The expression of chemerin and its receptors (CMKLR1, GPR1, CCRL2) in the porcine uterus during the oestrous cycle and early pregnancy and in trophoblasts and conceptuses

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Animal journal

Supplementary Table S1 *Body weights and number of corpus luteum, follicles and/or embryos in each research group of pigs*

Research group	Body weights [kg]	Number of CL, F and/or E
Days 2-3 of the oestrous cycle	132-136	13-15 CL
Days 10-12 of the oestrous cycle	132-136	13-15 CL
Days 14-16 of the oestrous cycle	135-138	13-15 CL
Days 17-19 of the oestrous cycle	135-137	15-17 F
Days 10-11 of pregnancy	133-135	13-15 CL
Days 12-13 of pregnancy	133-135	13-15 CL
Days 15-16 of pregnancy	131-133	13-15 CL
Days 27-28 of pregnancy	130-132	10-14 E
Days 30-32 of pregnancy	130-132	10-14 E

On days 10-16 of pregnancy finding the number of embryos is very difficult, which is why the numbers of CLs are given.
CL: corpus luteum; F: follicles; E: embryos.

Supplementary Table S2 *The results of the validation method of real-time PCR with the use of porcine tissues*

Gene	Slope	r^2	Efficiency [%]
RARRES2	-3.462	0.998	104.9
CMKLR1	-3.057	0.992	92.6
GPR1	-3.03	0.977	91.8
CCRL2	-3.23	0.984	97.8

RARRES2: chemerin; *CCRL2*: C-C motif chemokine receptor like 2; *CMLKR1*: chemokine-like receptor 1; *GPR1*: G protein-coupled receptor 1; r^2: correlation coefficients

Supplementary Table S3 *Repeatability (intraassay variation) for real-time PCR method with the use of porcine tissues*

Gene	Endometrium	Myometrium	Trophoblasts and conceptuses
RARRES2	0.48%	0.32%	0.16%
CMKLR1	0.66%	0.74%	0.76%
GPR1	0.63%	0.77%	0.45%
CCRL2	0.76%	0.49%	0.47%

RARRES2: chemerin; *CCRL2*: C-C motif chemokine receptor like 2; *CMLKR1*: chemokine-like receptor 1; *GPR1*: G protein-coupled receptor

Supplementary Table S4 *Quantitative information on the performance of Western blot analysis with the use of porcine tissues*

ENDOMETRIUM

Protein	Condition	Days	n	Normalized values	Mean	SEM
CHEMERIN	Oestrous cycle	2-3	1n	0.59		
			2n	0.43		
			3n	0.45	0.49	0.03
			4n	0.57		
			5n	0.41		
		10-12	1n	0.70		
			2n	0.88		
			3n	0.57	0.67	0.05
			4n	0.53		
			5n	0.66		
		14-16	1n	0.88		
			2n	0.60		
			3n	0.54	0.56	0.08
			4n	0.32		
			5n	0.44		
		17-19	1n	0.58		
			2n	0.45		
			3n	0.32	0.39	0.05
			4n	0.25		
			5n	0.33		
	Pregnancy	10-11	1n	0.62		
			2n	0.53		
			3n	0.23	0.76	0.18
			4n	1.12		
			5n	1.30		
		12-13	1n	0.70		
			2n	0.39		
			3n	0.23	0.46	0.09
			4n	0.32		
			5n	0.68		
		15-16	1n	0.45		
			2n	0.97		
			3n	0.65	0.63	0.08
			4n	0.59		
			5n	0.49		
		27-28	1n	2.49		
			2n	1.23		
			3n	0.81	1.40	0.35
			4n	0.39		
			5n	2.09		
		30-32	1n	0.67		
			2n	0.50		
			3n	1.01	0.69	0.18
			4n	0.73		
			5n	0.55		
CMKLR1	Oestrous cycle	2-3	1n	0.19		
			2n	1.18		
			3n	1.90	1.27	0.29
			4n	1.95		
			5n	1.12		
		10-12	1n	1.59		
			2n	0.89		
			3n	1.39	1.49	0.21
			4n	1.30		
			5n	2.29		
		14-16	1n	5.55		
			2n	6.02		
			3n	5.37	5.52	0.20
			4n	5.93		
			5n	4.73		
		17-19	1n	3.55		
			2n	2.13		
			3n	1.25	2.91	0.74
			4n	5.85		
			5n	1.77		
	Pregnancy	10-11	1n	1.49		
			2n	0.35		
			3n	0.38	0.62	0.20
			4n	0.63		
			5n	0.23		
		12-13	1n	0.21		
			2n	0.47		
			3n	0.11	0.18	0.07
			4n	0.07		
			5n	0.05		
		15-16	1n	0.69		
			2n	1.40		
			3n	0.84	0.99	0.11
			4n	0.98		
			5n	1.05		
		27-28	1n	1.00		
			2n	0.35		
			3n	0.34	0.80	0.23
			4n	1.69		
			5n	0.62		
		30-32	1n	1.24		
			2n	1.28		
			3n	1.13	1.66	0.27
			4n	1.93		
			5n	2.74		

