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In situ Identifizierung und Charakterisierung endothelialer Progenitorzellen im bovinen Ovar

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„Ärzte ohne Anatomie sind wie Maulwürfe, sie tappen im Dunkeln und ihrer Hände Arbeit sind Erdhügel.“

Friedrich Tiedemann (Heidelberger Anatom, 1781 - 1861)

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1 Abkürzungen und Formelzeichen

ACTB	Beta-actin
ACTG2	Actin, gamma-enteric smooth muscle
ANOVA	Analysis of variance
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum
CNRQ	Calibrated normalized relative quantities
Cq	Quantification cycle
Ct	Cycle threshold
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dT	Deoxythymine
EDTA	Ethylendiamintetraacetat
EPC	Endothelial progenitor cell
Fig.	Figure
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
H_2Odest	Destilliertes Wasser
H3F3B	H3 histone
HE	Hämatoxylin-Eosin
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horseradish-peroxidase
IgG	Immunglobulin G
IHC	Immunohistochemistry
KDR	Kinase insert domain receptor
M	Molare Masse
M-MuLV	Moloney murine leukemia virus
MIQE	Minimum information for publication of quantitative real-time PCR experiments
mRNA	Messenger ribonucleic acid
NF	Normalization factor
NTC	No template control
OD	Optical density
p-value	Significance level

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
POLR2C	Polymerase II polypeptide C
qPCR	Quantitative polymerase chain reaction
r	Pearson's correlation coefficient
r^2	Correlation coefficient
R^2	Coefficient of determination
RG	Referenzgen
RNA	Ribonucleic acid
RPL19	Ribosomal protein L19
RPS18	Ribosomal protein S18
RPS9	Ribosomal protein S9
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SD	Standard deviation
SDHA	Succinat dehydrogenase complex, subunit A
SMA	Smooth muscle actin
Tab.	Table
TBS	Tri-buffered-saline
Tris	Tris(hydroxymethyl)-aminomethan
U	Unit
UBA52	Ubiquitin A-52
UXT	Ubiquitously expressed transcript isoform 2
v/v	volume/volume (Volumenkonzentration)
VIP	Vascular initiation point
w/v	mass/volume (Massenkonzentration)
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooyxgenase activation protein, zeta polypeptide
ZP	Zona parenchymatosa ohne Corpus luteum
ZPCL	Zona parenchymatosa mit Corpus luteum
ZV	Zona vasculosa
VEGF-R2	Vascular endothelial growth factor-receptor 2

2 Einleitung und Ziele der Arbeit

Das Ovar ist ein komplexes Organ. Als Bestandteil des weiblichen Reproduktionstraktes unterliegt es erheblichen zyklischen Veränderungen, sowohl in morphologischer Hinsicht als auch auf molekularer Ebene. Beim monoovulatorischen Zyklus des Rindes entwickeln sich Keimzellen enthaltende Follikel. Mit dem sprungreifen Graaf-Follikel wird die Follikelreifung abgeschlossen. Nach dem Eisprung verbleibt eine Follikelhöhle. Die Wandzellen der Follikelhöhle, die sogenannten Granulosazellen, proliferieren jetzt enorm. Ein neuer Gelbkörper (Corpus luteum, CL) wächst heran [1-3]. Dieser nimmt während seiner Entwicklung eine unterschiedliche Morphologie an, nach denen das Stadium des Geschlechtszyklus eines Individuums bestimmt werden kann. Man unterscheidet folgende Lutealstadien: Anbildung, Blüte und Rückbildung sowie Trächtigkeit [4, 5]. Neben den periodisch auftretenden Veränderungen, kann das Ovar des Rindes morphologisch in die innen liegende Medulla (Zona vasculosa, ZV) und den äußeren Cortex (Zona parenchymatosa, ZP) eingeteilt werden. In der Zona parenchymatosa sind Funktionsgebilde, die Follikel bzw. Corpora lutea, lokalisiert. Die beiden Zonen ZV und ZP weisen jeweils ein heterogenes Zellbild auf. Insbesondere in der ZP wechselt der Anteil von Luteinzellen, Endothelzellen, Perizyten, glatten Muskelzellen, Fibroblasten und Fibrozyten im zyklischen Rhythmus. Die unterschiedlichen Lutealstadien und die mit ihnen einhergehenden Veränderungen der Zellzusammensetzung, führen zu einem sich immer wieder stark verändernden makroskopischen und mikroskopischen Erscheinungsbild des Ovars [6, 7].

Jedes Gewebewachstum führt zu einem erhöhten Bedarf an Nährstoffen und Sauerstoff, was Gefäßbildung erforderlich macht [8, 9]. Die Umwandlung der Wandung der Follikelhöhle in den Gelbkörper geht mit einer massiven Zellvermehrung und einem enormen Wachstum von Blutgefäßen einher. Im lutealen Stadium der Anbildung ist die Blutgefäßentwicklung mitunter intensiver als die in bösartigen Tumoren [10]. Die am Ende eines Zyklus folgende Luteolyse führt kontinuierlich zur Rückbildung von Blutgefäßen [1].

Zwei unterschiedliche Mechanismen sind für die Bildung der Blutgefäße verantwortlich. Zum einen Vaskulogenese, die de novo Differenzierung eines primitiven Gefäßnetzes aus endothelialen Vorläuferzellen, und zum anderen Angiogenese, die Sprossung von Kapillaren aus bereits bestehenden Blutgefäßen [1, 11]. Im Corpus luteum umfasst die sogenannte angiogene Phase, während derer es zur Bildung neuer Blutgefäße kommt, ein Drittel der gesamten Zykluslänge. Über 50 % der Zellen im Corpus luteum sind vaskulären Ursprungs [7, 12]. Bis jetzt gibt es weder eine Erklärung für das enorme angiogene Potential

im Corpus luteum noch für den Ursprung der proliferierenden Zellen. In den vergangenen Jahren veröffentlichte Studien lassen das Vorkommen von endothelialen Progenitorzellen bzw. Stammzellen als lokale Ressource für postnatale Vaskulogenese vermuten [13-17].
In einem vorangegangenen Projekt der Arbeitsgruppe um Prof. J. Plendl (Veterinär-Anatomie, Freie Universität Berlin) untersuchten Käßmeyer und Plendl [18] mikrovaskuläre Zellen in vitro, die aus dem Gelbkörper verschiedener Zyklusstadien des Rindes isoliert wurden. Sie beobachteten eine unterschiedliche angiogene Potenz der Zellen, sowohl Vaskulogenese als auch Angiogenese trat auf. Während isolierte Zellen aus dem Corpus luteum in Rückbildung Merkmale der angiogenen Kaskade zeigten, bildeten die aus dem Corpus luteum in Anbildung isolierten Zellen kapillarähnliche Strukturen via Vaskulogenese [18]. Aufgrund dieser Ergebnisse entstand die Hypothese der vorliegenden Arbeit, dass endotheliale Progenitorzellen im Ovar existieren und bei der Blutgefäßneubildung im Corpus luteum eine Rolle spielen könnten. Mit der vorliegenden Studie sollten die Ergebnisse der in vitro Modelle, die das Vorhandensein von endothelialen Progenitorzellen im Gewebe von Rinderovarien annehmen, in situ verifiziert werden.

Daraus ergab sich das erste Ziel dieser Arbeit:

1. Der qualitative und quantitative Nachweis von endothelialen Progenitorzellen in bovinen Ovarien.

Der qualitative Nachweis der endothelialen Progenitorzellen sollte über eine immun-histochemische Doppelmarkierung an Paraffinschnitten mittels der Marker KDR (vascular endothelial growth factor receptor 2, VEGF-R2) und CD34 erfolgen. Die Ko-Expression dieser beiden Marker ist allgemein für den Nachweis von endothelialen Progenitorzellen anerkannt [19-21]. Für die hier durchgeführten Untersuchungen kamen Ovarien von Schlachtrindern zum Einsatz. Die Ovarien wurden makroskopisch den verschiedenen Lutealstadien zugeordnet und anschließend erfolgte die Probenentnahme aus den Zonen ZV, wie auch ZP mit Corpus luteum (ZPCL) und ZP ohne Corpus luteum (ZP). Eine Überführung in die entsprechenden Fixantien erfolgte direkt nach der Probenentnahme. Die Quantität der Expression von *KDR*, *CD34* und auch des Stammzellmarkers *CD133* (*Prominin-1*[1]) [22-24] wurde während der unterschiedlichen Lutealstadien und innerhalb der verschiedenen Zonen

[1] zum Zeitpunkt der Untersuchungen war kein Antikörper gegen CD133, der mit bovinem Material kreuzreagiert, kommerziell erhältlich, daher konnte lediglich die quantitative Untersuchung des CD133 durchgeführt werden.

des Ovars auf molekularbiologischer Ebene via RT-qPCR (reverse transcription-quantitative polymerase chain reaction) Methode untersucht. Um verlässliche Ergebnisse mit Hilfe der RT-qPCR Methode erzielen zu können, muss eine Normalisierung der Ergebnisse mittels validierter Referenzgene erfolgen [25]. Für das Rinderovar standen für die geplante Untersuchung noch keine validierten und stabil exprimierten Referenzgene zur Verfügung, welche sowohl die morphologische als auch die zyklische Heterogenität des Organs berücksichtigten.

Vor diesem Hintergrund war das zweite Ziel dieser Arbeit:

2. Die Etablierung und Validierung adäquater Referenzgene für das bovine Ovar.

Die wesentliche Anforderung an Referenzgene ist eine stabile Expression in den zu untersuchenden Proben, unabhängig von Entwicklungsstadien und unabhängig von den zu untersuchenden Bedingungen [26] wie z. B. dem Lutealstadium oder der Lokalisation der Probenentnahme innerhalb des Ovars. Studien haben bereits belegt, dass viele „klassisch" genutzten Referenzgene schwankende Expressionen in unterschiedlichen Organen und Geweben zeigen [27-29]. Diese Schwankungen können zu beachtlichen Ungenauigkeiten während der Normalisierung führen [30]. Das heterogene morphologische Erscheinungsbild des Ovars [6, 31] lässt große dynamische Schwankungen in der Genexpression erwarten. Daher erfolgte in dieser Arbeit eine Analyse der Expressionsstabilität von 12 möglichen Referenzgenen mittels der Berechnungsalgorithmen GENORM [27], NORMFINDER [32] und BESTKEEPER [33]. Die Referenzgene sollten für die unterschiedlichen lutealen Stadien aus den Bereichen ZP, ZPCL und ZV validiert werden. Aus der Literatur wurden zwei Gruppen möglicher Referenzgene recherchiert und für die Untersuchungen herangezogen: Gruppe 1: bereits als stabil exprimiert für unterschiedliche Gewebe des Rindes empfohlen (*GAPDH*, *ACTB*, *YWHAZ*, *HPRT1*, *SDHA*, *UBA52*, *POLR2C*, *RPS9*) [33-36]; Gruppe 2: für andere Spezies als stabil exprimiert empfohlen, aber noch nicht an Rindergewebe getestet (*ACTG2*, *H3F3B*, *RPS18*, *RPL19*) [37-41]. Die in dieser Arbeit ermittelten stabil exprimierten Referenzgene erlaubten eine Normalisierung der Expression untersuchter endothelialer Progenitorzellen.

3 Publikationen

Die Forschungsergebnisse der vorliegenden Qualifikationsarbeit wurden als Originalartikel in internationalen Fachzeitschriften mit Gutachtersystem publiziert.

Identification of CD133-, CD34- and KDR-positiv cells in the bovine ovary: A new site of vascular wall resident endothelial progenitor cells

Kornelia Schoen, Ruth M. Hirschberg, Johanna Plendl and Sabine Kaessmeyer

Clinical Hemorheology and Microcirculation 52 (2012) 67–84

DOI 10.3233/CH-2012-1585

Identification of Stable Expressed Reference Genes for RT-qPCR Data Normalization in Defined Localizations of Cyclic Bovine Ovaries

K. Schoen, J. Plendl, C. Gabler and S. Kaessmeyer

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Beide Publikationen sind entsprechend der Vorgaben der jeweiligen Fachzeitschrift aufgebaut.

3.1 Identification of CD133-, CD34- and KDR-positive cells in the bovine ovary: A new site of vascular wall resident endothelial progenitor cells

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Abstract

Intense angiogenesis, vascular remodelling as well as regression of its vasculature are prerequisites for ovarian function with its cyclically developing and regressing follicles and corpora lutea. So far neither a stringent explanation for the enormous angiogenic potential of the ovary nor its cellular origins have been suggested. In an earlier study of our work group, endothelial cells were isolated from the bovine corpus luteum and cultivated *in vitro*. They performed vasulogenesis *in vitro* and showed properties of progenitor cells.

The present study aimed at *in situ* identification of endothelial progenitor cells (EPCs) in the bovine ovary. Immunohistochemical examinations, based on the detection of KDR and CD34 co-labelled cells – a marker combination that amongst others is commonly accepted as typical for EPC identification – was performed. Hormonal cycle dependent expression varieties were analysed by the measurement of mRNA amounts of *CD34* and *KDR* as well as the stem cell marker *CD133* (*Prominin-1*).

Ovarian samples comprising corpora lutea of varying stages (developing and mature corpus luteum, corpus luteum in regression, corpus luteum of pregnancy) from 17 adult cows were examined. Results show that specific mRNA of *CD133*, *CD34* and *KDR* was expressed in ovaries of all luteal stages. Expression data analysis revealed significant differences in *CD133* and *CD34* expression levels between the luteal stages but no significant differences in *KDR* expression. CD34/KDR co-immunoreactive cells were predominantly situated within the media of arterial vessel wall. The detection of ovarian EPCs represents an important step towards further understanding of the mechanisms involved in the reproductive biology and pathophysiology of the ovary.

Keywords: Adult endothelial progenitor cells, resident stem cells, adult vasculogenesis, Prominin-1, VEGF-R2, vessel wall, reproductive organ, hormonal cycle, qPCR, double immunohistochemistry

1. Introduction

The ovaries which are cytogenic and hormone-secreting organs undergo different functional stages: while initially providing an environment for the maturation of oocytes within follicles, after ovulation the newly formed corpora lutea develop, mature and finally regress [52]. Specific stages of the ovarian cycle are characterised by massive cell proliferations that are accompanied by an intense growth of blood vessels and radical changes of the vascular bed [6, 28, 30, 52]. Particularly the transition of the follicle into the corpus luteum displays a blood vessel development that is stronger than in highly malignant tumours [13]. More than 50 % of the proliferating cells during luteal growth are from vascular origin [21, 50]. Yet, neither a stringent explanation for the enormous angiogenic potential of this organ, nor the source of the proliferating cells has been suggested.

Two mechanisms account for the development of blood vessels, vasculogenesis and angiogenesis. The initial differentiation and assembly of endothelial precursor cells into a primitive vascular plexus is known as vasculogenesis. Expansion of this primitive vascular network depends on angiogenesis, in which new blood vessels sprout from pre-existing ones [16, 27, 52, 66].

In an earlier *in vitro* study of our work group [36], microvascular cells were isolated from the adult bovine corpus luteum. Those cell cultures showed morphologic properties of vasculogenic and angiogenic capillary development. Whereas the cells of cultures obtained from regressing corpus luteum displayed the features of the angiogenic cascade *in vitro* [7], cells isolated from a developing corpus luteum managed capillary tube formation according to vasculogenesis: Specific cells proliferated, while building cellular clusters which connected by bridging protoplasmatic protrusions and finally constructed a three-dimensional network of lumenised tubular structures [36]. These data lead us to the hypothesis that endothelial progenitor cells (EPCs) or stem cells could reside in ovaries and trigger the luteal neovascularisation. In the past decade several studies indicated the presence of EPCs and stem cells as a local reservoir for postnatal vasculogenesis in tissues, like the bone marrow, the peripheral blood and the connective tissue, in both adult animals and humans [12, 22, 44, 68, 75].

In order to verify this concept derived from our *in vitro* experiments, the present study was designed to examine the presence of endothelial progenitor cells within the ovary *in situ*.

Identification of vascular progenitor cells, also the visualisation of their distribution pattern was achieved by immunohistochemical examination that were based on labelling of vascular endothelial growth factor receptor 2 (KDR)- and CD34-co-expressing cells – two markers that amongst others are commonly accepted as typical for EPC detection (reviewed by: [37, 51, 53, 70]). For the analysis of hormonal cycle dependent expression varieties we measured mRNA amounts of the endothelial progenitor cell marker *CD34* and *KDR* as well as the stem cell marker *CD133* (*Prominin-1*) [42, 47, 72, 76], by qPCR. Therefore a representative number of ovaries, classified by their luteal stage (corpus luteum in development, mature corpus luteum, corpus luteum in regression and corpus luteum graviditatis) was collected and evaluated.

2. Materials and methods

2.1. Material

Ovaries were obtained from German Holstein Friesian dairy cows (Table 1) at a local abattoir within ten minutes after slaughtering. Only samples from individuals with healthy inner genital tract were accepted, which was verified by exterior and interior veterinarian examination. The ovaries were classified into four different luteal stages [32]: corpus luteum in development (haemorrhagicum), mature corpus luteum, corpus luteum in regression, identified by the presence of an early residual body (corpus albicans) and corpus luteum of pregnancy. The luteal stages were categorized by measuring the diameter of the corpora lutea and judging their typical morphological aspects [32]. Because only slaughterhouse waste was used for sample taking, neither institutional nor National Research Council guidelines had to be followed. For an overview of samples see Table 1.

2.1.1. Samples for qPCR

12 ovaries were collected, and from each luteal stage three samples were taken (Table 1). The ovaries were cut longitudinally and divided into three areas: a) the zona vasculosa (ZV), i.e. the highly vascular stroma in the center of the ovary, b) the part of the zona parenchymatosa containing the corpus luteum (ZPCL) and c) the part of the zona parenchymatosa surrounding the corpus luteum (ZP). From each area a sample of 5 mm in diameter was punched out and fixed in RNA later (QIAGEN, Hilden, Germany). The samples were immediately prepared and stored at 4 ºC until RNA extraction.

Table 1

Summarising of material (sample number, breed, and age of sampled cows) and stages of ovarian samples based on macroscopic examination according to Hünigen et al. [32].

	Sample no.	Breed	Age (month)	Staging based on macroscopic examination
Immunhistochemistry	IV	German HF	37	Regressing corpus luteum, late residual body / corpus albicans
	X	German HF	41	Corpus luteum of pregnancy
	XIII	German HF	42	Early regressing corpus luteum
	XIV	German HF	23	Mature corpus luteum
	CVII	Not documented	adult	Not documented
qPCR	13	German HF	12	Developing corpus luteum (corpus haemorrhagicum)
	14	German HF	12	Developing corpus luteum (corpus haemorrhagicum)
	15	German HF	12	Developing corpus luteum (corpus haemorrhagicum)
	16	German HF	61	Mature corpus luteum
	17	German HF	74	Mature corpus luteum
	18	German HF	45	Mature corpus luteum
	19	German HF	67	Regressing corpus luteum, early residual body / corpus albicans
	21	German HF	38	Regressing corpus luteum, early residual body / corpus albicans
	22	German HF	129	Corpus luteum of pregnancy
	23	German HF	109	Corpus luteum of pregnancy
	24	German HF	16	Corpus luteum of pregnancy
	26	German HF	140	Regressing corpus luteum, early residual body / corpus albicans

HF = Holstein Friesian

2.1.2. Samples for histology and immunohistochemistry

5 ovaries were obtained and cut into pieces of 1 cm^3, each specimen containing corpora lutea and follicles in varying stages of development (Table 1). The specimens were fixed in 4 % (v/v) neutral buffered formalin solution, then dehydrated in an ascending series of ethanol solutions and processed for embedding in paraffin wax according to standard procedures [57].

