glands of three lactating sows of commercial herds. Animal care and tissue collection was performed in compliance with the German Law of Animal Protection. The experimental protocol was approved by the Animal Care Committee of the Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany. Animals were exsanguinated following electronarcosis and dissected. Cryopreserved mammary epithelial cells were thawed and resuspended in complete medium consisting of Dulbecco's

Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, PAN Biotech, Aidenbach Germany), 10% fetal bovine serum (FBS, PAA, Cölbe, Germany), 1% Antibiotic/Antimycotic Solution (APS, 10,000 U/ml penicillin, 10 mg/ml streptomycin sulphate, 25 μg/ml amphotericin B, PAA), 10 μg/ml insulin (Sigma-Aldrich, Taufkirchen, Germany) and 1 μg/ml hydrocortisone (Sigma-Aldrich).

E. coli strain (gMEc240, sequence type 101, phylogroup B1, C+) used for this study is an isolate from milk of PDS-positive sows and was provided by the Institute of Microbiology and Epizootics, Department of Veterinary at the Freie Universität Berlin, Berlin, Germany. *E. coli* was grown in brain-heart-infusion-broth (BHB, Oxoid, Wesel, Germany) at 37 °C to the logarithmic phase of culture growth (Optical Density at 600 nm [OD_{600}] $0.5 \sim 5 \times 10^{7}$/ml). Bacteria were heat-inactivated at 80 °C for 1 h, spun down at 3000 rpm for 15 min, washed twice with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium and resuspended herein at a density of 1×10^{8}/ml.

The experimental design was identical with the procedure as described before [7]. Briefly, approximately 4.4×10^{5} cells from each 3 individual (3 biological replicates) were seeded and cultured in collagen-coated 6-well plates (1:10 collagen R in destilled water, Menal, Emmendingen, Germany) in complete medium without APS (three technical replicates per individual and treatment condition). After 24 h, medium was changed. Within the next 48 h after seeding, cells reached about 90% confluency. Then PMECs were challenged with 10^{7}/ml heat-inactivated *E. coli* for 3 h and for 24 h. Unchallenged cells were used as a negative control. After incubation periods, medium was discarded, and cells were washed three times with phosphate buffered saline (PBS, PAA) to remove the bacteria. Afterwards, cells were collected for isolation of total RNA and small RNA.

RNA isolation

Total RNA was isolated from each of the 27 samples using the TRI® reagent (Sigma-Aldrich) and extracted with phenol-chloroform according to the manufacturer's instructions. Isolated RNA was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany), and contaminating DNA was removed by DNase I digestion (Qiagen). Isolation and enrichment of small RNA was performed by using a miRNeasy kit and an RNeasy MinElute Cleanup kit (both from Qiagen) according to the manufacturer's protocols. RNA integrity and quantity were assessed with an Agilent 2100 Bioanalyzer using an RNA 6000 Nano kit for total RNA and a separate kit for small RNA (both from Agilent Technologies Inc., Santa Clara, CA, USA). The mean RIN and standard deviation for the samples used in the analysis was 9.72 ± 0.24.

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microRNA microarray analysis and statistics

microRNA expression profiling was performed using hybridization to GeneChip miRNA 3.0 Arrays (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's recommendations. The array has a very high sensitivity and specificity and offers 100% coverage of miRBase 17 (www.mirbase.org) by a one-color approach. In addition, the array contains 16,772 entries representing hairpin precursor, total probe set 19,724 for detection of 1733 mature miRNA, 2216 human snoRNA and scaRNA products in 153 species, and provides a greater than 3-log dynamic range with higher than 95% reproducibility and 85% transcript detection at 1.0 amol for a total RNA input of 130–500 ng.

In total 27 small RNA samples were isolated. Equivalent amount of small RNA from three technical replicates per individual was pooled. In total 9 pooled samples of small RNA were used for further experiment. An amount of 200 ng of each small RNA sample pool was labeled with the FlashTag Biotin HSR RNA Labeling kit (Genisphere, Hatfield, PA). Labeled RNA was hybridized for 16 h to the miRNA arrays according to the manufacturer's recommendations. A total of nine microarrays were obtained. Afterwards, arrays were washed and stained in a Fluidics Station 450 (Affymetrix), and scanned on the G3000 GeneArray Scanner (Affymetrix). Pre-processing of raw expression data was done using the Expression Console™ software (Affymetrix). Robust multi-array average (RMA) background correction, log-2 transformation, quantile normalization and statistical analysis were performed using SAS 9.4 containing JMP Genomics 7 (SAS Institute, Cary, NC, USA). The ANOVA procedure in JMP genomics 7 was performed to determine relative changes in miRNA levels, including effects mediated by 3 experimental group (before (0 h) and after challenged (3 h and 24 h) with each 3 replicates of each experimental group. Alterations in transcript abundances were considered to be statistically significant at $p < 0.05$. Subsequently, data were filtered by fold change >1.5 or < −1.5. The miRNA microarray data were deposited in the Gene Expression Omnibus (GEO) public repository (GEO accession number GSE88870: GSM2350436 -GSM2350444).