MYOMETRIUM

Protein	Condition	Days	n	Normalized values	Mean	SEM
CHEMERIN	Oestrous cycle	2-3	1n	0.46		
			2n	0.44		
			3n	0.76	0.58	0.07
			4n	0.81		
			5n	0.46		
		10-12	1n	0.28		
			2n	0.31		
			3n	0.28	0.29	0.02
			4n	0.34		
			5n	0.23		
		14-16	1n	0.51		
			2n	0.43		
			3n	0.11	0.29	0.07
			4n	0.21		
			5n	0.17		
		17-19	1n	0.12		
			2n	0.17		
			3n	0.07	0.11	0.02
			4n	0.12		
			5n	0.06		
	Pregnancy	10-11	1n	0.07		
			2n	0.10		
			3n	0.06	0.09	0.01
			4n	0.10		
			5n	0.14		
		12-13	1n	0.05		
			2n	0.03		
			3n	0.05	0.05	0.01
			4n	0.05		
			5n	0.07		
		15-16	1n	0.14		
			2n	0.26		
			3n	0.27	0.25	0.03
			4n	0.27		
			5n	0.32		
		27-28	1n	0.36		
			2n	0.21		
			3n	0.33	0.31	0.03
			4n	0.37		
			5n	0.27		
		30-32	1n	0.23		
			2n	0.33		
			3n	0.23	0.31	0.03
			4n	0.37		
			5n	0.39		
CMKLR1	Oestrous cycle	2-3	1n	0.07		
			2n	0.02		
			3n	0.02	0.04	0.01
			4n	0.07		
			5n	0.02		
		10-12	1n	0.08		
			2n	0.18		
			3n	0.04	0.08	0.02
			4n	0.09		
			5n	0.02		
		14-16	1n	0.01		
			2n	0.05		
			3n	0.04	0.04	0.01
			4n	0.02		
			5n	0.09		
		17-19	1n	0.19		
			2n	0.36		
			3n	0.20	0.26	0.03
			4n	0.31		
			5n	0.23		
	Pregnancy	10-11	1n	0.06		
			2n	0.03		
			3n	0.11	0.14	0.05
			4n	0.15		
			5n	0.34		
		12-13	1n	0.09		
			2n	0.12		
			3n	0.02	0.08	0.01
			4n	0.09		
			5n	0.07		
		15-16	1n	0.28		
			2n	0.22		
			3n	0.28	0.27	0.02
			4n	0.33		
			5n	0.25		
		27-28	1n	0.20		
			2n	0.01		
			3n	0.13	0.11	0.03
			4n	0.07		
			5n	0.15		
		30-32	1n	0.15		
			2n	0.19		
			3n	0.28	0.16	0.03
			4n	0.09		
			5n	0.09		