2.2. Methods

2.2.1. RNA extraction

Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany). 50 – 60 mg of each sample were homogenised with an Ultra Turrax homogeniser (IKA, Staufen, Germany). RNA concentration and purity were evaluated spectrophotometrically (BioPhotometer Eppendorf AG, Hamburg, Germany) by optical density (OD 260/280). Integrity of RNA was assessed using a 2 % denaturing agarose gel (Serva electrophoresis GmbH, Heidelberg, Germany). Until cDNA synthesis, the RNA samples were stored at −80 ºC.

2.2.2. Real-time PCR

Primer pairs for the different bovine (*Bos taurus forma domestica*) genes (*CD133*, *CD34* and *KDR*) as well as primer pairs for reference genes (*RPS9*, *RPS18* and *UBA52*) were designed using PRIMER3 software [58] and *in silico* verified via BLAST software [1]. Primer information (sequence, GenBank accession number and product size) is summarised in Table 2.

Table 2
Details of primers, GenBank accession numbers and amplicon sizes for all detected genes

Gene	Full gene name	GenBank accession number	Forward primer sequences (5´-3´) Reverse primer sequences (5´-3´)	Amplicon size (bp)
CD34	CD34 molecule	AB021662.1	AAGTGAAATTGGCCCACAGCATCTGC GCCTGTTTCTTCTGACACAGGAATCC	115
KDR	Kinase insert domain receptor	NM_001110000.1	CTGTGGCATCCGAAGGCTCAAACC GTGGTGTCCGTGTCGTCAGAATGG	73
CD133	Prominin 1	NM_001245952.1	ATCTGCAGTGGGTCGAGATGGC GGTCGACGATGTAGCTGCACAG	111
RPS18	Ribosomal protein S18	NM_001033614.1	GAGGAAAGCAGACATCGACCTCACC GGTGATCACACGTTCCACCTCATCC	73
RPS9	Ribosomal protein S9	NM_001101152.1	CGAGTATGGGCTCCGGAACAAACG GGGTCTTTCTCATCCAGCGTCAGC	105
UBA52	Ubiquitin A-52	NM_001076363.1	AGAAAGAGTCCACCCTGCACTTGG ACACTTGCGGCAGATCATCTTGTCG	113

Primers were ordered lyophilised from Eurofins (Eurofins MWG Operon, Ebersberg, Germany). The annealing temperatures were optimised by a gradient-PCR (Eppendorf Mastercycler Gradient, Hamburg, Germany) and the PCR products were additionally

sequenced (GenExpress, Berlin, Germany) to verify their specificity. Pooled cDNA of all samples was used for creation of standard curves (10-fold dilution series with five measuring points) to determine the slope and efficiency of the primer pairs.

An initial amount of 2 µg total RNA was used and treated with 2 U DNase I (Fermentas GmbH, St. Leon-Rot, Germany) for 30 min at 37 ºC. The reaction was stopped by heating for 10 min at 65 ºC after adding 2 µl 50 mM EDTA solution (Fermentas GmbH, St. Leon-Rot, Germany).

The Reverse Transcription (RT) mixture contained 4 µl 5× buffer, 20 U RNase Inhibitor, 2 µl 10 mM dNTP mix, 40 units M-MuLV reverse transcriptase, 0.5 µg oligo(dT) and 0.2 µg of random hexamer primers (all ingredients were obtained from First Strand cDNA Synthesis Kit, Fermentas). 9 µl from DNase I treated total RNA were added to 11 µl of the RT-mixture and incubated at 25 ºC for 5 min, followed by 60 min at 37 ºC for reverse transcription. Inactivation of the enzyme was achieved via heating (70 ºC) for 5 min. All samples including negative-controls were diluted to a ratio of 1:5 with DNase/RNase free water (Fermentas GmbH, St. Leon-Rot, Germany) and stored at −80 ºC for four weeks maximum.

The qPCR amplification was performed using a RotorGene 6000 Cycler (QIAGEN, Hilden, Germany). The reaction mix contained 10 µl Maxima SYBR Green qPCR Master Mix (Fermentas GmbH, St. Leon-Rot, Germany), 6.8 µl nuclease free water (Fermentas GmbH, St. Leon-Rot, Germany), 5 pmol of each primer and 2 µl 1:5 diluted cDNA in a total volume of 20 µl. All samples were analysed in triplicates and the mean of the triplicates were used for further calculations. Each run included a RT negative control and a non-template control with water instead of cDNA. All negative controls were also analysed in triplicates. Pooled cDNA was processed as an inter-run-calibrator according to Hellemans et al. [26]. To confirm the specificity of the PCR product a melting curve profile was generated at the end of each qPCR. The amplification started with an initial template denaturation at 95 ºC for 10 min, followed by 35 cycles with a melting step at 95 ºC for 15 s, primer annealing at 61 ºC for 30 s and elongation at 72 ºC for 20 s. After 35 cycles a final extension started at 72 ºC for 30 s. Subsequently, a melting curve analysis was performed starting at 72 ºC increased by 1 ºC each 5 seconds up to 95 ºC with continuous fluorescence measurement. The quantification cycles (Cq) and primer specific reaction efficiencies were entered in QBASEPLUS software (Biogazelle, Zwijnaarde, Belgium). The results were normalised with validated reference

genes (*RPS9*, *RPS18* and *UBA52*), which were stably expressed in all samples, independent of the luteal stage and localisation within the ovary.

The statistical analysis was performed using the software SPSS version 20 (IBM Deutschland GmbH, Ehningen, Germany). The raw data were grouped by the parameter luteal stage: with the levels – corpus luteum in development (haemorrhagicum) – mature corpus luteum – corpus luteum in regression – corpus luteum of pregnancy, as well as the parameter localisation: with the levels – zona vasculosa (ZV) – zona parenchymatosa containing corpus luteum (ZPCL) – zona parenchymatosa surrounding the corpus luteum (ZP). A two-way ANOVA test was performed for each primer pair to check whether there was a significant total effect of the parameters chosen. Data were logarithmically transformed and a normal distribution was assumed. The differences within the standard deviation were marginal and no weighting factors were calculated and additionally a bootstrap estimation for robust standard errors was realised.

Provided that there was a significant effect of the parameters, the *post-hoc* test Fisher-LSD was performed to investigate a pairwise comparison of the levels from the parameters stages and localisations. Statistical significance was set at p-value < 0.05. The correlations were calculated according to Pearson's correlation coefficient.

2.2.3. Histological staining and immunohistochemistry (IHC)

For histological staining and immunohistochemistry, from 5 ovaries (see Table 1) serial sections of approximately 5 to 6 µm thickness were cut on a microtome. These were dewaxed with xylene and rehydrated in a descending series of ethanol solutions. After the respective staining processes, the sections were again dehydrated in an ascending ethanol series, transferred into intermedium xylene and then covered with permanent mounting media (HistoPerm, HISTOPRIME®, Linaris Biologische Produkte, Dossenheim, Germany).

Sections from the 5 specimens collected for histological techniques were stained with haematoxylin and eosin (HE) according to standard procedures [57].

2.2.3.1. Antibodies

The expression of KDR receptor was investigated by immunohistochemistry using a mouse monoclonal primary antibody (Santa Cruz Biotechnology, Heidelberg, Germany) and a donkey-anti-mouse secondary antibody (Linaris Biologische Produkte GmbH, Dossenheim, Germany).

The detection of CD34 was performed by a monoclonal goat-anti-CD34 primary antibody (Santa Cruz Biotechnology, Heidelberg, Germany) and swine-anti-goat as secondary antibody (Linaris Biologische Produkte GmbH, Dossenheim, Germany). The commercially available CD34 antibody, which was used within this study, reacts with a human, murine and rat CD34-epitope and, according to the manufacturer, also with bovine and canine CD34-epitopes. However, some researchers regard cross-reactivity of the commercially available antibodies with bovine CD34-epitopes as uncertain [23, 49, 60] or even as unspecific while giving no details on antigen-retrieval [39, 63].

An antibody against CD133 (Prominin-1) antigen, which cross-reacts with bovine, was not commercially available.

2.2.3.2. Double immunostaining

Another series of the same 5 ovarian specimens (see Table 1) was submitted to double IHC against KDR and CD34 according to Table 3. Double IHC was repeated at least three times for each individual.

Negative controls were performed in which the primary antibody was replaced by (a) buffer and (b) non-immune serum (Table 3). Positive tissue controls were carried out on appropriate tissue sections (Table 3).

Sections were washed in citrate buffer (0.01 M, at pH 6.0) for 5 min at room temperature. Antigen retrieval was achieved by heating in citrate buffer for 20 min at 95 ºC. Sections were then cooled to room temperature in citrate buffer for 20 min and washed in aqua bidest. for 2 min followed by Tris-buffered-saline-(TBS)-buffer (0.05 M Tris-HCl pH 7.6 + 0.9 % w/v NaCl) for 2 × 3 min. Blocking of endogenous peroxidase activity was achieved by immersion in TBS-buffer with 0.6 % (v/v) H_2O_2 for 20 min, followed by washing in TBS-buffer plus 0.01 % (v/v) polysorbate 20 (Tween20, Sigma-Aldrich Chemie AG, Taufkirchen, Germany) 2 × 3 min.

Table 3

Antibodies for immunohistochemistry, dilutions, and controls used for double immunolabelling

Antibody	Item	Dilution	Manufacturer	Negative serum control	Positive tissue control
Anti-KDR: primary mouse monoclonal	A-3: sc-6251	1:50	Santa Cruz Biotechnology, Heidelberg, Germany	Mouse-IgG1 serum, 1:25 (x0931, Dako)	lung tissue, human kidney tissue, rat
secondary antibody: donkey anti-mouse IgG:HRP	ZMH2162	1:50	Linaris Biologische Produkte GmbH, Dossenheim, Germany		
Anti-CD34: primary goat monoclonal antibody	C-18: sc-7045	1:50	Santa Cruz Biotechnology, Heidelberg, Germany	Goat-IgG1 serum, 1:50 (sc-2028, Santa Cruz)	lung tissue, human skin tissue, human
secondary antibody: swine anti-goat IgG (H+L):HRP	ZZH5160	1:5	Linaris Biologische Produkte GmbH, Dossenheim, Germany		

The slides were then pre-incubated with 20 % (v/v) donkey IgG serum in incubation buffer A (0.05 M Tris-HCl pH 7.6 + 0.9 % w/v NaCl + 0.66 mM $MgCl_2$ + 1 % w/v BSA + 0.1 % w/v gelatin + 0.01 % v/v Tween20) for 20 min. Incubation with the KDR-antibody (1:50 in incubation buffer A + 2 % v/v donkey serum) was carried out overnight at 4 ºC. After triple washing in TBS-buffer for 5 min at room temperature, the slides were incubated with the donkey anti-mouse secondary antibody (1:50 in incubation buffer A + 2 % v/v donkey serum) for 30 min. The slides were then rinsed in TBS 2 × 3 min, followed by phosphate-buffered saline (PBS) according to Dulbecco (Serva 47402, Heidelberg, Germany), at pH 7.4 for 3 min. The enzyme-substrate reaction was visualised by diaminobenzidine (DAB) (Sigma-Aldrich Chemie, Deisenhofen, Germany) at room temperature for 20 min under microscopic control: 20 mg DAB powder solved in 200 ml PBS, filtered via SS 593 filter, with 50 µl H_2O_2 (30 % v/v) added before use at pH 7.6. The slides were then washed in PBS for 3 min and in TBS for 2 × 3 – 5 min followed by endogenous peroxidase blocking as already mentioned above for 10 min, followed by washing in TBS (0.05 M Tris-HCl pH 7.6 + 0.9 % w/v NaCl + 0.01 % v/v Tween 20) 2 × 3 – 5 min. Subsequent pre-incubation was carried out for 30 min at room temperature with 20 % (v/v) swine IgG serum in incubation buffer B (0.05 M Tris-HCl pH 7.6 + 0.9 % w/v NaCl + 0.66 mM $MgCl_2$ + 1 % w/v BSA + 0.1 % w/v

gelatine + 1 % w/v milk powder (milk powder, blotting grade; Roth, Karlsruhe, Germany) + 0.01 % v/v Tween20). Incubation with the CD34-antibody (1:50 in incubation buffer B + 2 % v/v swine serum) was carried out overnight at 4 ºC. After triple washing in TBS-buffer for 5 min at room temperature, the slides were incubated with the swine anti-goat secondary antibody (1:5 in incubation buffer B + 2 % v/v swine serum) for 30 min. The slides were then rinsed in TBS 2 × 3 min, followed by PBS according to Dulbecco for 3 min. Chromogen detection was achieved employing HistoGreen (5 – 10 min, HISTOPRIME®, Linaris Biologische Produkte, Wertheim, Germany) according to manufacturer's instructions, followed by consecutive washing in bi-distilled water and tap water, 30 s each. Nuclei were counterstained with haemalaun according to Mayer (Fluka-Chemie, Buchs, Switzerland) for 20 s, followed by washing in tap water for 2 × 20 s and tap water + 0.08 % v/v ammonia for 30 s.

2.2.4. Digital image recording

Representative images from the sections of each specimen were digitally recorded employing a light microscope (Axioskop, Carl Zeiss, Oberkochen, Germany), a digital camera (DS-Ri1, Nikon, Düsseldorf, Germany) and a PC-based laboratory imaging programme (NIS-Elements, Version 3.0, Nikon, Düsseldorf, Germany).

3. Results

3.1. Evaluation of qPCR

3.1.1. Quality controls

Optical density (OD) 260/280 was checked spectrophotometrically to control RNA purity. The mean ratio of all samples was 1.96. The RNA integrity of each sample for further analysis was tested electrophoretically with 2 % denatured agarose gel and the 28S rRNA signal was nearly doubly intense than the 18S rRNA signal related to luminance and thickness. The maximum RNA integrity has to have a ratio of 2.0 [10].

Standard curves were accomplished for each primer pair using pooled cDNA. The slopes of the standard curves were -3.336 (*CD133*), -3.406 (*CD34*) and -3.315 (*KDR*). The efficiencies of the primers pairs were 0.988 (*CD133*), 0.997 (*CD34*) and 0.998 (*KDR*). Results of amplification efficiencies, correlation coefficient and the slope of all primers including reference genes are summarized in Table 4.

Table 4
Slopes, correlation coefficients (r^2) and efficiencies from qPCR standard curves of all studied genes

Gene Symbol	Slope	Correlation coefficient (r^2)	Efficiency (%)
CD34	-3.406	0.997	97
KDR	-3.315	0.998	100
CD133	-3.336	0.988	99
RPS18	-3.339	0.999	99
RPS9	-3.358	0.998	99
UBA52	-3.280	0.999	102

RT-negative controls and non-template controls were realized in all runs. Cq values for these controls were negative or showed Cq values over 30.

Normalisation of the data was performed using the reference genes *RPS9*, *RPS18* and *UBA52*, stably expressed in all samples, independent of the luteal stage and localisation within the ovary.

3.1.2. Gene expression levels

Specific mRNA of *CD34*, *KDR* and *CD133* was expressed in all analysed luteal stages as well as in each localisation in the bovine ovary (presented in Figs. 1 and 2).

Expression of *CD34* mRNA showed varying levels in the different luteal stages (Fig. 1). High expressions were detected in ovaries of developing and mature corpus luteum whereas it was low in the luteal stages regression and pregnancy. The differences in *CD34* expression between all luteal stages revealed a significance of $p = 0.006$. Expression differences between the luteal stages mature and regression ($p = 0.003$), respectively mature and pregnancy ($p < 0.002$), were significant whereas mature and development were insignificant ($p = 0.093$). These results were confirmed by the 95 % confidence interval of the bootstrap-estimation for multiple comparisons. The *CD34* expression displayed no variation between ovarian localisations (p-value was < 0.591) (Fig. 2).

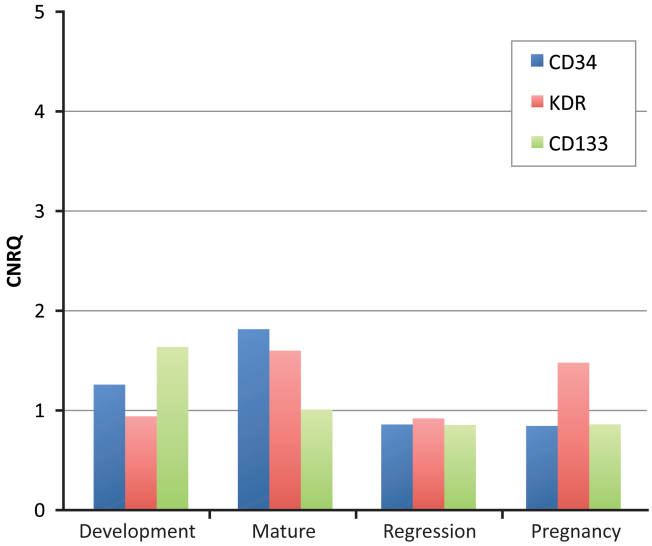

Fig. 1. Calibrated normalised relative quantities (CNRQ) of *CD34*, *KDR* and *CD133* merged by the stages of the corpora lutea (in development, mature, regression and pregnancy).

The mRNA expressions of *KDR* showed no significant variations in the four luteal stages ($p < 0.346$) (Fig. 1), whereas the p-value < 0.002 of the three localisations within the ovary indicated significant differences. The expression level in the localisation ZPCL was significantly higher than in ZV ($p < 0.004$) and in ZP ($p < 0.001$) (Fig. 2).

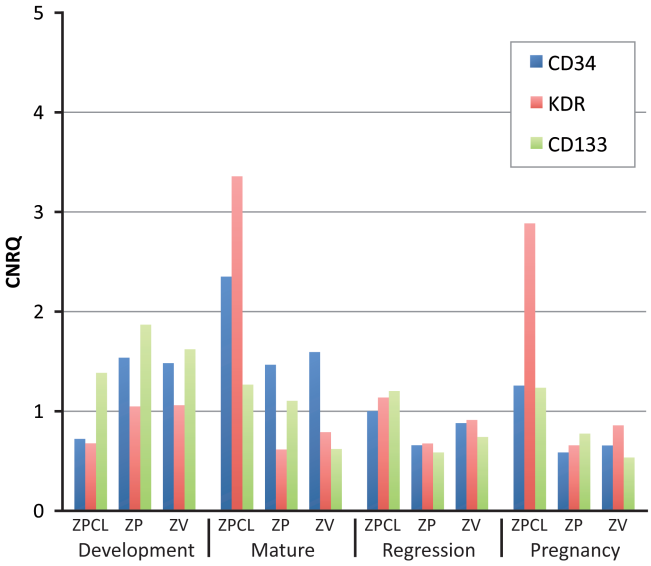

Fig. 2. Calibrated normalised relative quantities (CNRQ) of *CD34*, *KDR* and *CD133* merged by the stages of the corpus luteum (in development, mature, regression and pregnancy) and the localisations within the ovary, i.e., zona parenchymatosa with corpus luteum (ZPCL), zona parenchymatosa without corpus luteum (ZP) and central zona vasculosa (ZV).

Data analysis revealed high variation in *CD133* expression between the luteal stages, with a significance of $p = 0.001$. The luteal stage "development" showed a significantly ($p < 0.005$) higher expression of *CD133* than mature as well as regression and pregnancy (both < 0.001). The luteal stages mature, regression and pregnancy showed no significant variations among each other (Fig. 1).

Variation in *CD133* expression in the three ovarian localisations was slightly significant ($p = 0.041$). The *p*-value of the localisation ZV and ZPCL signalised a significant difference ($p = 0.012$), which was confirmed by the bootstrap-estimation for multiple comparisons (Fig. 2).

3.1.3. Pearson's correlation coefficient

Pearson's correlation coefficients (r) were calculated to detect possible linear relationships between the investigated markers. The highest correlation ($r = 0.641$) and highest significance ($p < 0.001$) were observed between *CD34* and *KDR*. *CD34* and *CD133* demonstrated a correlation of $r = 0.452$ with a significance of $p = 0.006$ whereas *CD133* and *KDR* showed the lowest correlation ($r = 0.385$) with $p = 0.021$ significance.

