REPRODUCTION
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Expression of atresia biomarkers in granulosa cells after ovarian stimulation in heifers

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Abstract

The use of younger gamete donors in dairy cattle genetic selection programs significantly accelerates genetic gains by decreasing the interval between generations. Ovarian stimulation (OS) and the practice of follicle-stimulating hormone (FSH) withdrawal, also known as coasting, are intensively used in pre-pubertal heifers without detrimental effects on subsequent reproductive performance but generally with lower embryo yields. However, recent data from embryo transfer programs showed similar embryo yields in younger and sexually mature animals but with a significant difference in the coasting period. The aim of the present study was to identify a set of granulosa cell biomarkers capable of distinguishing optimal follicle differentiation from late differentiation and atresia in order to assess the differences in coasting dynamics between pre- and post-pubertal donors. We integrated transcriptomic data sets from a public depository and used vote counting meta-analysis in order to elucidate the molecular changes occurring in granulosa cells during late follicle differentiation and atresia. The meta-analysis revealed the gene expression associated with follicle demise, and most importantly, identified potential biomarkers of that status in bovine granulosa cells. The comparison of the expression of six biomarkers between pre- and post-pubertal donors revealed that younger donors had more signs of atresia after the same period of coasting. We found different follicular dynamics following coasting in younger donors. It is possible that younger donors are less capable to sustain follicular survival most likely due to insufficient luteinizing hormone signaling. In summary, the pre-pubertal status influences follicular dynamics and reduces the oocyte developmental competence curve following OS and FSH withdrawal in heifers.

Reproduction (2018) **156** 239–248

Introduction

The manipulation of reproductive hormones requires knowledge of ovarian physiology, which is mainly based on a network of extracellular and intracellular molecular interactions controlled by follicle-stimulating hormone (FSH) and luteinizing hormone (LH). One of the functions of FSH is the tonic stimulation of granulosa cells via its receptor FSHR, which increases intracellular cAMP formation and activation of genes required for proliferation and differentiation (Hillier 2001). Then, tonic stimulation of granulosa cells from dominant follicles by LH via its receptor LHR prior to its surge mimics some of the FSH actions while its surge triggers ovulation (Hillier 2001, Nivet *et al.* 2017). Therefore, the fate of follicles is based on the granulosa cell responses to circulating hormones (Matsuda *et al.* 2012). Understanding these interactions is crucial to define new approaches for pharmaceutical manipulation of ovarian function and to improve fertility treatments.

In cattle, assisted reproductive technologies such as *in vitro* fertilization and embryo transfer were created and have been used for animals of high commercial value. One important discovery that has significantly increased embryo yields in dairy cows is ovarian stimulation (OS) followed by FSH withdrawal, also known as coasting (Sirard *et al.* 1999, Blondin *et al.* 2002). This technique is based on the maintenance of a high concentration of gonadotropin by injection of exogenous FSH for 3 days followed by a period of time with no exogenous FSH before the ovum pick-up (OPU) procedure. During this time, a temporary improvement in oocyte developmental competence generally occurs (Nivet *et al.* 2012). Indeed, the final oocyte competence acquisition occurs between the FSH decline and the pre-ovulation LH surge artificially induced by coasting (Sirard *et al.* 2006). Interestingly, oocyte quality increases at the beginning of coasting, then reaches a maximum after 2 days and plateaus, and then decreases as the follicle begins to undergo atresia and late differentiation (Nivet *et al.* 2012). However, coasting dynamics and the quality of oocytes following coasting vary significantly between animals and according to their sexual maturity (Landry *et al.* 2016, 2017).