CONCEPTUSES

Protein	Condition	Days	n	Normalized values	Mean	SEM
CHEMERIN	Pregnancy	15-16	1n	0.08		
			2n	0.07		
			3n	0.08	0.07	0.01
			4n	0.05		
			5n	0.07		
		27-28	1n	0.03		
			2n	0.11		
			3n	0.11	0.09	0.01
			4n	0.12		
			5n	0.08		
		30-32	1n	0.16		
			2n	0.11		
			3n	0.20	0.13	0.02
			4n	0.09		
			5n	0.10		
CMKLR1	Pregnancy	15-16	1n	0.35		
			2n	0.15		
			3n	0.23	0.24	0.05
			4n	0.37		
			5n	0.09		
		27-28	1n	1.19		
			2n	2.73		
			3n	1.05	2.32	0.55
			4n	4.41		
			5n	2.23		
		30-32	1n	1.13		
			2n	2.46		
			3n	1.38	1.80	0.27
			4n	1.44		
			5n	2.60		
GPR1	Pregnancy	15-16	1n	2.17		
			2n	2.47		
			3n	2.92	2.60	0.16
			4n	3.10		
			5n	2.33		
		27-28	1n	1.58		
			2n	1.40		
			3n	1.35	1.74	0.27
			4n	2.92		
			5n	1.47		
		30-32	1n	7.66		
			2n	11.22		
			3n	11.76	7.92	1.89
			4n	0.03		
			5n	8.98		
CCRL2	Pregnancy	15-16	1n	1.16		
			2n	1.41		
			3n	1.18	1.26	0.08
			4n	1.03		
			5n	1.54		
		27-28	1n	0.15		
			2n	0.18		
			3n	0.19	0.39	0.15
			4n	1.03		
			5n	0.41		
		30-32	1n	5.93		
			2n	9.49		
			3n	5.03	6.62	0.68
			4n	6.69		
			5n	5.98		

TROPHOBLASTS

Protein	Condition	Days	n	Normalized values	Mean	SEM
CHEMERIN	Pregnancy	27-28	1n	0.01		
			2n	0.11		
			3n	0.08	0.06	0.01
			4n	0.04		
			5n	0.08		
		30-32	1n	0.13		
			2n	0.25		
			3n	0.22	0.31	0.08
			4n	0.68		
			5n	0.30		
CMKLR1	Pregnancy	27-28	1n	0.21		
			2n	0.18		
			3n	0.37	0.26	0.03
			4n	0.26		
			5n	0.30		
		30-32	1n	3.30		
			2n	1.51		
			3n	2.04	3.10	0.56
			4n	5.08		
			5n	3.57		
GPR1	Pregnancy	27-28	1n	15.01		
			2n	8.54		
			3n	7.99	10.40	1.55
			4n	14.06		
			5n	6.42		
		30-32	1n	2.50		
			2n	0.79		
			3n	1.31	2.22	0.52
			4n	4.20		
			5n	2.28		
CCRL2	Pregnancy	27-28	1n	4.06		
			2n	3.09		
			3n	6.50	4.79	0.52
			4n	5.42		
			5n	4.90		
		30-32	1n	5.92		
			2n	16.07		
			3n	10.03	10.87	1.45
			4n	11.30		
			5n	11.00		