3.2. Evaluation of immunohistochemistry

3.2.1. Controls

No staining was visible in the negative buffer controls. Negative serum controls displayed very weak uniformly distributed brownish background staining (KDR-immunolabelling) as well as minimal green precipitations within larger vessel lumina and the periphery of the specimens (CD34-immunolabelling). Both these types of staining were non-cell-bound and thus regarded as non-specific. Immunoreactivity for CD34 was always assessed within areas that displayed no unspecific precipitations. The positive tissue controls (see Table 3) displayed immunoreactivity as expected.

3.2.2. Double-immunoreactivity

Double reactivity of both KDR and CD34 was detected only within tunica media cells of vascular walls (exemplary in Fig. 3a and with higher magnification in Fig. 3b), predominantly of arteries and arterioles and only sporadically of veins and venules. The co-labelled cells appeared either isolated or in small clusters in cross sections of the vessel media (Fig. 3c).

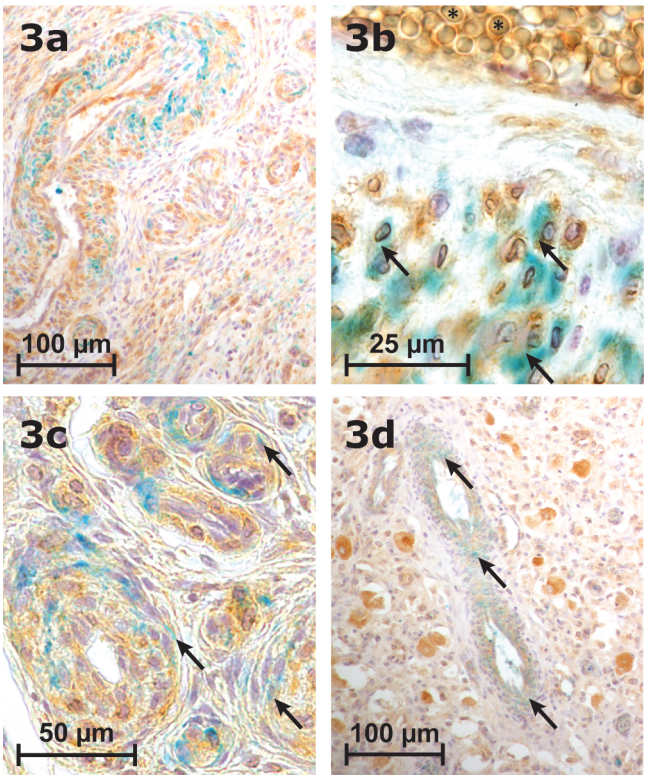

Fig. 3. 3a and b sample no. XIII, c sample no. IV and 3d sample no. CVII, all samples show ovaries with a corpus luteum in early regression. Double-IHC (KDR: brown + CD34: green); (3a) Stromal arteries of the ovarian tissue outside of the corpora lutea displaying co-reactive media cells, predominantly in the middle and outer layers of the media. (3b) Higher magnification of an arterial vessel wall displays media cells (arrows) that are immunoreactive for both, KDR and CD34. The lumen is filled with falsely immunopositive erythrocytes (asterisks, insufficient blocking of endogenous peroxidase). (3c) shows arterioles in which predominantly the outer media cells display double reactivity (arrow) for CD34 and KDR. (3d) Larger artery in longitudinal section showing double-reactive media cells in the course of the vessel (arrows).

Co-reactive cells were localised predominantly in bigger arteries supplying the tertiary follicles and corpora lutea, in smaller blood vessels surrounding these functional bodies but also within corpora lutea (Figs. 3d, 4a, b). No double reactivity within the smooth muscle cushions of sphincteric arteries or the (sub-) intimal cushions was detected (Fig. 4c). Nearly no microvascular endothelial CD34 staining was detected in our study within the examined ovaries.

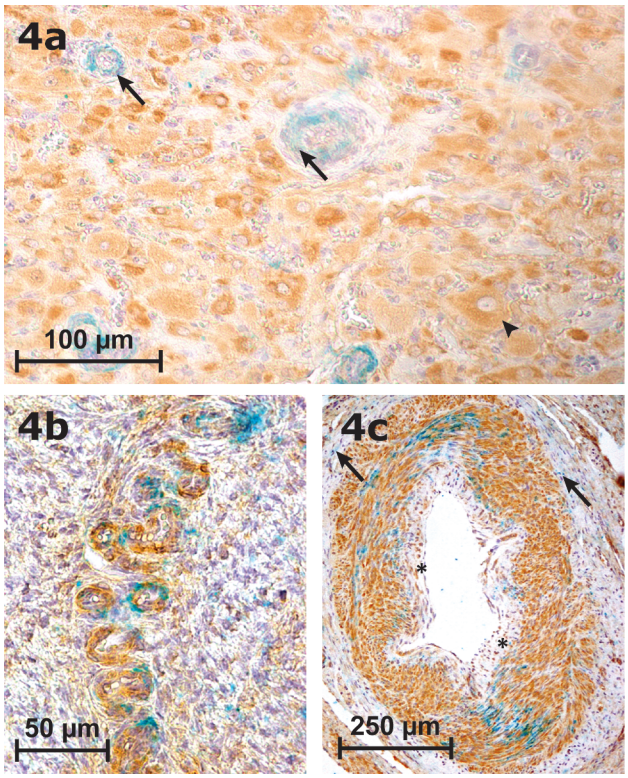

Fig. 4. 4a sample no. XIV with mature corpus luteum and 4b + 4c sample no. X with corpus luteum of pregnancy. Double-IHC (KDR: brown + CD34: green); (4a) higher magnification of corpus luteum vasculature displaying double-reactive media cells in cross sections (arrows) and luteinised cells (arrowhead) with only KDR positive immunoreactivity. (4b) Convoluted small stromal artery in vicinity to the corpus luteum displaying co-reactive cells within the media within the whole section. (4c) shows a bigger stromal artery in vicinity to the corpus luteum with an (sub-) intimal cushion (asterisks), smooth muscle strands in two differently orientated layers, clusters of co-reactive cells within the media and single CD34-reactive adventitial cells (arrows).

4. Discussion

The aim of this study was to investigate the presence of putative resident EPCs *in situ* within the bovine ovary as our work group had isolated endothelial cells from a developing corpus luteum that displayed vasculogenic characteristics *in vitro* in a previous study. The investigated cells *in vitro* displayed immunoreactivity to a canine CD34 antibody[1] and vascular endothelial growth factor receptor 2 (KDR) antibody[2] [36].

Our current study revealed mRNA expression of the stem cell marker *CD133* and the endothelial progenitor cell markers *CD34* and *KDR* in all luteal stages and in each analysed localisation of the bovine ovary *in situ*. The selected markers KDR and CD34 in combination

[1] Antibody no longer commercially available; originally selected because of high percentage of amino acid sequences identity in canine and bovine CD34 (see: Zhou et al., 2001) [77].

[2] Antibody obtained from Santa Cruz Biotechnology, Heidelberg, Germany.

are commonly regarded as typical for vascular progenitor cells, particularly for EPCs (e.g. [75], reviewed by: [17, 37, 41, 51, 70]). Moreover, *in situ* double immunostaining with different markers is considered particularly important in order to complement data gained on *in vitro* culture systems [18]. CD133 (Prominin-1) became a distinct molecular marker in identification and separation of stem and precursor cells due to its characteristic of down-regulated expression in differentiated cells [7, 6]. Yin et al. [72] firstly described that CD133-positive cells proliferated non-adherently in medium under selective conditions and could be stimulated to differentiate into CD133-negative, mature, adherent endothelial cells. Initially it was assumed that CD133-positive cells represent adult hemangioblasts [4, 8, 61, 73]. In the last years, CD133 expression has been detected in various sources like embryonic stem cells, permanent tissue stem cells and circulating endothelial progenitors as well as cancer stem cells, beside haematopoietic cells [45, 46, 74]. Today CD133 is considered to play an important role in specific sorting of stem cells as well as in the studies on stem cell related diseases [20, 45, 47, 76].

Thus, our results indicate the presence of stem cells or/and EPCs within the bovine ovary. Interestingly, older studies from the 30 s and 40 s of the last century already described the unique arrangement and morphology of the ovarian, and also the luteal, vasculature [11, 31] that even at that time were interpreted as postembryonal angioblasts – i.e., vascular progenitor cells – by some researchers [31, 40]. This old – until now unproven – hypothesis has nowadays gained new importance, since the concept of adult vascular stem and progenitor cells has been commonly accepted after the ground-breaking detection of adult EPCs in the 1990s [3, 62]. Since then, adult blood vessel development is conceived as a combination of vasculogenesis and angiogenesis (e.g. reviewed by [37, 54]).

The expression of *CD34* and *CD133* mRNA showed high variations between the luteal stages. Highest expression of both markers was detected in the luteal stages development and mature. Furthermore *CD133* revealed a significantly higher expression ($p < 0.005$) in the developmental luteal stage than in mature. In relation to the developmental stage *CD133* expression was significantly reduced in the luteal stages regression and pregnancy ($p < 0.001$). *CD34* gene expression differences were only significant between the luteal stages mature and regression ($p = 0.003$) respectively pregnancy ($p < 0.002$).

In an earlier study [36] on different forms of vascular development *in vitro* by microvascular cells derived from the developing and regressing bovine corpus luteum, specific cell groups

so-called “Vascular Initiation Points” (VIPs) were detected. These VIPs, found only in cultures derived from the developing corpus luteum, were highly proliferative and represented CD34 reactive cell clusters from which capillary-like tubes with an internal lumen developed *in vitro*, resembling the mechanism of vasculogenesis. This and the immunohistochemical characterisation of the VIPs suggested that these cells might represent EPCs [36]. Thus, the cells of the VIPs could relate to the CD34/KDR reactive cells detected *in situ* within developmental respectively mature luteal stages of the ovary, and probably constitute a source of EPCs. The VIP cells might also relate to the CD133 expressing stem cells now detected within ovaries of the developmental respectively mature stages *in situ*. The putative EPC nature of the doubly-labelled cells of the present study is further corroborated by the high correlation ($r = 0.641$) with a significance of $p < 0.001$ between *CD34* and *KDR* observed in our present study.

Expression of *KDR* showed no significant variations between the luteal stages. However, significant differences of expression were obvious between the localisations within the ovary. Zona parenchymatosa tissue samples including the corpus luteum (ZPCL) displayed highest expression levels of *KDR* ($p = 0.001$). The corpus luteum consists of different types of *KDR* expressing cells like EPC, endothelial cells and granulosa cells [24]. Therefore a high expression of *KDR* within the corpus luteum in our qPCR results is not remarkable. Several other studies confirm these results by immunohistochemical findings on the bovine ovary (e.g. [32, 33]) and the ovary of various other species, including human (e.g. [15]/human, [64]/rat, [43]/canine, [35]/porcine).

Co-expression of CD34 and KDR is a widely used surface marker combination to identify and purify cells via flow cytometry from various sources, which are able to generate endothelial cells *in vitro*, suggesting that CD34 positive cells contain EPCs [3, 5, 75]. Although numerous data are available on the isolation of EPCs by CD34/KDR-co-expression, the discussion upon the identification of EPCs is still controversial since CD34 positive and KDR positive cells also represent mature ECs. Besides being a marker for EPCs, CD34 is generally regarded as a marker for microvascular endothelial cells in most organs of primates and rodents (e.g. [67]), including the ovary (e.g. [14, 25, 71]). KDR has a crucial role in both EPC as well as endothelial cell biology and therefore it is ubiquitously detectable on those cells [65]. In our study microvascular endothelial cells were nearly never labelled immunohistochemically within the ovarian tissue. Several other groups studied the reactivity of CD34 in different tissues respectively organs and of different species. In summary, the expression pattern of

CD34 (in different splice-variants) seems to depend on the organ, the localisation within an organ, and it even changes from individual to individual of the same species, including bovine, e.g. [23, 49, 60, 67], thus backing our present results.

By common consent, CD34 phenotype cells that co-express CD133 are accepted to identify 'true EPCs' [47, 55]. Our conception on the presence of stem/progenitor cells within the ovarian tissues investigated is confirmed by the mRNA expression correlation between *CD34* and *CD133* of $r = 0.452$ with a significance of $p = 0.006$ while *CD133* and *KDR* showed only low correlation ($r = 0.385$) with $p = 0.021$ significance.

With regard to the enormous angiogenic potential of the ovary with its cycling functional bodies, the need for tissue-immanent stem cells/EPCs as a locally and thus rapidly accessible source for all neovascular processes appears plausible. In this regard, it is of particular importance to perceive that oestrogen mobilises inactive EPCs and thus initiates neovascularisation, and that serum oestradiol levels correlate significantly with the number of circulating EPCs ([34], reviewed by [37]). The follicular granulosa cells are the main production site of oestradiol within the female body. Oestradiol is also produced in arterial walls and evidently at least in humans most pronounced in the smooth muscle cells of the tunica media (e.g. [48]), while oestrogen synthesis and functional oestrogen receptors have been demonstrated in bovine aortic endothelial cells [9]. Thus, an oestradiol-regulated activation of stem cells/EPCs might be expected, particularly so within the ovary.

Immunohistochemical examination displayed co-labelled KDR- and CD34-reactive cells within the media of arterial and – only sporadically – venous vessel walls of the bovine ovary. The co-labelled cells appeared either isolated or in small clusters in cross sections of the vessel media. Thus, immunohistochemical results of the present study indicate the vessel walls of the bovine ovary as a possible source of the EPCs (Fig. 5, schematic overview of vessel wall layers). These co-reactive putative EPCs were localised predominantly in bigger arteries supplying tertiary follicles and corpora lutea, in smaller blood vessels surrounding these functional bodies, but also within corpora lutea. In another study of our work group on the alpha-Smooth Muscle Actin (SMA) reactivity of the cycling bovine ovary [29] distinct isolated non-SMA-reactive cells within the ovarian vessel wall media were reported as an ancillary finding – representing media cells not committed to a vascular smooth muscle cell but possibly to an endothelial progenitor cell differentiation. These results are further confirmed by findings of qPCR in the present study. No significant expression differences of

CD34 and *CD133* between the localisations ZV, ZPCL and ZP (more explanations see please in chapter 2.1.1.) within the ovary were detectable. Putative vascular progenitor cells detected in this study were primarily located in the tunica media (and only rarely in the adventitia). Other groups also detected cells with progenitor potential in this vessel wall region (reviewed by [70]). Sainz and co-workers [59] isolated side population cells with EPC- and/or smooth muscle PC-characteristics specifically from the tunica media of the murine aorta.

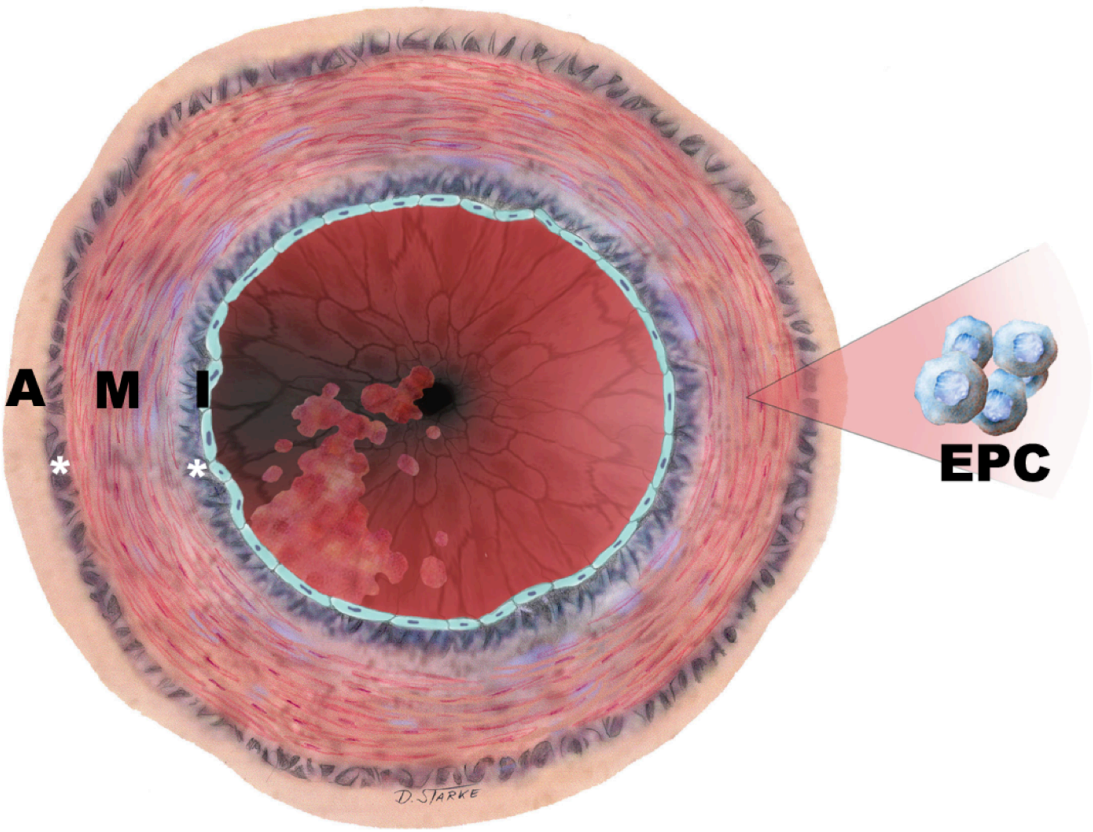

Fig. 5. Schematic drawing of the vessel wall layers displaying localisation of putative endothelial progenitor cells (EPCs) in the tunica media layer of arteries. Generally, the vessel wall consists of the tunica intima layer (I) comprising endothelial cells and pericytes, the tunica media layer (M) with vascular smooth muscle cells amongst connective tissue elements, and the adventitial layer (A) that consists mostly of connective tissue elements. Respective inner and outer elastic laminae (asterisks) separate these vessel wall layers.

Stem or progenitor cells residing in this peripheral so-called “vasculogenic niche” (e.g. reviewed and termed by [17]) have been suggested to be vascular wall endothelial progenitor cells, smooth muscle progenitor cells and mesenchymal stem cells. Those cells have been detected in embryonic aorta, adult larger blood vessels and adult intra organ arteries and veins (e.g. liver, heart, kidney, bladder, lung, brain) of varying calibre in different species, particularly human [19], reviewed by [37, 38, 51, 53, 70] – but, to the best of our knowledge,

not in the ovary of any species. Regarding the angiogenic potential of the corpus luteum the detection of vascular progenitor cells within this organ is of even greater significance.

Physiological blood vessel growth in the developing corpus luteum is more intense than in the highly malignant tumour glioblastoma multiforme and more than 50 % of the cells of the mature corpus luteum are of vascular origin [6, 21, 50]. Just recently, it has been described that a population of glioblastoma stem-like cells maintains glioblastoma tumours, because they displayed typical characteristics of stem cells. These tumour stem cells give rise to phenotypically and functionally endothelium-like cells of neoplastic origin [56]. An emerging role for stem-like cells in ovarian cancer has also been described (reviewed by [69]). These findings evoke the idea that comparable mechanisms might enable the enormous physiological neovascularisation potential of the ovary and its cycling functional structures.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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The manuscript was written in accordance with the ethical guidelines of *Clinical Hemorheology and Microcirculation* [2].