Prediction and functional annotation of miRNA target genes

We used our previous microarray-based mRNA expression data to integrate with the DE miRNAs and scan for potential target genes [7]. TargetScan was used to detect predicted target genes based on seed complementarity on both 3′-, 5′-UTR and coding sequences of the porcine mRNA sequences (*Sus scrofa* 10.2 download from NCBI: http://www.ncbi.nlm.nih.gov/ on 1.9.2015) and miRNA sequences [86]. RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) was used for direct prediction of multiple, energetically most favorable

potential binding sites of our differentially expressed miRNAs [87, 88]. microRNAs were tested due to the following default parameters: (i) number of hits per target, 1; (ii) energy cutoff, −25 kcal/mol; (iii) maximal internal or bulge loop size per side, 4. Pearson correlation of miRNA and mRNA expression levels was calculated based on the predicted miRNA-mRNA relationships. We selected only negative correlation pairs of miRNA-mRNA with $p < 0.05$. The most affected biological processes and molecular functions as well as enriched KEGG pathways of predicted target genes were determined using the Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID) [89]. In addition, the top canonical pathways, miRNA targets are involved in, were identified using the Ingenuity Pathway Analysis software ($p \leq 0.05$, Fisher's exact test; IPA, Ingenuity Systems, Redwood City, CA, USA).

Validation of microRNA microarray results by Fluidigm real-time quantitative PCR

Transcript levels of seven miRNAs were measured by RT-qPCR using the Fluidigm BioMark HD System (Fluidigm Corporation, San Francisco, CA, USA). First, 200 ng of miRNA was converted to cDNA using the Megaplex RT Primers, Human Pool Kit v2.1/v3.0 (Thermo Fisher Scientific, Waltham, MA, USA). The pre-amplification sample mixtures were prepared containing 1.25 μl of diluted cDNA, 2.5 μl of 2× Taqman pre-amplification master mix (Applied Biosystems, Waltham, MA, USA), and 0.5 μl of the primer pool (200 nmol each primer/μl; Promega, Mannheim, Germany; Additional file 6). The pre-amplification PCR program consisted of a 10 min 95 °C denaturation step and 14 cycles of 15 s at 95 °C and 4 min at 60 °C. The pre-amplified reactions were diluted 10 times in Tris-EDTA (TE) buffer. Five microliters from sample mix containing pre-amplified cDNA, pooled primers and EvaGreen master mix (Fluidigm BioMark) were combined as outlined in the manufacturer's protocol. The 48.48 dynamic array chip (Fluidigm Corporation) was first primed with control line fluid and then loaded with the sample and assay mixtures via the appropriate inlets using an IFC controller. The array chip was placed in the Fluidigm BioMark Instrument for PCR at 95 °C for 10 min, followed by 30 cycles at 95 °C for 15 s and 60 °C for 1 min. The samples were investigated in duplicate, and data were analyzed with RT-PCR analysis software in the BioMark HD instrument. The mean Ct values of the different miRNAs were normalized to U6 snRNA and miR-24-3p (internal controls), and then subjected to analysis using the $2^{-\Delta\Delta Ct}$ method [90]. Both internal controls were selected, because their transcript levels were most stable over time and treatment in PMECs. In addition, U6 snRNA is known as one of the most commonly used internal control for the normalization of miRNA qPCR data.

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Additional files

Additional file 1: Significantly DE miRNA probesets from microarray analysis in PMECs at 3 hpc and 24 hpc with *E. coli* compared with unchallenged controls. (XLS 78 kb)

Additional file 2: Significantly DE miRNA probesets and their predicted target genes (negatively correlated) from microarray analysis in PMECs at 3 hpc with *E. coli* compared with unchallenged controls. (XLS 82 kb)

Additional file 3: Significantly DE miRNAs and their predicted target genes (negatively correlated) from microarray analysis in PMECs at 24 hpc with *E. coli* compared with unchallenged controls. (XLS 208 kb)

Additional file 4: Significantly enriched GO functional annotation of predicted target genes in PMECs at 3 hpc and 24 hpc with *E. coli*. (XLS 92 kb)

Additional file 5: KEGG pathways and canonical pathways significantly enriched by predicted target genes in PMECs at 3 hpc and 24 hpc with *E. coli*. (XLS 34 kb)

Additional file 6: Verification of selected miRNA expression by RT-qPCR. (XLS 29 kb)

Abbreviations
DE: Differentially expressed; miRNA: microRNA; PDS: Postpartum dysgalactia syndrome; PMECs: Porcine mammary epithelial cells

Availability of data and materials
The availability of expression data are in the Gene Expression Omnibus public repository with the GEO accession number GSE88870: GSM2350436 -GSM2350444).

Authors' contributions
AJ and KW sampled the tissue probes during the slaughter process. NK and LBA collected and classified the bacteria samples. LBA prepared the bacteria for experimental study. AJ and SP designed the study. AJ carried out the cell culture experiments. SP, AJ and EM performed the statistical and bioinformatics analyses of microarray data. FH helped in target prediction analysis and created the heat map. AJ interpreted the data, drafted the manuscript and prepared figures and Tables. FH, NK, EM, KW and SP critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval
The German Landrace pigs were born and reared at the experimental pig farm of the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf (Germany). The experimental protocol was approved by the Animal Care Committee of the Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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