ENDOMETRIUM

Gene	Phase	Days		Normalized values	Mean	SEM
GPR1	Oestrous cycle	2-3	1n	2.76	2.75	0.06
			2n	2.95		
			3n	2.85		
			4n	2.57		
			5n	2.62		
		10-12	1n	5.71	3.83	0.71
			2n	1.54		
			3n	2.45		
			4n	4.35		
			5n	5.10		
		14-16	1n	3.01	3.49	0.77
			2n	4.03		
			3n	1.52		
			4n	6.54		
			5n	2.33		
		17-19	1n	1.78	1.31	0.16
			2n	1.34		
			3n	1.23		
			4n	0.68		
			5n	1.52		
	Pregnancy	10-11	1n	2.30	2.94	0.25
			2n	3.52		
			3n	2.51		
			4n	2.70		
			5n	3.66		
		12-13	1n	0.14	0.33	0.10
			2n	0.70		
			3n	0.47		
			4n	0.29		
			5n	0.05		
		15-16	1n	0.91	0.96	0.05
			2n	1.06		
			3n	1.06		
			4n	0.75		
			5n	1.02		
		27-28	1n	1.15	0.96	0.26
			2n	0.46		
			3n	0.33		
			4n	0.92		
			5n	1.96		
		30-32	1n	0.89	1.71	0.29
			2n	2.51		
			3n	2.35		
			4n	1.70		
			5n	1.08		
CCRL2	Oestrous cycle	2-3	1n	0.62	1.56	0.39
			2n	1.27		
			3n	3.09		
			4n	0.89		
			5n	1.92		
		10-12	1n	0.98	1.51	0.24
			2n	1.66		
			3n	1.60		
			4n	2.39		
			5n	0.93		
		14-16	1n	0.46	0.78	0.21
			2n	0.77		
			3n	0.67		
			4n	0.31		
			5n	1.68		
		17-19	1n	3.24	2.94	0.18
			2n	3.50		
			3n	2.70		
			4n	2.33		
			5n	2.92		
	Pregnancy	10-11	1n	0.98	3.58	0.85
			2n	2.39		
			3n	6.32		
			4n	3.13		
			5n	5.08		
		12-13	1n	2.35	1.16	0.30
			2n	0.80		
			3n	0.84		
			4n	0.45		
			5n	1.40		
		15-16	1n	6.06	9.75	1.55
			2n	8.11		
			3n	6.83		
			4n	13.07		
			5n	14.70		
		27-28	1n	5.76	5.34	0.83
			2n	4.54		
			3n	2.94		
			4n	4.87		
			5n	8.58		
		30-32	1n	1.31	2.69	0.74
			2n	3.45		
			3n	1.72		
			4n	1.37		
			5n	5.59		

MYOMETRIUM

Gene	Phase	Days		Normalized values	Mean	SEM
GPR1	Oestrous cycle	2-3	1n	0.13	0.20	0.07
			2n	0.09		
			3n	0.18		
			4n	0.50		
			5n	0.09		
		10-12	1n	0.42	0.28	0.06
			2n	0.12		
			3n	0.45		
			4n	0.14		
			5n	0.28		
		14-16	1n	0.02	0.14	0.06
			2n	0.02		
			3n	0.09		
			4n	0.16		
			5n	0.41		
		17-19	1n	0.48	0.67	0.08
			2n	0.99		
			3n	0.50		
			4n	0.70		
			5n	0.69		
	Pregnancy	10-11	1n	0.20	0.19	0.04
			2n	0.31		
			3n	0.13		
			4n	0.06		
			5n	0.24		
		12-13	1n	0.10	0.09	0.03
			2n	0.05		
			3n	0.03		
			4n	0.05		
			5n	0.23		
		15-16	1n	0.03	0.04	0.01
			2n	0.04		
			3n	0.04		
			4n	0.05		
			5n	0.03		
		27-28	1n	0.38	0.35	0.05
			2n	0.20		
			3n	0.49		
			4n	0.24		
			5n	0.42		
		30-32	1n	0.08	0.15	0.03
			2n	0.09		
			3n	0.26		
			4n	0.22		
			5n	0.11		
CCRL2	Oestrous cycle	2-3	1n	0.12	0.21	0.05
			2n	0.26		
			3n	0.13		
			4n	0.40		
			5n	0.12		
		10-12	1n	0.08	0.35	0.08
			2n	0.61		
			3n	0.22		
			4n	0.41		
			5n	0.40		
		14-16	1n	0.29	0.49	0.06
			2n	0.66		
			3n	0.47		
			4n	0.45		
			5n	0.58		
		17-19	1n	0.55	0.70	0.17
			2n	0.61		
			3n	1.45		
			4n	0.44		
			5n	0.46		
	Pregnancy	10-11	1n	0.05	0.06	0.01
			2n	0.05		
			3n	0.08		
			4n	0.03		
			5n	0.07		
		12-13	1n	0.01	0.14	0.07
			2n	0.01		
			3n	0.01		
			4n	0.38		
			5n	0.30		
		15-16	1n	0.91	1.21	0.16
			2n	0.84		
			3n	1.34		
			4n	1.85		
			5n	1.13		
		27-28	1n	1.06	0.76	0.09
			2n	0.58		
			3n	0.88		
			4n	0.52		
			5n	0.77		
		30-32	1n	0.68	1.18	0.20
			2n	1.23		
			3n	1.78		
			4n	1.52		
			5n	0.69		