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3.2 Identification of Stably Expressed Reference Genes for RT-qPCR Data Normalization in Defined Localizations of Cyclic Bovine Ovaries

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Summary

Ovaries are highly complex organs displaying morphological, molecular and functional differences between their cortical zona parenchymatosa and medullary zona vasculosa, and also between the different cyclic luteal stages. Objective of the present study was to validate expression stability of twelve putative reference genes (RGs) in bovine ovaries, considering the intrinsic heterogeneity of bovine ovarian tissue with regard to different luteal stages and intra-ovarian localizations. The focus was on identifying RGs, which are suitable to normalize RT-qPCR results of ovaries collected from clinical healthy cattle, irrespective of localization and the hormonal stage. Expression profiles of twelve potential reference genes (*GAPDH*, *ACTB*, *YWHAZ*, *HPRT1*, *SDHA*, *UBA52*, *POLR2C*, *RPS9*, *ACTG2*, *H3F3B*, *RPS18* and *RPL19*) were analysed. Evaluation of gene expression differences was performed using GENORM, NORMFINDER, and BESTKEEPER software. The most stably expressed genes according to GENORM, NORMFINDER and BESTKEEPER approaches contained the candidates *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C* and *UBA52*. Of this group, the genes *YWHAZ*, *H3F3B* and *RPS9* could be recommended as best-suited RGs for normalization purposes on healthy bovine ovaries irrespective of the luteal stage or intra-ovarian localization.

Introduction

Ovaries are cytogenic and hormone-secreting organs that provide an environment for the development of oocytes within follicles. Ovaries undergo various functional stages and consequently display a complex macroscopical and microscopical anatomy, which frequently is not considered in studies on ‘the ovary’. After ovulation, the corpus luteum develops from residual follicular cells (corpus haemorrhagicum). Primary function of the highly vascularized mature corpus luteum is secretion of progesterone, a hormone that is required for maintenance of pregnancy. If fertilization does not occur, the corpus luteum regresses and finally is transformed into an avascular scar-like tissue, the corpus albicans (reviewed by Plendl, 2000).

Bovine ovaries can be categorized according to their functional stages by the diameter of their corpus luteum and its typical morphological characteristics (Hünigen et al., 2008). Accordingly, the following stages are distinguished: developing corpus luteum (haemorrhagicum), mature corpus luteum, regressing corpus luteum and corpus albicans. Furthermore, in condition of gravidity, a corpus luteum of pregnancy can be identified. Each luteal stage goes along with the secretion of a specific combination of gonadal hormones and

massive modifications of cellular composition within the functional bodies and the surrounding stroma, characterized by proliferation, differentiation and apoptosis of cells (Schams and Berisha, 2004; Kaessmeyer and Plendl, 2009; Sakumoto et al., 2010).

In addition to cyclical changes, ovaries display regional variations of their anatomical architecture (Davis and Rueda, 2002; Kliem et al., 2007). Thus, most mammalian ovaries, including bovine ovaries, are divided into an inner zona vasculosa or medulla (ZV) and a surrounding outer zona parenchymatosa or cortex. Only the latter contains follicles and corpora lutea. Thus, it can be further subdivided into a zona parenchymatosa either with a corpus luteum (ZPCL) or without corpus luteum (ZP). Meandering arteries and veins are found in the ZV within a network of loosely arranged connective tissue and elastic fibres. In contrast, spinocellular connective tissue forms a grid-like structure in the ZP. In this zone, metabolically active stroma cells and oocytes of various developmental stages are present. Thus, each region is comprised of characteristic cell populations. Oocytes, follicular epithelial cells, luteal granulosa and theca cells are located exclusively in the cortex. Endothelial cells, pericytes, smooth muscle cells, and fibroblasts or fibrocytes are located in both regions (Young, 1911; Plendl, 2000; Wendl et al., 2012). Function and morphology of the ovary are controlled by a highly regulated expression and interaction of multiple genes in its different localizations and cyclical stages (Bonnet et al., 2008; Kenngott et al., 2013). Accordingly, specific activation of primordial follicles or the immense neovascularization of the corpus luteum during its development or apoptosis of cells in the regression stage can only be explained by site-specific stimulating or inhibitory factors (Klagsbrun and D'Amore, 1991; Plendl, 2000). Regarding the outstanding intrinsic heterogeneous nature of the ovarian tissue (Skarzynski et al., 2009; Aerts and Bols, 2010), it is obvious that considerable dynamic biological ranges in gene expression occur during luteal stages and also in different localizations (e.g. within different types of cells or follicles) of the ovary. Thus, various functional differences related to hormonal regulation and specific heterogeneity of this organ with regard to localization have to be considered when planning an experiment.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is an efficient method for quantification of gene expression and – from the viewpoint of an anatomist – can be regarded as a molecular scalpel to obtain most exact results. However, one weakness of RT-qPCR remains the difficulty in determination of the most optimal normalization strategy for mRNA quantification (Huggett et al., 2005), especially for comparative samples displaying intrinsic tissue heterogeneity. Reference genes (RGs), also called internal control

genes or housekeeping genes, are generally used to normalize the amount and quality of starting material for cDNA synthesis (Nygard et al., 2007). Selection of proper RGs for the tissues of interest is an essential step in the experimental design. It is recommended to use more than one single RG for normalization (Vandesompele et al., 2002). Characteristics of optimal RGs include their stable expression in comparative samples unrelated to tissue differences, e.g. through developmental and life stages, and that they are not affected by experimental conditions or treatments (Walker et al., 2009; Rekawiecki et al., 2012). Objective of the present study was to validate stably expressed RGs, considering the intrinsic heterogeneity of bovine ovarian tissue with regard to different luteal stages and intra-ovarian localizations. Even though excellent studies evaluating reference genes in bovine corpora lutea are available (Pfaffl et al., 2004; Rekawiecki et al., 2012), RGs for expression analysis throughout the different localizations during both pregnancy and the cycling bovine ovary have not been examined until now. Thus, expression stability of twelve candidate RGs was investigated within the localizations ZV, ZP and ZPCL of ovaries at different luteal stages, i.e. stage of development, maturity, regression and pregnancy. Twelve putative reference genes were selected from a nearly infinite pool of potential RGs described in the literature. Eight of these selected genes showed a stable expression in several different bovine tissues and were subsequently recommended (*GAPDH*, *ACTB*, *YWHAZ*, *HPRT1*, *SDHA*, *UBA52*, *POLR2C* and *RPS9*) (Pfaffl et al., 2004; Bionaz and Loor, 2007; Lisowski et al., 2008; Hosseini et al., 2010). Four genes had been evaluated as reference genes for other species but had not been tested on bovine tissues yet (*ACTG2*, *H3F3B*, *RPS18* and *RPL19*) (van Wijngaarden et al., 2007; Facci et al., 2011; Li et al., 2011; Peletto et al., 2011; Zhang et al., 2012). Systematic evaluation was performed using the algorithms GENORM, NORMFINDER and BESTKEEPER.

Material and Methods

Sample preparation and RNA extraction

Ovaries were obtained from Holstein dairy cows at the local abattoir within 10 min after slaughtering. Only samples from individuals with healthy genital tracts were taken, which was confirmed by veterinarian examination. Ovaries were classified into four different luteal stages, according to Hünigen et al. (2008): developing corpus luteum (haemorrhagicum) day 1–7, mature corpus luteum day 8–17, regressing corpus luteum day 18–21 and corpus luteum of pregnancy. Luteal stages were categorized by measuring the diameter of the dominant

corpus luteum and assessing typical morphological characteristics of the ovary and especially of the corpora lutea. Three ovaries were collected from each of the four luteal stages. As only abattoir waste was used for sample collection, institutional or national research council guidelines did not have to be adhered to. Each ovary was cut longitudinally and divided into the three localizations: zona parenchymatosa without corpus luteum (ZP), zona vasculosa (ZV) and zona parenchymatosa containing a corpus luteum (ZPCL) (see Fig. 1).

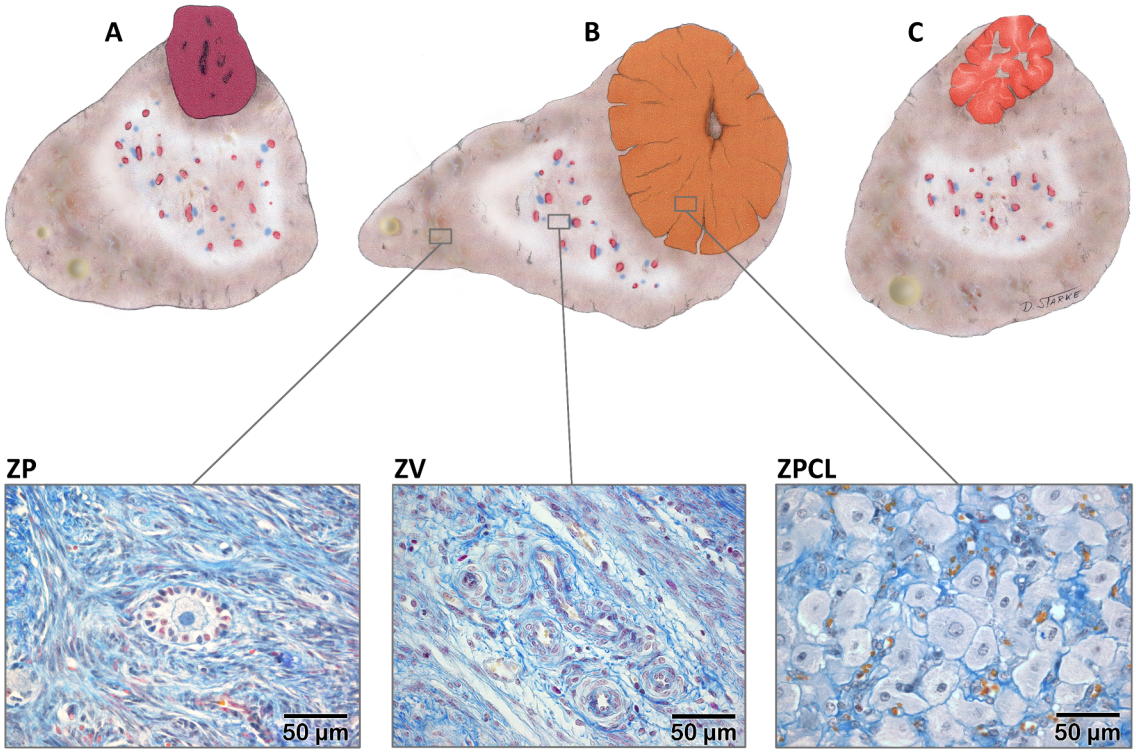

Fig. 1. Luteal stages and localizations of the ovary. Schematic drawing to illustrate the varying macroscopical and microscopical structure of ovaries during the different luteal stages [(A) developing, (B) mature and (C) regressing corpus luteum]. The histological sections, stained according to Ladewig show the typical structural architecture from different regions (from left to right: zona parenchymatosa (ZP) with a follicle, zona vasculosa (ZV) with arteries and veins integrated in a network of connective tissue and elastic fibers, and zona parenchymatosa with a corpus luteum (ZPCL) containing luteal cells of different sizes surrounded by small blood vessels).

A piece of 5 mm in diameter was punched out from each localization and fixed in RNAlater (QIAGEN, Hilden, Germany). In total, 36 samples were collected, processed immediately and stored at 4 °C until RNA extraction. All these samples were subsequently used for RT-qPCR analysis considering the MIQE guidelines (Bustin et al., 2009).

Total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the instructions provided by the manufacturer. Fifty to sixty milligrams of each sample was homogenized twice for 10 s with an Ultra Turrax homogenizer (IKA, Staufen, Germany) in RLT lysis

buffer, with a pause of 60 s for cooling on ice in between. RNA quality and quantity were examined prior to further analysis. Samples were mixed with Tris-HCl buffer (pH 8.0) (Carl Roth, Karlsruhe, Germany) for spectrophotometrical analysis using a BioPhotometer (Eppendorf, Hamburg, Germany). RNA concentrations were measured at 260 nm. Only samples with an OD 260/280 ratio of 1.9–2.0 were used for further analysis. An OD ratio > 1.8 is generally accepted as an indicator for sufficient RNA quality (Fleige and Pfaffl, 2006). RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) by loading 1 µl total RNA onto a RNA 6000 Nano Chip (Agilent) according to manufacturer's instructions. RNA samples were stored at -80 °C after extraction.

Samples for histological staining were fixed in 10 % neutral-buffered formalin solution for at least 24 h. Subsequently, samples were dehydrated in an ascending series of ethanol solutions and xylene for embedding in paraffin wax according to standard procedures (Romeis, 2010). Serial sections of approximately 5–6 mm thickness were sliced and dewaxed with xylene and rehydrated in a descending series of ethanol solutions and finally in tap water. Sections were stained in Weigert's iron hematoxylin (Merck, Darmstadt, Germany) for 5 min, rinsed briefly in H_2O_{dest}, transferred into 5 % phosphotungstic acid (Honeywell-Riedel-de Haën, Seelze, Germany) for 3 min and rinsed again briefly in H_2O_{dest}. This was followed by 4 min of staining in aniline blue – acid fuchsine – gold orange (aniline blue from Merck; acid fuchsine and gold orange from Chroma, Münster, Germany) and rinsed for 1 s in H_2O_{dest}. Finally, residuals of aniline blue were washed out in 96 % ethanol for 1 min. After dehydration in absolute ethanol solution and the intermedium xylene, the sections were embedded in Roti-Histokit (Roth).

cDNA synthesis

DNase treatment was performed prior to cDNA synthesis to remove any genomic DNA. DNase digestion was carried out in a total volume of 10 µl containing 1 µg total RNA, 1 U DNase I (Fermentas, St. Leon-Rot, Germany), 1 µl 10x DNase reaction buffer (Fermentas) and DNase/RNase-free water (Fermentas). The reaction mixture was incubated at 37 °C for 30 min. Subsequently, 1 µl 25 mM EDTA solution (Fermentas) was added, followed by heating at 65 °C for 10 min to inactivate DNase I. The reaction mixture was placed on ice immediately. Reverse transcription was performed with First Strand cDNA Synthesis Kit (Fermentas) as follows: A Master Mix was prepared, which contained 4 µl 5x reaction buffer, 20 U RNase Inhibitor, 1 mM dNTP mix, 40 U M-MuLV reverse transcriptase, 1 µg oligo(dT)

and 0.5 µg of random hexamer primers in a total volume of 11 µl per sample. 9 µl DNase I treated RNA was added to the Master Mix and incubated at 25 °C for 5 min (primer annealing), followed by 60 min at 37 °C and 5 min at 70 °C to inactivate the enzyme. RT-negative controls (DNase I treated RNA and Master Mix without RT-enzyme) were performed to examine the success of DNA digestion. Moreover, no template controls (NTC) with water instead of RNA were incorporated in each run to exclude the presence of any contaminations. cDNA and negative controls were diluted with DNase/RNase-free water (Fermentas) to a ratio of 1:5. A five-point standard curve was prepared using a 10-fold dilution of cDNA from a pool of all samples to determine the slope and efficiencies of all primers. cDNA aliquots were stored at -80 °C without repeated thawing and refreezing.

Primer design and qPCR

All primers for the twelve candidate RGs were newly designed using PRIMER3 software (Rozen and Skaletsky, 2000) and are intron-spanning except for *HPRT1*, *RPL19*, *SDHA* and *RPS9*. All primer sequences were verified *in silico* by checking specificity of the primer sequences using BLAST software (Altschul et al., 1990). In preliminary experiments, optimal annealing temperature was determined by a gradient-PCR with a Mastercycler gradient (Eppendorf) to be 61 °C for all primer pairs. Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All PCR products were sequenced (GenExpress, Berlin, Germany). They showed a 100 % homology of the amplicon as compared with the known bovine sequences. The primer sequences, GenBank accession numbers, function, expected product sizes and melting points are presented in Table 1. In addition, all primer sequences were uploaded to the public database RTprimerDB, where primers can be accessed for free to prevent time-consuming primer design (Pattyn et al., 2003). One of the quality control factors in the RT-qPCR phase is the calculation of a standard curve for each primer pair, which consists of a 10-fold dilution series with five measuring points derived from the formula $E = 10^{(1/-slope)} - 1$. The correlation coefficient (r^2) demonstrates the linearity of the standard curve. To determine the amplification efficiency, the slope of the standard curve was calculated.

qPCR amplification was performed using a RotorGene 6000 Cycler (QIAGEN). All samples were analysed in triplicates. The means of the triplicates were used for further calculations. Each run included RT-negative controls and non-template controls as triplicates. According to Hellemans et al. (2007), pooled cDNA of all samples was processed as an inter-run-

calibration to correct possible run-to-run differences. The reaction mix contained 10 µl Maxima SYBR Green qPCR Master Mix (Fermentas), 2 µl 1:5 diluted cDNA, 5 pmol of each primer and nuclease-free water (Fermentas) in a total volume of 20 µl. Amplification started with an initial activation at 95 °C for 10 min, followed by 35 cycles with a melting step at 95 °C for 15 s, primer annealing at 61 °C for 30 s and elongation at 72 °C for 20 s.

Table 1. Details of primers, amplicons and melting points for the 12 candidate reference genes

Gene	Full gene name	Accession number/ Reference	Function	Forward primer sequences (5´→3´) Reverse primer sequences (5´→3´)	Amplicon size (bp)	Melting points (°C)
ACTB	Beta-actin	NM_173979.3	Cytoskeletal structural protein	GAGAAGAGCTACGAGCTTCCTGACG AGGATTCCATGCCCAGGAAGGAAGG	106	84
ACTG2	Actin, gamma-enteric smooth muscle	NM_001013592.1	Component of the cytoskeleton; mediator of internal cell motility	CTCTATGCTTCTGGCCGCACAACG TCAAGGCGCATGATGGCATGAGG	113	82.8
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034.1	Glycolytic enzyme	AAGGGCATTCTAGGCTACACTGAGG ACAAAGTGGTCGTTGAGGGCAATGC	113	82.6
H3F3B	H3 histone	NM_001242571.1	Involved in structure of chromatin and nucleosomes	TCCAGAGGTTGGTGAGGGAGATCG ACCCACCAGATACGCTTCGCTAGC	107	83.6
HPRT1	Hypoxanthine phosphoribosyl-transferase 1	NM_001034035.1	Catalyses purine recycling in all cells	GTGCTTAGGAAAGTAGCGAGAGGC ATGCGGTCTCTCTGACACAATGCC	75	80.2
POLR2C	Polymerase II polypeptide C	NM_001034212.1	Involved in synthesizing messenger RNA	TGACAATGCCCTGAGGCACACG AACCTCTCTGGCTTGCCGTTGG	123	83.8
RPL19	Ribosomal protein L19	NM_001040516.1	Encodes a ribosomal protein	TCGCTGTGGCAAGAAGAAAGTCTGG AGCCCATCTTTGATCAGCTTCCG	102	81.7
RPS18	Ribosomal protein S18	Schoen et al. (2012)	Encodes a ribosomal protein	GAGGAAAGCAGACATCGACCTCACC GGTGATCACACGTTCCACCTCATCC	73	83.5
RPS9	Ribosomal protein S9	Schoen et al. (2012)	Encodes a ribosomal protein	CGAGTATGGGCTCCGGAACAAACG GGGTCTTTCTCATCCAGCGTCAGC	105	81.7
SDHA	Succinate dehydrogenase complex, subunit A	BC105357.1	Mitochondrial protein, catalyses the oxidation of succinate	GACAACTGGCGGTGGCACTTCTACG CCTGCTCCGTCATGTAGTGGATGGC	88	83.7
UBA52	Ubiquitin A-52	Schoen et al. (2012)	Protein degradation	AGAAAGAGTCCACCCTGCACTTGG ACACTTGCGGCAGATCATCTTGTCG	113	84.1
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxy-genase activation protein, zeta polypeptide	NM_174814.2	Mediates signal transduction	ACCGCTACTTGGCTGAGGTTGC GCTGTGACTGGTCCACAATCCC	63	79

After 35 cycles, a final extension started at 72 °C for 30 s. Subsequently, a melting curve followed each RT-qPCR to confirm specificity of the PCR product. The melting curve started

at 72 °C. Every 5 s the temperature increased by 1 °C up to 95 °C with fluorescence measuring continuously. Cq values of the results were exported from the rotor-gene 6000 software (QIAGEN) into QBASEPLUS (Biogazelle, Zwijnaarde, Belgium), NORMFINDER and BESTKEEPER. BESTKEEPER uses raw Cq values, whereas for NORMFINDER and GENORM analysis the raw Cq values were transformed to relative quantities by delta-Ct method.

Software approaches

Expression stability value (M-value) was calculated according to the GENORM algorithm (Vandesompele et al., 2002), which is implemented in the QBASEPLUS software. The M-value was calculated as average pairwise variation of one RG compared with all other RGs. M-value allows ranking and sorting of the candidate RGs based on their expression stability. Lower values are assigned to the most stable genes. The next step for selection of a proper set of RGs was the stepwise exclusion of the least stably expressed candidate reference genes, i.e. those with the highest M-value. Subsequently, new M-values were calculated for the remaining RGs. This procedure was repeated until the average M-value of the remaining RGs was below 0.5. The optimal number of the RGs for normalization was calculated as GeNormV value (Vandesompele et al., 2002). The GeNormV value indicates the effect of including an additional gene into the calculation for normalization factors and is helpful to minimize the number of required RGs. The GeNormV value is the pairwise variation of the resultant normalization factors (NFs) between n and $n + 1$ RG in each bar and should be below the critical limit of 0.15.

NORMFINDER uses a model-based approach (Andersen et al., 2004), which compares expression values of all candidates by variance analysis. All genes were used first for estimation of intragroup variations, followed by estimation of inter-group expression variations. NORMFINDER combines the intragroup and inter-group expression variation to calculate a stability value. This stability value allows for ranking of candidate RGs by their expression stability. Lower values indicate an increased stability in gene expression. An overall sample size of 36 was grouped by the luteal stages development, maturity, regression and pregnancy, with nine samples each. Alternatively, samples were also grouped according to their localizations, i.e. ZV, ZPCL and ZP, with 12 samples each. After excluding the least stably expressed genes *ACTG2*, *SDHA*, *HPRT1* and *GAPDH*, a recalculation was started and the order of RGs changed accordingly.

The excel-based tool BESTKEEPER (Pfaffl et al., 2004) uses raw Cq values and calculates the standard deviation (SD) for each candidate RG. A pairwise correlation analysis was performed to investigate inter-gene relations of all possible RG combinations. Candidate RGs examined here with SD > 1 can be considered as least stably expressed, whereas the most stably expressed RGs showed the lowest SD. A BESTKEEPER Index was calculated from stably expressed RGs (SD < 1) as a geometric mean of the Cq values from a maximum of ten RGs. The Pearson correlation coefficients were calculated by both pairwise correlation analysis between each RG and between the BESTKEEPER Index and each RG. The results of the latter correspond to the BESTKEEPER correlation coefficient. There was no specific threshold for the BESTKEEPER correlation coefficient with higher values, indicating suitable RGs.

Results

The amplification efficiencies of the used primer pairs ranked from 97 to 102 %. The coefficient of determination (R^2) varied between 0.988 and 0.999. Quantification cycle (Cq) values ranged between 10 and 23 (see Fig. 2).

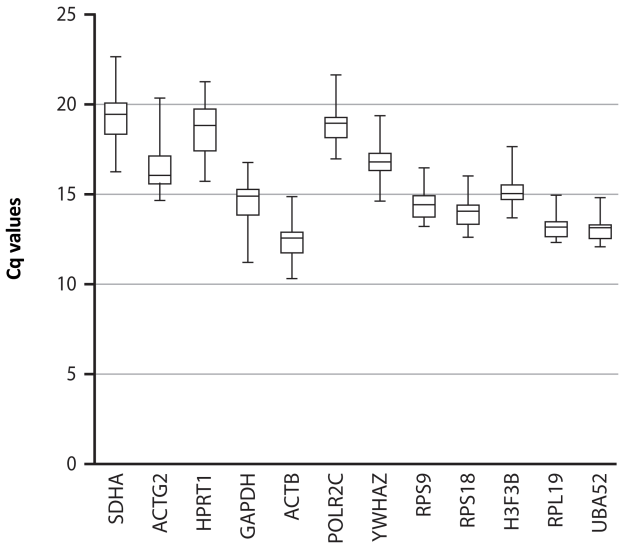

Fig. 2. Boxplot of Cq values. The variations of the quantification cycle (Cq) values are shown for all examined genes from bovine ovary samples (sorted by expression range). Each box represents the 50 % quartile, whereas the median is figured as the line in each box. The whiskers depict the 25 % minimum and maximum range of the Cq values.

Candidate RGs had been included in the GENORM calculation. The average M-value was below 0.5 when first excluding *ACTG2*, followed by *SDHA*, *HPRT1*, *GAPDH* and finally *ACTB*. The remaining seven genes with an average M-value below 0.5 were the following: *POLR2C*, *YWHAZ*, *H3F3B*, *RPL19*, *UBA52*, *RPS9* and *RPS18* (see Fig. 3a). In this study, the

GeNormV value of the first bar V2/3 (NFs of *RPS18* and *RPS9* compared to NFs of *RPS18*, *RPS9* and *UBA52*) was 0.095 and therefore below the critical limit of 0.15 (Fig. 3b). Thus, *RPS18* and *RPS9* were selected as adequate RGs using GENORM.

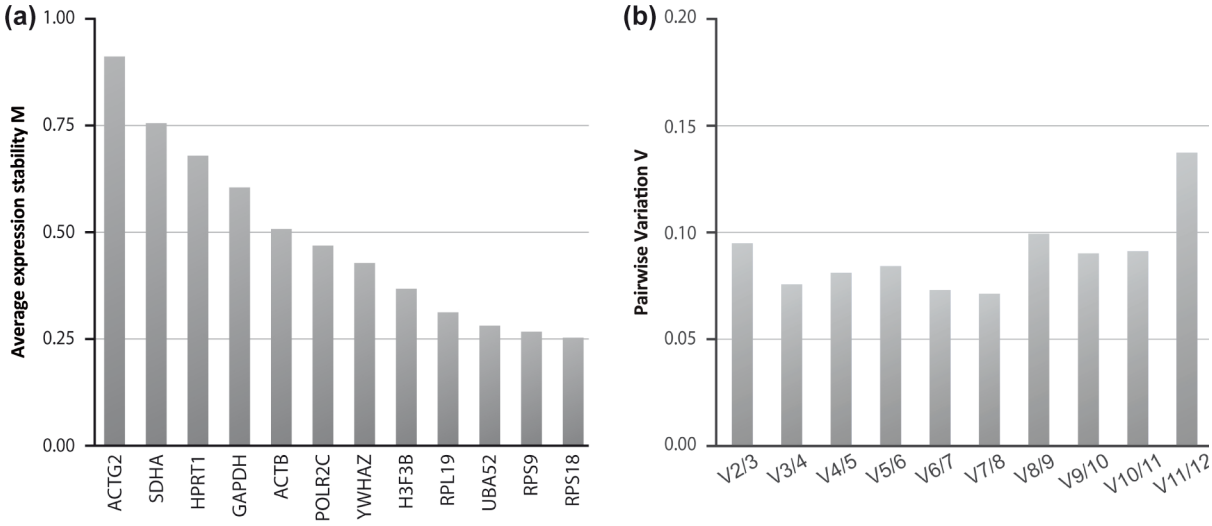

Fig. 3. (a) GeNorm M expression stability. GENORM algorithm ranked candidate RGs by pairwise variation according to an average expression stability M (M-value), which was calculated by stepwise exclusion of the least stably expressed RGs until the M-value of the remaining genes was below 0.5. The figure shows the order of RGs according to their expression stability from left (least stably expressed) to right (stably expressed). (b) Determination of the optimal number of reference genes for normalization. The GeNormV value shows the pairwise variation between the normalization factors of RGs (V_n/V_{n+1}) and demonstrates the effect of including an additional RG into the calculation for normalization factors. Values below the critical limit of 0.15 indicate an optimal number of RGs for normalization.

NORMFINDER identified *ACTG2*, *SDHA*, *HPRT1* and *GAPDH* as the least stably expressed genes. *H3F3B*, *YWHAZ*, *RPS9*, *POLR2C*, *RPS18* and *UBA52* were classified as most stably expressed genes in varying order, which depend on setting parameters such as group formation and number of analysed RGs. *YWHAZ* and *H3F3B* were selected as optimum pair of RGs (stability value 0.061) when implementing all investigated genes into the calculation and taking the different localizations ZV, ZP and ZPCL (i.e. without differentiation of the luteal stages) into account (see Fig. 4a). *POLR2C* and *RPS18* were identified as best gene combination (stability value 0.071), when taking different luteal stages (i.e. comparison of all luteal stages not differentiating the localizations) into account (see Fig. 4b). The genes *ACTG2*, *SDHA*, *HPRT1* and *GAPDH*, which showed the highest stability values (considering luteal stages > 0.237; considering localizations > 0.508) were removed from the panel of analysed genes. Subsequent reanalysis of the remaining RGs showed changes of the best gene combinations. *RPL19* and *YWHAZ* (stability value 0.067) were identified as best RG

combination considering the localizations, respectively, *RPS9* and *RPS18* (stability value 0.076), considering the luteal stages.

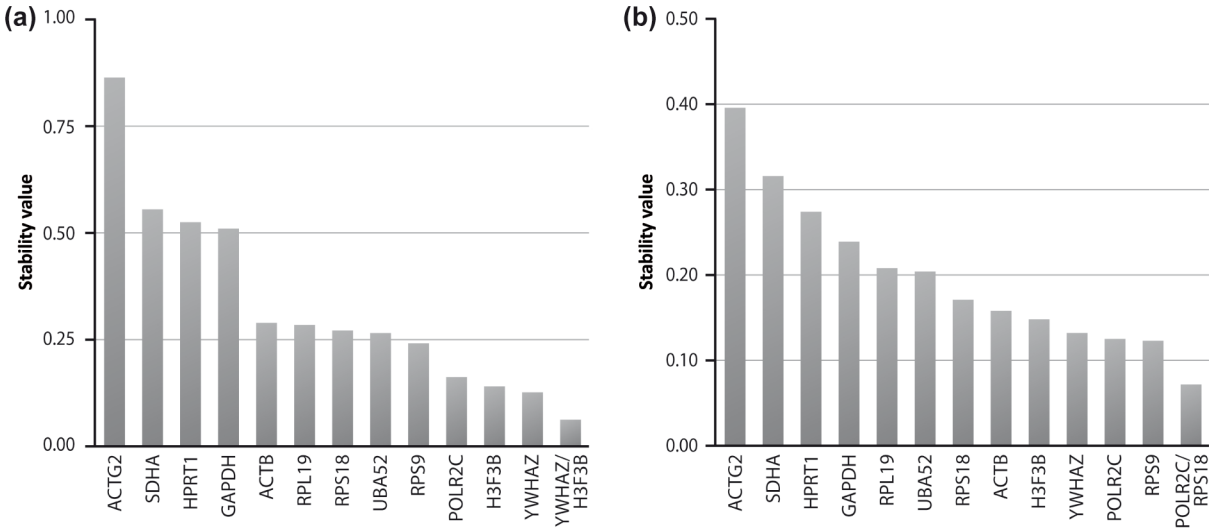

Fig. 4. NORMFINDER calculations. NORMFINDER algorithm ranked genes by calculated stability values, whereas lower stability values indicated greater expression stability. Calculating intra- and inter-group variations: (a) NORMFINDER results considering different localizations (zona vasculosa, zona parenchymatosa with a corpus luteum and without corpus luteum); (b) NORMFINDER results considering different luteal stages (stage of development, maturity, regression and pregnancy).

BESTKEEPER calculated a SD > 1 for the genes: *SDHA* (1.28), *HPRT1* (1.26), *ACTG2* (1.18) and *GAPDH* (1.07). All other RGs showed a SD < 1 and were included in further calculations: *ACTB* (0.83), *POLR2C* (0.76), *YWHAZ* (0.72), *RPS9* (0.6), *RPS18* (0.59), *H3F3B* (0.52), *RPL19* (0.5) and *UBA52* (0.44). In addition, up or down regulation of RG expression was calculated as the SD of the absolute regulation coefficient: *ACTB* (±1.77), *POLR2C* (±1.70), *YWHAZ* (±1.65), *RPS9* (±1.52), *RPS18* (±1.51), *H3F3B* (±1.43), *RPL19* (±1.41) and *UBA52* (±1.36).

The pairwise correlation analyses were realized between each RG pair ($0.872 < r < 0.958$), as well as each RG and the BESTKEEPER Index ($0.87 < r < 0.96$) (see Fig. 5). The results indicate high correlations combined with high significance levels ($P < 0.001$).

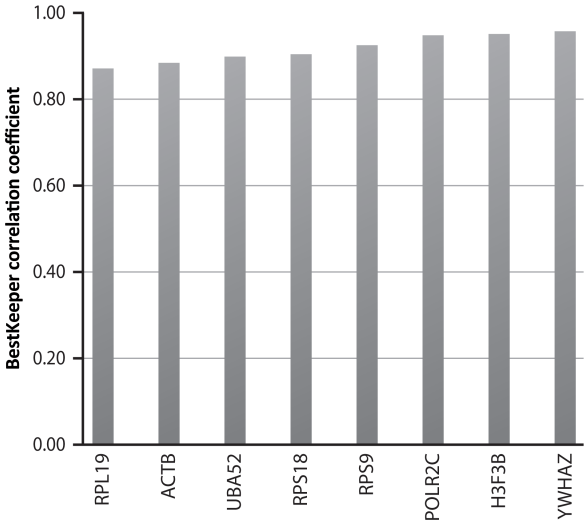

Fig. 5. BESTKEEPER calculation. BESTKEEPER ordered candidate RGs by the determined BESTKEEPER correlation coefficient, whereas only genes with a SD < 1 were included into the calculation. Lower values indicate least stably expressed genes, and higher values indicate more stably expressed genes (ordered from left to right).

Discussion

The heterogeneous nature of the ovary is manifested by its specific cellular composition within the localizations ZV, ZP and ZPCL as well as different luteal stages concerning the corpus luteum, i.e. development, maturity, regression and pregnancy. By creating the experimental design of comparative gene expression studies, consideration of those specific intra- and inter-ovarian features is indispensable to obtain reliable results. Focus of this study was the evaluation of RGs, which show stable expression independent of intra- and inter-ovarian cellular or ovarian cyclic differences. Stability rankings, obtained with GENORM, NORMFINDER and BESTKEEPER approaches, showed the candidate RGs *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C* and *UBA52* as suited best for normalization. The three approaches displayed differences in the ranked order only.

RPS9 and *RPS18* were identified by GENORM as best RGs for normalization purposes. The order of the remaining stably expressed genes corresponds to that resulting from the range of Cq values (see Fig. 2). Expression of these genes does not appear to be related to either the luteal stage or localization. RPS9 and RPS18 belong to a group of ribosomal protein genes and are involved in ribosome production (Robledo et al., 2008). However, prerequisite of GENORM calculation is that the candidate genes are not co-regulated, because a possible co-regulation of candidate genes could influence the efficiency of this algorithm due to the use of pairwise comparisons (Andersen et al., 2004; Żyżyńska-Granica and Koziak, 2012). A co-regulation of the ribosomal genes *RPS9* and *RPS18* is likely and could be the reason for the

selection by GENORM. On the other hand, various ribosomal protein genes are both suitable RGs (de Jonge et al., 2007; Popovici et al., 2009; Serrano et al., 2011) and significantly varying genes (Thorrez et al., 2008). The alleged drawback of a potential co-regulation of stably expressed ribosomal protein genes which are used as RGs could not be confirmed in various other studies either (Bionaz and Loor, 2007; Ahn et al., 2008). Moreover, NORMFINDER takes co-regulation into account, and selected also *RPS9* and *RPS18* in this study as best-suited RGs when sorted according to different luteal stages after excluding least stably expressed RGs from the calculation. Bionaz and Loor (2007) described UXT (ubiquitously expressed transcript isoform 2), *RPS9*, and *RPS15* as being stably expressed in the bovine mammary gland and as suitable RGs. During the lactation cycle, the mammary gland is characterized by cellular and molecular changes, which compares with changes in the ovary during the estrous cycle. However, results on RGs of the mammary gland cannot be transferred directly to the ovary. In addition, dilution effects of genes or RGs have been described previously for the bovine mammary gland (Sorensen et al., 2006; Bionaz and Loor, 2007) and may also be present in ovarian tissue. The GENORM algorithm allows for a potential dilution effect on gene expression (Bionaz and Loor, 2007).

In addition to intra-group variations, the NORMFINDER algorithm also takes inter-group variation into account (Andersen et al., 2004), which are defined by the parameters luteal stage and localization in this study. NORMFINDER selected *YWHAZ* and *H3F3B* as well as *POLR2C* and *RPS18* as best gene combination for normalization. *YWHAZ* and *H3F3B* are also identified as best-suited RGs based on BESTKEEPER, whereas *POLR2C* and *RPS18* are ranked on the third and fifth position, respectively. A benefit of the NORMFINDER algorithm is that this software avoids misinterpretations caused by artificial selection of co-regulated genes due to its intra- and inter-group variation measurements (Andersen et al., 2004). Thus, NORMFINDER calculations are not affected significantly by co-regulation of candidate reference genes (Beekman et al., 2011). On the other hand, NORMFINDER results strongly depend on the number of samples and candidates analysed (Andersen et al., 2004). Mehta et al. (2010) reported that their NORMFINDER results varied with an increase in sample size and corresponded better to GENORM analysis. Andersen et al. (2004) recommended to choose genes with no expression difference between groups so that the average expression level shows correspondence between different groups. Thus, RGs with significant differences between groups were excluded from the calculation. Further evaluation showed that ranking of the candidates changed when reanalysed. Exclusion of least stably expressed genes resulted

in NORMFINDER selecting the same RGs as best combination as GENORM, i.e. the ribosomal genes *RPS9* and *RPS18*, when grouped by different luteal stages. It is remarkable that GENORM also identified two ribosomal genes as best-suited RGs. Thus, in contrast to GENORM analysis, the NORMFINDER approach serves as an effective method to avoid the effect of co-regulation (Mehta et al., 2010).

The BESTKEEPER algorithm has the advantage to determine a standard deviation (SD) for each investigated RG. A normal distribution is a prerequisite for further analysis (Khanlou and Van Bockstaele, 2012). However, BESTKEEPER software can only compare ten RGs at a time. BESTKEEPER determined a high SD for *SDHA*, *HPRT1*, *ACTG2* and *GAPDH*, which corresponds to the calculations of GENORM and NORMFINDER. BESTKEEPER determined the genes *YWHAZ*, *H3F3B*, *POLR2C*, *RPS9*, *RPS18* and *UBA52* (from most to least stable) as best-suited RGs. Only the order of RG varied when compared to the other algorithms. *YWHAZ*, which is the best-ranked RG by BESTKEEPER, is ranked on the sixth position by GENORM and on the fourth or second position by NORMFINDER depending on the samples grouped either according to luteal stages or localizations. Other studies demonstrate high expression stability of *YWHAZ* in different pre-implantation embryonic stages (Goossens et al., 2005), polymorphonuclear lymphocytes (De Ketelaere et al., 2006), peripheral lymphocytes (Spalenza et al., 2011), liver, or kidney (Lisowski et al., 2008) of bovine species. H3F3B is involved in the structure of chromatin and nucleosomes (Frank et al., 2003) and had not been tested in the bovine yet. The highest expression of *H3F3B* was found in the ZPCL, the most active component of the ovary with regard to metabolism. NORMFINDER ranked this gene at all parameter settings from the first to the fourth, BESTKEEPER on the second and GENORM on the fifth position. The high ranking by all used algorithms showed *H3F3B* as an appropriate RG. Thus, in this study *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C* and *UBA52* were considered as appropriate RGs to normalize mRNA expression of targeted genes within ovaries in comparative studies. The results are obtained from individuals with healthy genital tracts. However, results from cystic or inflamed ovaries might yield different stably expressed genes.

Moreover, all algorithms identified *ACTG2*, *SDHA*, *HPRT1* and *GAPDH* as the least stably expressed genes and ranked them in a corresponding order. GENORM calculated M-values much higher than 0.5 for these genes, and NORMFINDER showed highest variations between their expression levels grouped by luteal stages and localizations. High inter-group variations indicate a high bias, whereas the intra-group variation represents the confidence interval.