CCRL2: C-C motif chemokine receptor like 2; CMLKR1: chemokine-like receptor 1; GPR1: G protein-coupled receptor 1

Supplementary Material S1

1. *Quantitative real-time PCR*

Total RNA was extracted from endometrial, myometrial, trophoblast and conceptus samples using the peqGold TriFast isolation system (Peqlabs, Erlangen, Germany). Concentration and quality of RNA were determined specrometrically (Infinite M200 Pro, Tecan, Männedorf, Switzerland). Only RNA with purity (A_{260}/A_{280}) in the 1.8-2.0 range were used for further analysis. Synthesis of cDNA was carried out using the Omniscript RT Kit (205113; Qiagen, Hilden, Germany) with 1 µg of RNA, 0.5 µg oligo(dT)15 (Roche, Basel, Switzerland) in a total volume of 20 µL at 37°C for 1 h. Reaction was terminated by the incubation at 93°C for 5 min.

Quantitative real-time PCR analysis was conducted using a PCR System 7300 (Applied Biosystems, Foster, CA, USA) with 7500 Software v2.0.6 (Applied Biosystems, Foster, CA, USA).

A calibration curve was made for qPCR validation. Five dilutions of cDNA were used for this purpose (150 ng, 30 ng, 6 ng, 1.2 ng, 0.24 ng). Real-time PCR reaction was carried out with the same conditions as for the tested samples. The obtained results are presented in Supplementary Table S2.

Relative expression levels of chemerin (**RARRES2**), chemokine-like receptor 1 (**CMKLR1**), G protein-coupled receptor 1 (**GPR1**) and C-C chemokine receptor-like 2 (**CCRL2**) genes were calculated based on the comparative cycle threshold method (**ΔΔCT**; Livak and Schmittgen, 2001) and normalised using the geometrical means of reference genes expression levels: cyclophilin A (**PPIA**) and β-actin (**ACTB**). The calculation of *RARRES2*, *CMKLR1*, *GPR1* and *CCRL2* genes expression was conducted with the equation $2^{-\Delta\Delta CT}$, where ΔCT was obtained by subtracting the corresponding geometrical means of reference genes (*PPIA* and *ACTB*) CT value from the specific CT of the target (*RARRES2*, *CMKLR1*, *GPR1* and *CCRL2*), and ΔΔCT was determined by subtracting the ΔCT of each experimental sample from ΔCT of the reference sample, called the calibrator. Calibrator was ΔCT from tissue with the lowest expression.

2. *Western blot*

The samples of porcine endometrium, myometrium, trophoblasts and conceptuses were homogenized on ice within a cold buffer (50 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.02% sodium azide, 100 µM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 2 µg/mL leupeptin, 2 µg/mL aprotinin, 1 µg/mL pepstatin, pH=7.6) and incubated on ice for 30 min. The tissue lysates were centrifuged two times (10000 x g, 10 min, 4°C) to obtain purified supernatant. Probes were stored at -80°C for further analysis. Protein concentrations were measured by the Bradford dye-binding procedure with 0.5 mg/mL BSA as a standard. Just before the SDS-PAGE electrophoresis, equal amounts of tissue lysates were solubilized in a sample buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.2% bromophenol blue and 200 mM dithiothreitol, pH=6.8) and heated (3 min, 99.9°C). Electrophoresis was

performed at 200 V and 15 mA/gel for 5 min followed by 80 V and 20 mA/gel for 3 h in Tris-glycine buffer (pH=8.3; electrophoresis apparatus, Pharmacia Biotech, Piscataway, NJ, USA).