BESTKEEPER determined high SDs for these genes, which indicate least stably expression. The only difference between BESTKEEPER and GENORM/NORMFINDER results was the ranking position of *ACTG2*. ACTG2, which is a component of the cytoskeleton and functions as mediator of internal cell motility in most cell types (Selga et al., 2011), had not been tested in bovine tissues yet. For example, GENORM results showed that *ACTG2* was the gene with the highest expression variation. Both, high variation and localization dependency are characteristics of non-suitable reference genes. *SDHA*, *HPRT1* and *GAPDH* are commonly used RGs not only for different bovine tissues, but also in other species and organs. These candidates, which fall roughly into the category of metabolism-related genes (Radonić et al., 2004; Lisowski et al., 2008), also displayed considerable expression ranges between different ovarian localizations and luteal stages. Data analysis demonstrated that *GAPDH*, which is used most frequently as RG (Barber et al., 2005), were variably expressed. These results correspond to Rekawiecki et al. (2012), who evaluated expression stability of RGs in bovine corpora lutea of cyclic or pregnant cows and described *ACTB* and *GAPDH* as moderate stably expressed in the corpus luteum. Based on an oestrogen-affected expression (Schroder et al., 2009), they did not recommend these genes as suitable RGs either. Moderate expression stability of *GAPDH* in metabolically active tissues is possible. Cell synthesis activity and tissue growth result in an increased usage of energy, which is provided by glucose (DeBerardinis et al., 2007). GAPDH catalysis is an important step of glycolysis. Thus, its high expression in developing and mature corpus luteum and in corpus luteum of pregnancy reflects an intense metabolic rate of these luteal stages. Expression of *GAPDH* decreased within the ZPCL during regression of the corpus luteum. This might cause controversies about using *GAPDH* as reference gene between high and low metabolic tissues. *ACTB*, which is a structure-related gene (Radonić et al., 2004), is considered as suitable (Pfaffl et al., 2004; Lisowski et al., 2008) or as co-regulated and least stably expressed (Kadegowda et al., 2009) gene. Expression of *ACTB* is likely to be affected by changes of the cytoskeleton, such as changes in cell structure related to cell motility or maintenance of cell integrity.

Especially the example *GAPDH* shows clearly that the expression of commonly used RGs can vary in different tissues and organs or during experimental treatment (Dheda et al., 2005; Robinson et al., 2007; Li et al., 2011). We are conforming to Vandesompele et al. (2002) who stress the importance of using only validated RGs for normalization. Moreover, a minimum of technical information in accordance to the 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments' (MIQE) guidelines should be given in each qPCR

study (Bustin et al., 2009) as this was performed in the present study. The availability of essential technical information and transparent reporting is very important to obtain reliable and reproducible RT-qPCR results. However, Bustin et al. (2013) reported that there is still a lack of adherence to the MIQE guidelines in qPCR studies.

Conclusions

In general, it is strongly recommended to evaluate stably expressed RGs to achieve reliable results in RT-qPCR experiments for each new experimental study design. The bovine ovary comprises a multitude of heterogeneous cell populations within its different localizations, which are affected by the estrous cycle and pregnancy on a morphological and molecular level. The present study compared the suitability of twelve potential RGs for normalization of RT-qPCR results within the bovine ovary. Results obtained here, which are based on different algorithms and analytical procedures, identified a pool of suitable RGs: *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C* and *UBA52*. Of this pool, *YWHAZ*, *H3F3B* and *RPS9* could be recommended as best-suited RGs for normalization purposes on healthy bovine ovaries irrespective of specific intra-ovarian localizations and luteal stages. Still, these validated RGs should be revalidated for further experiments and cannot be used as gold standard. The Software tools GENORM, NORMFINDER, and BESTKEEPER have different benefits. Each currently available software approach includes advantages and disadvantages in determination of stably expressed genes. At least two different methods should be used to gain reliable results. If the set-up includes samples from different groups, inter-group variations should be considered, which is only implemented within the NORMFINDER approach.

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Conflict of Interest

The authors declare no conflict of interest.

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4 Diskussion

4.1 Anatomische Forschung heute

Die Anatomie gilt als eine der ältesten naturwissenschaftlichen Disziplinen der Medizin und kann auf eine rund 2000 Jahre alte Geschichte zurückblicken. Aus dem griechischen übersetzt bedeutet Anatomie "zerschneiden - zergliedern". Eine Definition, die zunächst hauptsächlich auf die makroskopische Anatomie zutrifft. Diese beschreibt die Lage, Struktur und Gestalt von Körperteilen, Organen und Geweben, die mit dem bloßen Auge sichtbar sind [42]. Mit dem Feinbau von Organen und Geweben, unterhalb des mit bloßem Auge sichtbaren Bereiches, beschäftigt sich die mikroskopische Anatomie und Histologie. Die Lehre der makroskopischen und mikroskopischen Anatomie stellt einen zentralen Punkt in der tierärztlichen Ausbildung dar. Gleichzeitig sind das anatomische Wissen und die entsprechenden methodischen Fertigkeiten ebenso in der Forschung von größter Bedeutung. In diesem Zusammenhang werden heute die Ziele moderner anatomischer Forschung von der Anatomischen Gesellschaft weiter gefasst:

"Ziel anatomischer Forschung ist die Klärung der Funktion von Zellen, Geweben und Organen unter besonderer Berücksichtigung ihrer Struktur".

Dieser Strukturbegriff ist mittlerweile bis in den molekularbiologischen Bereich vorgedrungen [43]. Molekularbiologische Methoden haben in fast allen Forschungsbereichen einen wichtigen Stellenwert und gewinnen auch in der anatomischen Forschung immer mehr an Gewicht. Insbesondere in der tiermedizinischen Forschung dienen molekularbiologische Methoden als gute Ergänzung zur Immunhistochemie, die bei der oft unzureichenden Verfügbarkeit geeigneter Antikörper für das Zielgewebe noch schnell an ihre Grenzen gerät. Mit Hilfe der Immunhistochemie ist eine Identifizierung einzelner Gewebe und Zellen sicher möglich. Die eindeutige Kennzeichnung einzelner Zellen mittels Antikörpermarkierung gelingt dabei durch die spezifische Bindung der Antikörper an Proteine, Polysaccharide oder andere Makromoleküle [44]. Eine Einschränkung im Bereich der Immunhistochemie stellt allerdings nicht nur die Verfügbarkeit geeigneter Antikörper dar, sondern auch deren zeit- und kostenintensive Etablierung für jedes zu untersuchende Gewebe. Diesen Einschränkungen unterliegt die PCR nur teilweise, denn die Herstellung tierartspezifischer Primer ist kommerziell kostengünstig möglich und es besteht keine Limitierung in der Verfügbarkeit [45]. Trotz dieser Vorteile ist die PCR, z. B. durch den Einsatz von homogenisierten Proben, bezüglich einer Aussage auf Zellebene eingeschränkt. Hier kommt

wieder die Immunhistochemie zum Zuge, die eine qualitative Analyse der positiven Ergebnisse ermöglicht.

Summa summarum führt die Kombination und der Einsatz verfeinerter und universell einsetzbarer Methoden zu einer enormen Zunahme an Wissen über die Komplexität von Strukturen [46] und damit auch zu einer Spezialisierung auf Teilbereiche.

4.2 Neue Erkenntnisse: Vaskulogenese im bovinen Ovar

Eine von vielen Forschergruppen mit großem Interesse verfolgte Fragestellung betrifft die Bildung neuer Blutgefäße. Nicht nur bei der Tumorentstehung, sondern auch bei der physiologischen Gefäßneubildung, z. B. während der Wundheilung, kommt diesem Forschungsgebiet eine große Bedeutung zu. Das spiegelt sich auch in der großen Anzahl von Veröffentlichungen in der Datenbank von Pubmed wieder. Hier finden sich zu Stichwörtern wie "blood vessel" (712.794), "neovascular" (6.280) und "vasculogenesis" (2.553) eine große Anzahl von Einträgen.

Ein enormes Neovaskularisationspotential zeigt das Ovar in den sich zyklisch entwickelnden Corpora lutea und stellt deshalb ein ausgezeichnetes Modell für die physiologische Blutgefäßentwicklung dar [1, 47]. Nach in vitro Versuchen von Käßmeyer und Plendl entstand die Hypothese, dass für das enorme Neovaskularisationspotential neben Angiogenese auch Vaskulogenese eine Rolle spielen könnte [18].

Diese in vitro Ergebnisse zu verifizieren war das Ziel der vorliegenden Studie, nämlich der qualitative und quantitative Nachweis endothelialer Progenitorzellen im bovinen Ovar in situ. Mittels spezifischer Marker erfolgte die Identifizierung immunhistochemisch (Anti-CD34, Anti-KDR) und mittels RT-qPCR (CD34, KDR und CD133). Um verlässliche RT-qPCR Ergebnisse zu erlangen, war im Vorfeld die Etablierung stabil exprimierter Referenzgene zur Normalisierung der Ergebnisse nötig.

4.3 Grundlegende Vorarbeiten: Etablierung stabil exprimierter Referenzgene für das bovine Ovar

4.3.1 Notwendigkeit der Validierung von Referenzgenen

Die "quantitative polymerase chain reaction" (qPCR) kombiniert mit der "Reversen Transkription" (RT-qPCR), ist eine sehr effektive und häufig genutzte Methode für Genexpressionsanalysen [48]. Eine hohe Sensitivität und Spezifität, gepaart mit der

Möglichkeit einer relativ schnellen Ergebniserzielung, sowie ein breites Einsatzgebiet stellen die Vorteile dieser Methode dar [49]. Auf der anderen Seite verlangt die Durchführung von der RNA-Isolierung über den DNase-Verdau, die cDNA-Synthese bis zu der eigentlichen qPCR aber auch größte Sorgfalt. Während über die beste Vorgehensweise der genannten Versuchsschritte größtenteils Einigkeit herrscht, ist die Auswertung der qPCR-Daten noch immer eine häufige Fehlerquelle und damit in Diskussion [50].

Für die Datenanalyse stehen verschiedene Quantifizierungsstrategien zur Verfügung, wenngleich die relative Quantifizierung die am weitesten verbreitete Methode ist. Bei der relativen Quantifizierung wird sowohl die Expression der Zielgene als auch die der Referenzgene in jedem Versuchsdurchlauf erfasst. Sowohl die Zielgene wie auch die Referenzgene werden während des Versuchsablaufs in gleicher Weise beeinflusst. Setzt man die Expression der Referenzgene und Zielgene ins Verhältnis, ist es möglich, experimentell und technisch induzierte Variationen von „wirklichen“ biologischen Variationen zu unterscheiden [51]. Dieses Vorgehen wird als Normalisierung bezeichnet.

Die wichtigste Anforderung an gute Referenzgene für eine akkurate Normalisierung ist ihre stabile Expression in dem untersuchten Gewebe. Diese sollte unabhängig sein von hormonellen und metabolischen regulatorischen Einflüssen, sowie dem jeweiligen Entwicklungszustand des Gewebes [26]. Zusätzlich sollte die Lokalisation der Probenentnahme im Organ ohne Auswirkung auf die Expressionshöhe der ausgewählten Referenzgene bleiben. Die Verwendung von Referenzgenen ohne den Nachweis einer stabilen Expression ist häufig die Ursache von Normalisierungsfehlern [50], wodurch es zu einer signifikanten Verzerrung von Ergebnissen kommen kann [52]. Untersuchungen belegen, dass auch vielfach "universell" oder „klassisch“ eingesetzte Referenzgene je nach Gewebe, Spezies und Versuchsaufbau eine wechselnde Expressionsstabilität zeigen [26, 35, 53]. Um diese Fehlerquelle möglichst auszuschließen, wurde in der vorliegenden Arbeit eine Validierung möglicher Referenzgene unter Berücksichtigung der jeweiligen Versuchsbedingungen durchgeführt.

4.3.2 Etablierung von Referenzgenen für das bovine Ovar

Es lagen keine Untersuchungen zu geeigneten Referenzgenen am Rinderovar vor, die sowohl die verschiedenen Zyklusstadien wie auch die unterschiedlichen Lokalisationen berücksichtigten. Alle in dieser Studie ausgewählten und untersuchten Referenzgene zeigten in Studien an anderen Spezies und Organen bereits eine stabile Expression. Die Einteilung der

ausgewählten potentiellen Referenzgene erfolgte in zwei Gruppen: (1) Gene die bereits in unterschiedlichen Geweben des Rindes eine stabile Expression zeigten (*GAPDH*, *ACTB*, *YWHAZ*, *HPRT1*, *SDHA*, *UBA52*, *POLR2C*, *RPS9*) [33-36], sowie (2) Gene die ausschließlich in "nicht Rindergewebe" getestet wurden (*ACTG2*, *H3F3B*, *RPS18*, *RPL19*) [37-41]. Für alle Referenzgene wurden rinderspezifische Primer neu konzipiert.

GENORM, NORMFINDER und BESTKEEPER stellen die am häufigsten verwendeten Softwareprogramme zur Beurteilung der Expressionsstabilität von Referenzgenen dar [54, 55]. Die in der vorliegenden Arbeit verwendeten Berechnungsalgorithmen basieren auf unterschiedlichen Prinzipien, woraus sich programmspezifische Vorteile wie auch Schwächen ergeben:

Eine Berücksichtigung möglicher Verdünnungseffekte auf die Genexpression ist mittels des GENORM Algorithmus gegeben [34, 56]. Der Verdünnungseffekt kann bei einer Zunahme der RNA-Gesamtkonzentration in einem Gewebe auftreten. Dabei erscheint die konstante RNA-Konzentration des Zielgens relativ zur gesamt RNA-Konzentration erniedrigt, wodurch Verzerrungen der Ergebnisse möglich sind. Eine Normalverteilung der Daten ist bei GENORM nicht zwingend erforderlich, wohingegen die untersuchten Gene keine Ko-Regulation zeigen sollten [57]. Von Ko-Regulation spricht man, wenn mehrere Gene in ähnlicher Weise auf einen Reiz reagieren [34, 58]. Durch den paarweisen Vergleich von GENORM würde es dadurch zu einer Übervorteilung ko-regulierter Gene bei der Ermittlung stabil exprimierter Referenzgene kommen.

Bei NORMFINDER wird das Ergebnis hingegen nur unwesentlich durch eine mögliche Ko-Regulation der untersuchten Gene beeinflusst. Die Proben werden hierbei unter studienspezifischen Gesichtspunkten zuerst in Gruppen eingeteilt. Für die Berechnung des Stabilitätswertes werden dann sowohl Variationen innerhalb einer Gruppe (intra) wie auch zwischen einzelnen Gruppen (inter) berücksichtigt. Der hieraus berechnete Stabilitätswert wird nur unwesentlich durch systematische Fehler, wie beispielsweise eine Ko-Regulation von Genen, beeinflusst [32, 59].

Für die BESTKEEPER Software ist eine Normalverteilung der Cq-Werte nötig, da zur Berechnung der Pearson-Korrelations-Koeffizient benutzt wird. Dieser statistische Test verlangt die Normalverteilung der genutzten Rohdaten. Darüber hinaus ist bei dieser Excel-basierten Berechnung ein Vergleich von maximal zehn Genen durchführbar, wobei durch die

Ermittlung der Standardabweichung schon zu Beginn Gene mit hoher Standardabweichung aus der Berechnung ausgeschlossen werden sollten [33, 60].

Durch eine parallele Nutzung der Algorithmen ergeben sich daher einige Vorteile bei der Ermittlung stabil exprimierter Referenzgene. Im Folgenden sind die Grundlagen der einzelnen Algorithmen und die Ergebnisse ausführlich dargestellt.

Beim paarweisen Vergleich des GENORM Algorithmus dient ein für jedes Referenzgen ermittelter M-Wert zur Beurteilung der Expressionsstabilität [27]. Ein M-Wert < 0,5 deutet auf stabil exprimierte Referenzgene hin. Darüber hinaus erfolgt die Bildung einer Stabilitätsrangfolge aller untersuchten Referenzgene [61]. In dieser hier ermittelten Rangfolge war *ACTG2* das am wenigsten stabil exprimierte Gen, gefolgt von *SDHA*, *HPRT1*, *GAPDH*, *ACTB*, *POLR2C*, *YWHAZ*, *H3F3B*, *RPL19*, *UBA52*, *RPS9* und *RPS18*; letzteres zeigte die stabilste Expression. Ab *POLR2C* zeigten alle Referenzgene einen M-Wert < 0,5. Die optimale Anzahl an Referenzgenen wird anschließend durch den V-Wert berechnet. Dieser ermöglicht die Ermittlung der minimalen Anzahl nötiger Referenzgene, wobei mehr als ein Referenzgen zur Normalisierung eingesetzt werden sollte [27]. Mit einem M-Wert von 0,266 und einem V-Wert von < 0,15 sind die Referenzgene *RPS9* und *RPS18* laut GENORM für die Normalisierung von Proben aus dem gesamten Rinderovar geeignet – unabhängig vom Zyklusstadium und der Lokalisation.

Beide Gene *RPS9* und *RPS18* gehören zur Gruppe ribosomaler Proteine und sind an der Herstellung von Ribosomen beteiligt [62]. Der GENORM Algorithmus setzt voraus, dass die untersuchten Gene nicht ko-reguliert sind [32, 63]. Eine mögliche Ko-Regulation kann die Zuverlässigkeit dieses Berechnungsalgorithmus beeinflussen [64]. Eine Ko-Regulation der ribosomalen Gene *RPS9* und *RPS18* ist wahrscheinlich und könnte ein möglicher Grund für die Auswahl durch GENORM sein. Die Anwendung ribosomaler Gene zu Normalisierungszwecken wird kontrovers diskutiert, sie werden sowohl als stabil exprimierte und geeignete Referenzgene [29, 65, 66] als auch mit signifikant schwankender Genexpression [67] beschrieben. Der vermeintliche Nachteil einer Nutzung von ko-regulierten stabil exprimierten ribosomalen Genen als Referenzgene konnte in einigen Studien nicht bestätigt werden [34, 68]. Bionaz und Loor beschrieben *UXT* (ubiquitinously expressed transcript isoform 2), *RPS9* und *RPS15* als stabil exprimiert in der Milchdrüse des Rindes [34]. Während des Laktationszyklus ist die Milchdrüse durch vergleichbare intensive zelluläre und molekulare Veränderungen gekennzeichnet wie das Ovar während der lutealen

Stadien. Obgleich die Ergebnisse aus den Versuchen mit Milchdrüsengewebe nicht ohne weiteres auf das Ovar übertragbar sind, unterliegen doch beide Gewebe einem nicht unerheblichen zyklischen Einfluss. Bei der Evaluierung von Referenzgenen sollten diese Einflüsse zwingend Berücksichtigung finden. Darüber hinaus wurde für die Milchdrüse des Rindes ein Verdünnungseffekt von Genen beschrieben, der durch die Zunahme der RNA Konzentration in einem Gewebe entsteht [34, 56]. Das Auftreten dieses Phänomens ist im ovariellen Gewebe ebenso denkbar. Der GENORM Algorithmus jedoch berücksichtigt einen möglichen Verdünnungseffekt auf die Genexpression [34].