The semi-dry electroblotting onto membranes was performed at 80 V and 38 mA for 1.5 h in 48 mM Tris-39 mM glycine buffer containing 0.037% SDS and 20% methanol. Membranes were washed after each step with 10 mM Tris, 150 mM NaCl buffer (pH=8.0) containing 0.05% Tween-20 (v/v).

References:
Livak KJ and Schmittgen TD 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.
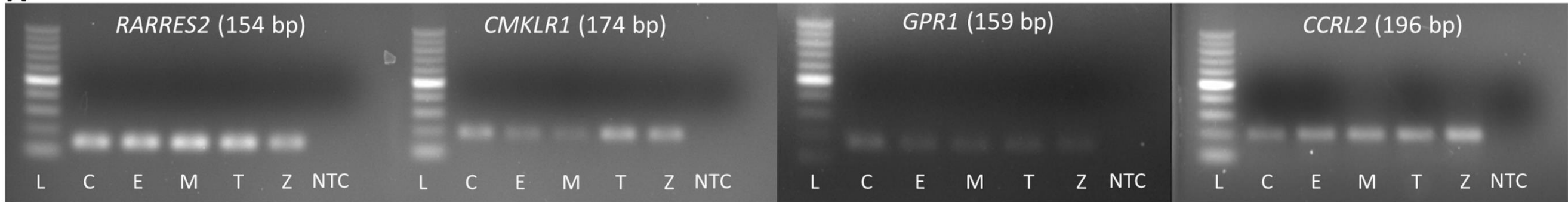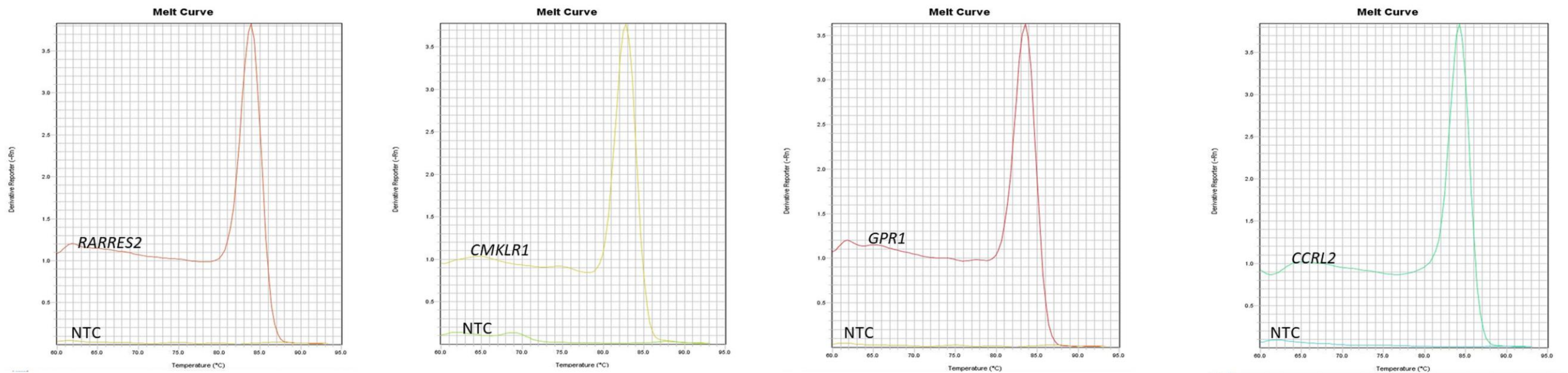
Supplementary Figure S1 Results of quality control for real-time PCR: (A) real-time PCR products electrophoresis in 1% agarose gel; (B) representative melting curves for each gene. *RARRES2*: chemerin; *CCRL2*: C-C motif chemokine receptor like 2; *CMLKR1*: chemokine-like receptor 1; *GPR1*: G protein-coupled receptor 1; L: 100-bp DNA lader; C: positive control (porcine liver for chemerin, *GPR1* and *CCRL2*, porcine spleen for *CMKLR1*); E: endometrium; M: myometrium; T: trophoblast; Z: conceptuses; NTC: no template control; bp: base pair