Eine Bestimmung der Expressionsstabilität potentieller Referenzgene erfolgte zusätzlich durch den NORMFINDER Algorithmus [32]. Der Berechungsalgorithmus dieses modellbasierten Ansatzes bildet mittels Varianzanalyse einen Stabilitätswert, aus dem sich eine Rangfolge der untersuchten Gene ableiten lässt. Eine studienabhängige Definition von Gruppen (hier: Lokalisation bzw. luteales Stadium) zur Errechnung der intra- und inter-Gruppen-Variationen ist notwendig. Aus den Variationen innerhalb der einzelnen Gruppen (intra) und zwischen verschiedenen Gruppen (inter) errechnet sich der Stabilitätswert [32]. Die Auswahl geeigneter Referenzgene erfolgt bei NORMFINDER nicht ausschließlich aufgrund ähnlicher Expressionslevels, sondern auch entsprechend der Expressionsmuster. Daher ist eine Berechung mittels NORMFINDER nur unwesentlich durch eine mögliche Ko-Regulation von Referenzgenen beeinflusst [69] und stellt somit eine hervorragende Ergänzung zum GENORM Algorithmus dar. NORMFINDER ermittelte, wie auch GENORM und BESTKEEPER, *ACTG2*, *SDHA*, *HPRT1* und *GAPDH* als die am unstabilsten exprimierten Gene. Am stabilsten waren hier die Gene *H3F3B*, *YWHAZ*, *RPS9*, *POLR2C*, *RPS18* und *UBA52* exprimiert. Unter Berücksichtigung der intra- und inter-Gruppen-Variationen der verschiedenen Lokalisationen ZV, ZP und ZPCL (ohne Berücksichtigung der lutealen Stadien) wurden *YWHAZ* und *H3F3B* (Stabilitätswert 0,061) als das stabilste Referenzgenpaar ermittelt. Eine Ermittlung der stabilsten Referenzgene unter Berücksichtigung der intra- und inter-Gruppen-Variationen der unterschiedlichen lutealen Stadien (ohne Berücksichtigung der verschiedenen Lokalisationen) ergab für *POLR2C* und *RPS18* den höchsten Stabilitätswert (0,071). Andersen et al. [32] empfehlen die Auswahl von Genen, die keine Expressionsunterschiede zwischen den Gruppen erwarten lassen. Folglich sollten unstabil exprimierte Gene von der weiteren Berechnung ausgeschlossen werden. In der anschließenden Neuberechnung, ohne Berücksichtigung von Genen mit hohen Zwischengruppen-Variationen (*ACTG2*, *SDHA*, *HPRT1* und *GAPDH*), ergab sich ein divergierendes Ergebnis, wobei es zu einer

Verschiebung der Stabilitätsreihenfolge kam. Der Ausschluss von unstabil exprimierten Genen aus der NORMFINDER-Berechnung führt unter Berücksichtigung der verschiedenen Lokalisationen zur Ermittlung von *RPL19* und *YWHAZ* als geeignete Referenzgene (Stabilitätswert 0,067). Bei der Einbeziehung lutealer Stadien in die Gruppenbildung werden, wie auch bei GENORM, die Gene *RPS9* und *RPS18* als geeignet identifiziert (Stabilitätswert 0,076). Eine Beeinflussung der NORMFINDER-Ergebnisse ist generell durch die Anzahl der untersuchten Referenzgene und Proben möglich [32]. Metha et al. [57] berichten, dass sich bei einem größeren Probenumfang die NORMFINDER-Ergebnisse änderten, was zu einer Annäherung an die Ergebnisse der GENORM-Berechnung führte.

Schließlich erfolgte eine Berechnung der Expressionsstabilität potentieller Referenzgene mittels der Excel basierten BESTKEEPER Software [33]. Hiermit ist ein Vergleich von maximal zehn Genen möglich. Für jedes Referenzgen wird auf Grundlage der ermittelten Cq-Werte aller untersuchten Proben die Standardabweichung (SD) berechnet. Referenzgene mit einer Standardabweichung > 1 werden als unstabil bewertet. In diesen Untersuchungen zeigten die Gene *SDHA* (SD 1,28), *HPRT1* (SD 1,26), *ACTG2* (SD 1,18) und *GAPDH* (SD 1,07) eine Standardabweichung über dem Grenzwert und wurden in den weiteren Berechnungen nicht berücksichtigt. Alle anderen untersuchten Gene zeigten eine Standardabweichung < 1: *ACTB* (SD 0.83), *POLR2C* (SD 0,76), *YWHAZ* (SD 0,72), *RPS9* (SD 0,6), *RPS18* (SD 0,59), *H3F3B* (SD 0,52), *RPL19* (SD 0,5) und *UBA52* (SD 0,44). Hierbei deutet die geringste Standardabweichung auf die am stabilsten exprimierten Referenzgene hin. Anschließend wird der BESTKEEPER-Index als geometrischer Mittelwert aller in die Berechnung einfließenden Cq-Werte berechnet und eine paarweise Korrelationsanalyse aller möglichen Referenzgen-Kombinationen durchgeführt. Der Pearson-Korrelations-Koeffizient wird zwischen jedem Referenzgen und dem BESTKEEPER-Index berechnet und als BESTKEEPER-Korrelations-Koeffizient bezeichnet. Daraus ergibt sich eine Rangfolge der untersuchten Gene entsprechend ihrer ermittelten Stabilität. Ein hoher BESTKEEPER-Korrelations-Koeffizient deutet auf eine stärkere Korrelation und damit auf stabiler exprimierte Referenzgene hin: *YWHAZ* (0,958), *H3F3B* (0,952), *POLR2C* (0,949), *RPS9* (0,926), *RPS18* (0,906), *UBA52* (0,900), *ACTB* (0,885) und *RPL19* (0,872). Ein unterer Grenzwert ist hierbei nicht definiert. *YWHAZ* wird von BESTKEEPER als am stabilsten exprimiert bewertet. Verglichen mit den anderen hier angewandten Softwareprogrammen zur Beurteilung der Expressionsstabilität ordnete GENORM *YWHAZ* als viertstabilstes Gen ein und NORMFINDER als das zweitstabilste Gen. Auch andere Studien beschreiben eine hohe Expressionsstabilität für *YWHAZ* wie zum

Beispiel in unterschiedlichen embryonalen Präimplantationsstadien [70], in peripheren Lymphozyten [71], sowie in der Leber und in der Niere [35] von Rindern. Eine Auswahl der stabilsten Gene erfolgte in der vorliegenden Studie bis zu einem Korrelations-Koeffizient von 0,9, welcher von den Genen *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C*, und *UBA52* erreicht wurde. Damit unterscheiden sich die BESTKEEPER Ergebnisse nur von der GENORM Auswahl in Bezug auf *POLR2C* (bei GENORM nicht unter den sechs besten) sowie *RPL19* (bei GENORM an vierter Stelle der stabil exprimierten Gene). Eine denkbare Ursache wäre die zu gute Bewertung von *RPL19* durch GENORM aufgrund der wahrscheinlichen Ko-Regulation ribosomaler Gene.

Der Einsatz der drei am häufigsten verwendeten Berechnungsalgorithmen GENORM, NORMFINDER und BESTKEEPER erlaubte eine zuverlässige Ermittlung von geeigneten Referenzgenen. Aufgrund ihrer programmspezifischen Vor- und Nachteile, scheint die parallele Nutzung von GENORM, NORMFINDER als auch von BESTKEEPER die spezifischste Vorgehensweise zur Ermittlung stabil exprimierter Referenzgene zu sein. Die Gene *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C* und *UBA52* zeigten die höchste Expressionsstabilität entsprechend der drei eingesetzten Berechnungsalgorithmen, lediglich die Reihenfolge variierte. Folglich stellen die Gene *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C* und *UBA52* einen Pool der am besten geeigneten Referenzgene dar, um die mRNA Expression von Ziel-Genen aus dem bovinen Ovar zu normalisieren. In der Literatur wird empfohlen, möglichst drei stabil exprimierte Referenzgene zur Normalisierung einzusetzen [72]. Um die drei stabilsten Referenzgene aus diesem Pool zu ermitteln, wurde jedem Gen in den einzelnen Algorithmen ein Rang zugeordnet. Daraus konnte durch die Bildung der Mittelwerte eine Rangfolge der Referenzgene unter Einbeziehung aller Algorithmen bestimmt werden. Aus diesem Pool zeigen *YWHAZ*, *H3F3B* und *RPS9* die höchste Stabilität und können zur Normalisierung von RT-qPCR Ergebnissen unabhängig von der Probenlokalisation oder dem lutealen Stadium empfohlen werden. Eine Revalidierung vor jedem neuen Experiment ist trotzdem unumgänglich. Es ist außerdem zur Kenntnis zu nehmen, dass für die vorliegende Arbeit ausschließlich Ovarien von Rindern mit nicht pathologisch verändertem Genitaltrakt zur Evaluierung stabiler Referenzgene eingesetzt wurden.

4.4 Endotheliale Progenitorzellen im bovinen Ovar: ein qualitativer und quantitativer Nachweis

Das Ziel dieser Untersuchung war endotheliale Progenitorzellen in situ im Ovar des Rindes qualitativ und quantitativ nachzuweisen. In der Arbeitsgruppe um Prof. J. Plendl, wurden in früheren Versuchen mikrovaskuläre Zellen aus dem Corpus luteum in Anbildung isoliert [18, 73]. Diese Zellen bildeten in vitro gefäßähnliche Strukturen. Der Ursprung ging von spezifischen Zellen, den so genannten "Starting Points" oder "Vascular-Initiation-Points" aus, diese konnten morphologisch Progenitorzellen zugeordnet werden. Diese Zellen proliferierten intensiv und bildeten Zellcluster, die sich zu kapillarähnlichen Strukturen differenzierten [18]. Diese "Vascular-Initiation-Points" waren ausschließlich in Zellkulturen des Corpus luteum in Anbildung nachweisbar. Sie stellten sich als hoch proliferativ heraus und bildeten in vitro CD34 und KDR positive Zellcluster, aus welchen kapillarähnliche Strukturen mit einem Lumen entstanden. Dieser Mechanismus ähnelte der Vaskulogenese und ließ zusammen mit der immunhistochemischen Charakterisierung der "Vascular-Initiation-Points" vermuten, dass diese Zellen endotheliale Progenitorzellen repräsentieren [18]. Daraus war die Hypothese entstanden, dass die Entwicklung der Blutgefäße im sich anbildenden Corpus luteum eine Kombination aus Vaskulogenese und Angiogenese darstellt, was auch in anderen Organen bereits beschrieben worden ist [73, 74]. Um die Ergebnisse der in vitro Versuche in situ zu verifizieren, erfolgte zunächst eine immunhistochemische Doppelmarkierung von Paraffinschnitten boviner Ovarien mittels Anti-CD34 und Anti-KDR.

Der Nachweis einer Ko-Expression von CD34 und KDR stellt eine häufig genutzte Markerkombination dar, um endotheliale Progenitorzellen zu identifizieren und zu isolieren. In vitro differenzieren diese Zellen zu maturen Endothelzellen [13, 20, 75]. Einzeln exprimiert wird CD34 auch auf aktivierten mikrovaskulären Endothelzellen beschrieben, wodurch eine eindeutige Abgrenzung dieser Zellen zu den endothelialen Progenitorzellen erschwert ist. In den meisten Geweben gilt CD34 daher sowohl als Marker für endotheliale Progenitorzellen als auch für mikrovaskuläre Endothelzellen [76-80]. KDR spielt sowohl bei endothelialen Progenitorzellen als auch bei Endothelzellen eine entscheidende Rolle und ist daher ubiquitär auf diesen Zellen nachweisbar [81].

Der qualitative Nachweis der endothelialen Progenitorzellen im bovinen Ovar wurde immunhistochemisch durchgeführt. Die Ergebnisse der hier vorliegenden Arbeit zeigten für KDR und CD34 doppelt markierte Zellen in bestimmten Wandschichten von Arterien und Arteriolen ein spezifisches Verteilungsmuster. Die doppelt markierten Zellen waren in der

arteriellen Tunica media auszumachen, sowie - jedoch seltener - in der Tunica adventitia der ovariellen Arterien. Einzelne Venen zeigten ebenfalls KDR und CD34 doppelt markierte Zellen im Bereich der Tunica media. Die endothelialen Progenitorzellen traten überwiegend vereinzelt oder in kleinen Gruppen auf. Die Lokalisation bezog sich vorrangig auf Arterien und kleinere Blutgefäße innerhalb des Corpus luteum und auf Gefäße, welche die Funktionskörper des Ovars (tertiäre Follikel, Corpus luteum) umgaben. Im übrigen Ovargewebe ließen sich darüber hinaus wenige CD34 positive und KDR negative Zellen nachweisen. Diese Zellen konnten mikrovaskulären Endothelzellen zugeordnet werden und waren vorrangig weiter entfernt vom Corpus luteum und den anderen Funktionskörpern zu finden. Die Ergebnisse anderer Studien legen nahe, dass das Expressionsprofil von CD34 (mit unterschiedlichen Splicevarianten) nicht nur von dem Organ, sondern auch von der Lokalisation innerhalb eines Organs abhängig zu sein scheint. Zusätzlich treten interindividuelle Unterschiede im Expressionsprofil auf, welche auch für das Rind nachgewiesen werden konnten [79, 82-84].

Die Ergebnisse dieser Arbeit deuten darauf hin, dass die Blutgefäßwand eine Quelle von Progenitorzellen im Rinderovar ist. Die Blutgefäßwand als Ressource von Stamm- oder Progenitorzellen - außerhalb von Knochenmark und peripherem Blut - wurde originär von Ingram et al. [85] beschrieben. Auch andere Autoren haben die Blutgefäßwand als Nische der endothelialen Progenitorzellen beobachtet. Die endothelialen Progenitorzellen wurden dabei in der Tunica media [86] und auch in der sogenannten "vaskulogenen Zone" zwischen der Tunica media und der Tunica adventitia identifiziert [13, 87]. Aus Blutgefäßwänden stammende Stamm- oder Progenitorzellen könnten sowohl endotheliale Progenitorzellen, glatte Muskelzellprogenitoren wie auch mesenchymale Stammzellen darstellen [87]. Für verschiedene Spezies und Organe sind diese bereits in Gefäßen adulter Individuen nachgewiesen worden [88-91], bisher aber noch nicht im Ovar – insbesondere nicht im Ovar des Rindes. Die hier errungenen Ergebnisse bestätigen die in vitro Beobachtungen von Käßmeyer und Plendl, welche aus dem Corpus luteum des Rindes Zellen isolierten, die spezifische Eigenschaften der endothelialen Progenitorzellen zeigten [18]. Allerdings blieb bei den früheren Untersuchungen offen, wo die endothelialen Progenitorzellen residierten. Diese Frage konnte mit den neuen Ergebnissen der vorliegenden Arbeit gelöst werden.

Für den quantitativen Nachweis von endothelialen Progenitorzellen in bovinen Ovarien wurden Initial die Referenzgene *RPS18*, *RPS9* und *UBA52* zur Normalisierung der Ergebnisse ausgewählt. Diese Auswahl erfolgte mittels des GENORM Algorithmus, welcher das am

häufigsten benutzte Programm darstellt und als der Goldstandard zur Normalisierung in der Expressionsanalytik bezeichnet wird [50]. Für die Ermittlung stabil exprimierter Referenzgene werden von anderen Forschergruppen häufig zusätzliche Algorithmen wie NORMFINDER und BESTKEEPER zur Verifizierung der GENORM Ergebnisse eingesetzt [92]. In nachfolgenden Versuchen wurden daher Referenzgene für das bovine Ovar zusätzlich mittels der Programme NORMFINDER und BESTKEEPER ermittelt. Der Pool an stabil exprimierten Referenzgenen blieb dabei unverändert, allerdings kam es zu einer Verschiebung der Rangfolge. *YWHAZ*, *H3F3*B und *RPS9* zeigten jetzt, wie oben beschrieben, die stabilste Expression. Eine anschließende stichprobenartige Überprüfung der Ergebnisse des Nachweises endothelialer Progenitorzellen mittels der Referenzgene *YWHAZ*, *H3F3B* und *RPS9* führte lediglich zu marginalen quantitativen Veränderungen, welche jedoch die qualitativen Aussagen nicht beeinflussten. Das lässt die Schlussfolgerung zu, dass alle Gene aus dem Pool stabil exprimierter Referenzgene eine adäquate Normalisierung von Zielgenen im Ovar erlauben.

Für den Nachweis endothelialer Progenitorzellen kamen neben den Progenitorzellmarkern CD34 und KDR, auch der Marker hämatopoetischer Stammzellen CD133 (Prominin-1) [18-20] zum Einsatz. Eine Expression dieser Marker war in allen untersuchten Proben aus dem Ovar des Rindes detektierbar. Die Expression von *CD34* und *CD133* mRNA zeigte große Variationen zwischen den lutealen Stadien. Die höchste Expression beider Marker wurde im lutealen Stadium der Anbildung und Blüte nachgewiesen, wobei *CD133* eine signifikant höhere Expression ($p < 0{,}005$) im lutealen Stadium der Anbildung als während der Blüte zeigte. Im Vergleich zum lutealen Stadium der Anbildung war die *CD133* Expression während des lutealen Stadiums der Rückbildung und Trächtigkeit ($p < 0{,}001$) signifikant reduziert. Ein signifikanter Unterschied zeigte sich bei der Expression von *CD34* nur zwischen den lutealen Stadien der Blüte und Rückbildung ($p < 0{,}003$) beziehungsweise zwischen Blüte und Trächtigkeit ($p < 0{,}002$). *CD133* stellt durch seine herunterregulierte Expression in differenzierten Zellen einen eindeutigen Marker für die Identifizierung von Stamm- und Progenitorzellen dar [93]. Yin et al. [22] beschrieben, dass sich CD133 positive Zellen unter bestimmten Bedingungen zu CD133 negativen Endothelzellen differenzieren. Zunächst wurde vermutet, dass CD133 positive Zellen ausschließlich adulte Hämangioblasten repräsentieren [94-97]. In den letzten Jahren gelang jedoch der Nachweis einer CD133 Expression in Zellen unterschiedlichen Ursprungs. Dazu gehören neben hämatopoetischen Zellen auch embryonale Stammzellen, Tumorstammzellen, ortsständige Stammzellen in Geweben und zirkulierende endotheliale Progenitorzellen [98-100]. Heute wird vermutet,

dass CD133 eine wichtige Rolle als Oberflächenmarker bei der gezielten Sortierung dieser Stamm- und Progenitorzellen wie auch bei bestimmten Erkrankungen spielt [24, 93, 98, 101].

Die Expression von *KDR* zeigte keine signifikanten Variationen zwischen den lutealen Stadien. Allerdings wurden signifikante Unterschiede der Expression zwischen den verschiedenen Lokalisationen im Ovar nachgewiesen. Proben aus ZPCL zeigten die höchste Expression für *KDR*. Die ZPCL besteht aus unterschiedlichen Arten von KDR exprimierenden Zellen wie endotheliale Progenitorzellen, Endothelzellen und Granulosazellen [102, 103]. Da diese Zellen den größten Anteil im Corpus luteum stellen, ist eine hohe Expression von *KDR* in der ZPCL nicht verwunderlich. Verschiedene andere Studien bestätigen in immunhistochemischen Versuchen am Ovar des Rindes [4, 104] und anderer Spezies ([105]/Mensch, [106]/Ratte, [107]/Hund, [108]/Schwein) das zahlreiche Vorkommen dieser KDR positiven Zellen im Corpus luteum. Darüber hinaus zeigt die Expression der Marker *CD34* und *KDR* eine hohe Korrelation ($r = 0{,}641$) mit einer Signifikanz von $p < 0{,}001$. Die hohe Korrelation dieser Marker lässt vermuten, dass es sich bei den mit Anti-CD34 und Anti-KDR immunhistochemisch markierten Zellen um dieselben Zellen handeln könnte. Den RT-qPCR Ergebnissen entsprechend, könnten die Zellen der "Vascular-Initation-Points" den in situ CD34/KDR reaktiven Zellen innerhalb der lutealen Stadien Anbildung bzw. Blüte zuzuordnen sein, welche somit eine Quelle für endotheliale Progenitorzellen darstellen. Die Zellen der "Vascular-Initation-Points" könnten ebenso in Verbindung mit den *CD133* exprimierenden Zellen stehen, welche vor allem in Ovarien des lutealen Stadiums der Anbildung bzw. Blüte nachgewiesen wurden.

Die Isolierung von endothelialen Progenitorzellen mittels einer Ko-Expression von CD34 und KDR ist vielfach beschrieben [16, 18, 109-112]. Dessen ungeachtet wird diese Markerkombination immer wieder kontrovers diskutiert, da sowohl CD34 als auch KDR auf reifen Endothelzellen exprimiert werden. Der momentane Konsens besteht darin, dass CD34-positive Zellen, die zusätzlich CD133 exprimieren, gesichert endotheliale Progenitorzellen darstellen [24, 112, 113]. Die Hypothese, dass Stamm-/Progenitorzellen im Ovargewebe vorkommen, wird durch die Korrelation der Expression von *CD34* und *CD133* ($r = 0{,}452$) mit einer Signifikanz von $p = 0{,}006$ untermauert, während die Expression von *CD133* und *KDR* eine niedrigere Korrelation ($r = 0{,}385$) mit einer Signifikanz von $p = 0.021$ aufwies. Unter Berücksichtigung des enormen angiogenen Potentials des Ovars, scheint die Notwendigkeit von gewebseigenen Stammzellen/endothelialen Progenitorzellen als eine lokale und daher schnell verfügbare Quelle für alle gefäßneubildenden Prozesse sehr wahrscheinlich. Es konnte

gezeigt werden, dass ortsständige endotheliale Progenitorzellen aus Organen wie der Leber und dem Darm auch an der Bildung des zirkulierenden Pools der endothelialen Progenitorzellen beteiligt sind. Der Mechanismus der Mobilisierung ist bisher unbekannt [114]. In dieser Hinsicht ist es von besonderer Bedeutung, dass Östrogen inaktive endotheliale Progenitorzellen mobilisieren und so eine Gefäßneubildung initiieren kann. Der Serum-Östradiol-Spiegel korreliert offenbar signifikant mit der Anzahl zirkulierender endothelialer Progenitorzellen [18, 115, 116]. Die follikulären Granulosazellen sind der Hauptproduktionsort für Östradiol im weiblichen Organismus. Zusätzlich wird Östradiol in der Wand von Arterien gebildet, im menschlichen Organismus beispielsweise in den glatten Muskelzellen der Tunica media [117]. Beim Rind ist der Nachweis von Östrogenrezeptoren wie auch die Östrogensynthese in Endothelzellen der Aorta belegt [118]. Daher kann eine Östradiol-regulierte Aktivierung von endothelialen Progenitorzellen, besonders innerhalb des Ovars, erwartet werden.

4.5 Schlussfolgerung

Die Ermittlung stabil exprimierter Gene mittels GENORM, NORMFINDER und BESTKEEPER ergab, trotz unterschiedlicher Herangehensweisen der Berechnungsprogramme, weitgehend einheitliche Ergebnisse. Die Ergebnisse zusammengefasst, ergaben für die Gene *POLR2C*, *YWHAZ*, *H3F3B*, *UBA52*, *RPS9* und *RPS18* die höchste Expressionsstabilität. Diese Gene stellen somit einen Pool an Referenzgenen für die Normalisierung von RT-qPCR Ergebnissen für das bovine Ovar dar. Aus diesem Pool können, unabhängig von der Probenlokalisation oder dem lutealen Stadium, *YWHAZ*, *H3F3B* und *RPS9* zur Normalisierung von qPCR Daten empfohlen werden. Eine Revalidierung dieser Referenzgene ist trotzdem für jedes neue Experiment empfehlenswert.

In der vorliegenden Studie konnte weiter gezeigt werden, dass ortsständige endotheliale Progenitorzellen im Ovar des Rindes während des gesamten Zyklus wie auch während der Trächtigkeit vorkommen. Besonders das Stadium der Anbildung zeigte eine signifikant erhöhte Anzahl an endothelialen Progenitorzellen. Dies bestätigt die den in vitro Versuchen zugrundeliegende Vermutung [18], dass die beschriebenen Zellen den "Vascular-Initiation-Points" entsprechen. Eine Eingrenzung der Lokalisation der endothelialen Progenitorzellen erfolgte immunhistochemisch auf die Bereiche der Tunica media und der Tunica adventitia in Gefäßen des Ovars.

5 Zusammenfassung

In situ Identifizierung und Charakterisierung endothelialer Progenitorzellen im bovinen Ovar

Das Ovar unterliegt im Laufe des Zyklus starken Veränderungen, die sich unter anderem in einem intensiven Blutgefäßwachstum während der Anbildung des Corpus luteum und einer Rückbildung von Blutgefäßen während der Luteolyse äußern. Die Neovaskularisationsprozesse umfassen ein Drittel der gesamten Zykluslänge und sind intensiver als in malignen Tumoren. Der Ursprung der stark proliferierenden Endothelzellen im Corpus luteum ist bis heute nicht gänzlich geklärt. Die Hypothese der vorliegenden Arbeit besagt, dass endotheliale Progenitorzellen (endothelial progenitor cells, EPCs) als lokale Ressource dienen und somit maßgeblich an Neovaskularisationsprozessen im Ovar beteiligt sind. Das Ziel dieser Arbeit war daher der qualitative und quantitative Nachweis von endothelialen Progenitorzellen im Ovar des Rindes.

An Paraffinschnitten des Rinderovars konnten mittels Anti-KDR und Anti-CD34 immunhistochemisch doppelt markierte Zellen nachgewiesen werden. KDR und CD34 repräsentieren die am häufigsten genutzte Marker-Kombination, um endotheliale Progenitorzellen zu identifizieren. Die markierten Zellen waren in der Tunica media und selten in der Tunica adventitia von Arterien, sowie lediglich sporadisch in venösen Gefäßen, nachweisbar. Vorrangig befanden sich die markierten Blutgefäße innerhalb des Corpus luteum und in der direkten Umgebung der Funktionskörper des Ovars. Diese Ergebnisse deuten auf die Blutgefäßwand als mögliche Quelle von endothelialen Progenitorzellen im Ovar des Rindes hin. Um die immunhistochemischen Ergebnisse zu untermauern, erfolgte zusätzlich mittels RT-qPCR ein Nachweis der Progenitorzellmarker *KDR* und *CD34* als auch des Stammzellmarkers *CD133* (*Prominin-1*). Aus dem Ovar des Rindes waren hierfür während verschiedener lutealer Stadien Proben aus unterschiedlichen Lokalisationen entnommen und untersucht worden. Mittels der RT-qPCR Ergebnisse konnte eine Korrelation der Marker *CD34* und *KDR* nachgewiesen werden, wodurch der mögliche endotheliale-Progenitorzell-Charakter der immunhistochemisch mit Anti-KDR und Anti-CD34 markierten Zellen unterstützt wurde. *CD133* zeigte sein Expressionsmaximum im lutealen Stadium der Anbildung. Während dem lutealen Stadium der Rückbildung und der Trächtigkeit war die Expression dieses Markers signifikant reduziert. Die hohe Korrelation der Expression von *CD34* und *CD133* bestätigte zusätzlich die Hypothese, dass Stamm-/Progenitorzellen im

Ovargewebe vorkommen. Das Vorhandensein ortsständiger endothelialer Progenitorzellen innerhalb des Ovars scheint daher sehr wahrscheinlich.

Grundlage für die Erzielung verlässlicher molekularbiologischer Ergebnisse mittels RT-qPCR, ist eine Normalisierung der Zielgenexpression mit Hilfe von stabil exprimierten Referenzgenen (RG). Auf diese Weise ist es möglich, experimentell induzierte Variationen von tatsächlichen biologischen Variationen zu unterscheiden. Das bovine Ovar enthält eine Vielzahl heterogener Zellpopulationen innerhalb der unterschiedlichen Lokalisationen. Diese unterliegen sowohl auf morphologischer wie auch auf molekularbiologischer Ebene einer Beeinflussung durch den Sexualzyklus und die Trächtigkeit. Zum Zeitpunkt der Untersuchung standen keine validierten Referenzgene zur Verfügung, welche sowohl die morphologische als auch die zyklische Heterogenität des Organs berücksichtigen. Daher wurden insgesamt zwölf potentielle Referenzgene auf ihre Eignung mittels unterschiedlicher Berechnungsalgorithmen (GENORM, NORMFINDER, BESTKEEPER) untersucht. Die Referenzgene *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C* und *UBA52* waren in dieser Untersuchung die am stabilsten exprimierten Gene. Damit stellten diese Gene einen Pool an Referenzgene zur Normalisierung von RT-qPCR Ergebnissen für das bovine Ovar dar. Aus diesem Pool können für bovine Ovarien des gesunden Rindes *YWHAZ*, *H3F3B* und *RPS9* zur Normalisierung von RT-qPCR Ergebnissen unabhängig von der Probenlokalisation oder dem lutealen Stadium empfohlen werden. Trotzdem ist eine Revalidierung der Referenzgene für jedes neue Experiment empfehlenswert. Die Software Programme GENORM, NORMFINDER und BESTKEEPER haben unterschiedliche Vorteile und auch Schwachpunkte bei der Ermittlung stabil exprimierter Referenzgene. Deshalb ist es empfehlenswert, mindestens zwei unterschiedliche Berechnungsalgorithmen anzuwenden, wobei eine Berücksichtigung von Variationen zwischen unterschiedlichen Probengruppen nur mittels des NORMFINDER Algorithmus möglich ist.

6 Summary

In situ identification and characterization of endothelial progenitor cells in the bovine ovary

In the course of the reproductive cycle, ovaries are subject to considerable changes, which result in an abundant growth of blood vessels during the formation of the corpus luteum and the regression of blood vessels during luteolysis. The process of neovascularization comprises one third of the total length of the ovarian cycle and is more vigorous than in malignant tumors. The origin of these proliferating endothelial cells has not been completely clarified yet. The enclosed doctoral thesis supports the hypothesis that endothelial progenitor cells (EPCs) act as a local resource and thus are instrumental in the process of neovascularization in the ovaries. Therefore, the objective of this doctoral thesis was to detect the quality and quantity of endothelial progenitor cells in bovine ovaries.

By using a combination of anti-KDR and anti-CD34, it was possible to display immunohistochemically co-labeled cells in paraffine sections of the bovine ovary. KDR and CD34 are the most frequent combination*s* of markers used to identify endothelial progenitors. The co-labeled cells were detectable in the Tunica media but only rarely in the Tunica adventitia of arteries, and only sporadically in venous blood vessels. The co-labeled blood vessels were found predominantly in the corpus luteum and in close vicinity of the ovarian functional bodies. These findings indicate that a vascular wall may be the source of the endothelial progenitor cells in bovine ovaries. In order to verify these immunohistochemical findings, the measurement of mRNA amounts of the endothelial progenitor cell marker *CD34* and *KDR* as well as the stem cell marker *CD133* (*Prominin-1*) was carried out by qPCR. Therefore, specimens from various locations of the bovine ovary, who were taken during different luteal phases, were examined. The mRNA expression of the endothelial progenitor cell markers *CD34* and *KDR* revealed a correlation, which supported the hypothesis that the cells co-labeled with anti-KDR and anti-CD34 had characteristics of endothelial progenitor cells. *CD133* showed its maximum expression during the developmental luteal stage. During the luteal stages regression and pregnancy, the expression of *CD133* was notably reduced. The significant correlation of *CD34* und *CD133* confirmed the hypothesis that stem/progenitor cells are present in ovarian tissue. For this reason, the presence of resident endothelial progenitor cells within the ovaries is very likely.

The foundation for obtaining reliable gene expression results in RT-qPCR experiments is the normalization of the expression of target genes by means of stably expressed reference genes. Thus, it is possible to distinguish variations that have been induced by experimental treatment from their actual biological variations. Bovine ovaries contain multiple heterogeneous cell populations in their different localizations. Both on a morphological and on a molecular biological level, these cell populations are under the influence of both the estrous cycle and pregnancy. At the time of the study, no validated reference genes were available that take both the morphological and the cyclic heterogeneity of the organ into account. Using various calculation algorithms (GENORM, NORMFINDER, BESTKEEPER), a total of 12 potential reference genes were therefore assessed for their suitability. In the course of the assessment, the reference genes *H3F3B*, *RPS9*, *YWHAZ*, *RPS18*, *POLR2C*, and *UBA52* proved to be the most stable of the expressed genes. Based on this result, these genes present a pool of reference genes that can be used to normalize RT-qPCR findings for bovine ovaries. From this pool of genes, *YWHAZ*, *H3F3B*, and *RPS9* can be recommended to normalize RT-qPCR findings irrespective of the location of the specimen or the luteal stage. Still, it is advisable to revalidate the reference genes for each new experiment. Each of the software programmes GENORM, NORMFINDER and BESTKEEPER has their individual advantages but also limitations when establishing the most stable expressed reference genes. Thus, it is recommended to use at least two different calculation algorithms, taking into account that variations between different groups of specimens can only be determined by applying the NORMFINDER algorithm.

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8 Publikationsliste

8.1 Originalartikel

Schoen, K.; Hirschberg, R.M.; Plendl, J.; Kaessmeyer, S. Identification of CD133-, CD34- and KDR-positive cells in the bovine ovary: A new site of vascular wall resident endothelial progenitor cells. Clinical Hemorheology and Microcirculation **2012**, *52*, 67-84, DOI 10.3233/CH-2012-1585

Schoen, K.; Plendl, J.; Gabler, C.; Kaessmeyer, S. Identification of stably expressed reference genes for RT-qPCR data normalization in defined localizations of cyclic bovine ovaries. Anatomia, Histologia, Embryologia **2015**, *44*, 200-211, DOI: 10.1111/ahe.12128

8.2 Beteiligung der Autoren an den Originalartikeln

8.2.1 Identification of CD133-, CD34- and KDR-positive cells in the bovine ovary: A new site of vascular wall resident endothelial progenitor cells

K.S. war an der Konzeption des Versuches beteiligt und entwickelte überwiegend das Versuchsdesign. Es oblag K.S. die molekularbiologischen Proben vollständig zu sammeln. Die molekularbiologische Methodenentwickung ist vollständig, sowie die immunhistochemische Methodenentwicklung überwiegend von K.S. durchgeführt worden. Die molekularbiologischen Daten sind vollständig von K.S. erhoben worden und an der Erhebung der immunhistochemischen Daten war K.S. ebenso beteiligt. Die Auswertungen der molekularbiologischen Daten sind überwiegend von K.S. durchgeführt worden und an der Auswertung der immunhistochemischen Daten war K.S. beteiligt, wie auch an der Ergebnisdiskussion und an der Erstellung des Manuskriptes. **R.H.** oblag überwiegend die immunhistochemische Datenerhebung und -auswertung. Außerdem war R.H. an der Ergebnisdiskussion und der Erstellung des Manuskriptes beteiligt. **J.P.** war an der Konzeption des Versuches, der Ergebnisdiskussion und der Erstellung des Manuskriptes beteiligt. **S.K.** war an der Konzeption des Versuches und dessen methodischer Umsetzung beteiligt. Ebenso hat S.K. das Versuchsdesign mit erarbeitet. S.K. war an der molekularbiologischen und immunhistochemischen Datenauswertung beteiligt, wie auch an der Ergebnisdiskussion und Erstellung des Manuskriptes. **Alle Autoren** waren an der kritischen Durchsicht und Überarbeitung des Manuskriptes beteiligt.

8.2.2 Identification of stably expressed reference genes for RT-qPCR data normalization in defined localizations of cyclic bovine ovaries

K.S. war an der Konzeption des Versuches und dem Versuchsdesign überwiegend beteiligt. Die Proben sind vollständig von ihr gesammelt worden. Die Methodenentwicklung und Datenerhebung wurde vollständig von K.S. durchgeführt. Die Datenauswertung führte K.S. überwiegend durch. An der Ergebnisdiskussion und der Erstellung des Manuskriptes war K.S. überwiegend beteiligt. **J.P.** war an der Konzeption des Versuches, dem Versuchsdesign, der Ergebnisdiskussion sowie der Erstellung des Manuskriptes beteiligt. **C.G.** war an der Konzeption des Versuchs, dem Versuchsdesign, der Ergebnisdiskussion sowie der Erstellung des Manuskriptes beteiligt. **S.K.** war an der Konzeption des Versuches und dessen methodischer Umsetzung beteiligt. Ebenso hat S.K. das Versuchsdesign mit erarbeitet. S.K. war an der Methodenentwicklung und Datenauswertung beteiligt, wie auch an der Ergebnisdiskussion und Erstellung des Manuskriptes. **Alle Autoren** waren an der kritischen Durchsicht und Überarbeitung des Manuskriptes beteiligt.

8.3 Kongressbeiträge

Quitmann K. Identifizierung und Charakterisierung endothelialer Progenitoren im bovinen Corpus luteum in situ. 4. Doktorandensymposium u. DRS Präsentationsseminar "Biomedical Sciences" am Fachbereich Veterinärmedizin der Freien Universität Berlin. Freie Universität Berlin, 06.11.2009

Quitmann K. In situ Identifizierung und Charakterisierung endothelialer Progenitoren im bovinen Corpus lutem cyclicum. 6. Doktorandensymposium u. DRS Präsentationsseminar "Biomedical Sciences" am Fachbereich Veterinärmedizin der Freien Universität Berlin, 1.7.2011

Kaessmeyer, S.; Hirschberg, R.; Schoen, K.; Plendl, J. The adult bovine ovary: a new niche of putative endothelial progenitor cells. 45. Jahrestagung Physiologie und Pathologie der Fortpflanzung und 37. Veterinär-Humanmedizinische Gemeinschaftstagung,
Berlin 29.2. - 2.3. 2012

Schoen, K.; Plendl, J.; Hirschberg, R.M.; Kaessmeyer, S**.** Lokalisation endothelialer Progenitorzellen im Ovar**,** 17. Conference of the European Society of Hemorheology and Microcirculation and 31. Annual Conference of the German Society of Hemorheology and Microcirculation. Halle/Germany, 15. - 16.6.2012

Schoen, K.; Plendl, J.; Kaessmeyer, S. Identifizierung stabil exprimierter Referenzgene im Rinderovar. 7. Doktorandensymposium u. DRS Präsentationsseminar "Biomedical Sciences" am Fachbereich Veterinärmedizin der Freien Universität Berlin, 13.7.2012

Schoen, K.; Plendl, J.; Kaessmeyer, S. Candidate reference genes for the bovine, 29. Congress of European Association of Veterinary Anatomists, Stara Zagora, Bulgaria, 25. - 28.07.2012

Schoen, K.; Plendl, J.; Gabler, C.; Kaessmeyer, S. Evaluation of reference genes for the bovine corpus luteum, 2nd Joint German-Polish Conference on Reproductive Medicine, 46th Annual Conference of Physiology and Pathology of Repdoduction, 38th Joint Conference on Veterinary and Human Reproductive Medicine, Gdansk/Poland, 27.02 - 01.03.2013

Schoen, K.; Plendl, J.; Dietze, K.; Kaessmeyer, S. Investigation of the expression of endothelial progenitor markers in the bovine ovary, 2nd Joint German-Polish Conference on Reproductive Medicine, 46th Annual Conference of Physiology and Pathology of Repdoduction, 38th Joint Conference on Veterinary and Human Reproductive Medicine, Gdansk/Poland, 27.02 - 01.03.2013

Schoen, K.; Plendl, J.; Dietze, K.; Kaessmeyer, S. Expression of endothelial progenitor markers in defined stages of the bovine corpus luteum, Annual meeting of the "Anatomische Gesellschaft", Magdeburg/Germany, 22. - 25.03.2013

Schoen, K.; Plendl, J.; Dietze, K.; Kaessmeyer, S. Beeinflusst Östrogen die Entwicklung endothelialer Stammzellen? 32. Annual Conference of the German Society of Hemorheology and Microcirculation, Dresden/Germany, 24. - 25.05.2013

Schoen, K.; Plendl, J.; Dietze, K.; Kaessmeyer, S. Expression of estrogen receptor 1 and endothelial progenitor markers in the bovine ovary, 7th Meeting of the Young Generation of Veterinary Anatomists, Leipzig/Germany, 17. - 20.07.2013

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10 Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 25. Februar 2015

Kornelia Schoen