Although OS is possible in peri-pubertal or even pre-pubertal heifers, it is well documented that

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-18-0186
Online version via www.reproduction-online.org

embryo yields are significantly lower compared to sexually mature cows (Gandolfi *et al.* 1998, Salamone *et al.* 2001, Kauffold *et al.* 2005, Landry *et al.* 2016). Multiple essential biological functions such as cell differentiation, cell survival and death, inflammation and apoptosis signaling are affected in peri-pubertal heifers (Landry *et al.* 2017). Knowing that basal LH is lower before puberty and gradually increases as heifers reach puberty (Rodriguez & Wise 1989, Day & Nogueira 2013), it was proposed that the unsatisfactory reproduction rates in peri-pubertal heifers is possibly related to the insufficiency in LH signaling (Landry *et al.* 2017). To support this concept, it is possible to identify a number of genes that were associated with atresia and late differentiation in previous studies (Nivet *et al.* 2013, Douville & Sirard 2014, Girard *et al.* 2015). By performing a meta-analysis of transcriptomic data from optimal and late differentiated and atretic follicles, we discovered a number of potential biomarkers to assess the follicular status during coasting. These targets were validated on granulosa cell samples from animals of different ages and different coasting times to test the hypothesis. We found that, indeed, insufficient LH signaling in younger donors during coasting forces follicles to undergo atresia much sooner compared to follicles from sexually mature cows during the same period of coasting. Thus, the optimal window of oocyte competence following coasting is reduced and moved earlier in time.

Materials and methods

Ethics statement

The clinical procedures and industrial practices used at Boviteq follow the established cattle reproduction management practices, which have been approved by the College of Veterinary Surgeons of Quebec (OMVQ), the Canadian Embryo Transfer Association, and the International Embryo Transfer Society. This company follows the Canadian Council on Animal Care guidelines for farm animals and the research projects do not involve the use of exclusive animals for research purposes or the implementation of new animal procedures, other than the ones used in their routine commercial activities, to obtain additional biological samples. This study did not require handling animals on university premises.

Data retrieval

Microarray gene expression data from earlier studies of granulosa cells (Gene Expression Omnibus number: GSE110578, GSE40916, GSE63904, GSE63918, GSE63919 and GSE56145) were retrieved from the ELMA database and pooled together for meta-analysis. All datasets were generated using the EmbryoGENE bovine microarray and were analyzed using the following contrasts: heifers with low blastocyst yields vs cows with optimal blastocyst yields, 44 vs 92 h, and 68 vs 92 h of FSH withdrawal, plateau vs atretic follicles of 6–9 mm and >9 mm in diameter. All microarray experiments were validated in the original studies (Nivet *et al.* 2013, Douville & Sirard 2014, Girard *et al.* 2015). The data from each microarray were subjected to a simple background subtraction, normalized within array (Loess) and between array (Quantile), and analyzed statistically with the Limma package using FlexArray 1.6.1 (http://genomequebec.mcgill.ca/FlexArray).

Meta-analysis of biomarkers of atresia

Microarray meta-analysis was performed using the vote counting method (Tseng *et al.* 2012, Rikke *et al.* 2015). Briefly, differentially expressed (DE) genes are first selected based on a threshold to obtain a list of DE genes from each study. The vote for each gene is then calculated as the total number of times it occurs in all DE lists, and the final DE genes are selected based on the minimal number of votes set by the user. In this study, DE genes with a threshold P value lower than 0.1 from five different arrays were used in the vote counting meta-analysis. The vote counting strategy used in this study ranked biomarkers on the basis of one principal and two secondary criteria. The principal criterion was the number of supporting studies in which each microarray showed significant differential expression in the same direction for a given biomarker, and this counted as a vote in favor of that biomarker being real. Because vote counting frequently leads to ties, we also used two secondary criteria. One being that the first vote should be from the study contrasting good (over 70% embryo yield) and poor donors (lower than 35% embryo yield), in order to find biomarkers that are expressed in poor donors and in association with different stages of atresia. The second was the computation of a simple fold change average between all counts for a given biomarker to differentiate the most promising ones in the full set. The vote counting method was previously tested and approved as a strategy to rank biomarker candidates and is indeed an effective method for selecting biomarkers (Rikke *et al.* 2015).

Analysis of biological functions and upstream regulators

In order to determine which biological functions were affected by the candidate biomarkers, the meta-analysis datasets were subjected to functional analysis using Ingenuity Pathway Analysis (IPA). Briefly, the lists of differentially expressed genes from the meta-analysis were uploaded into IPA and analyzed for major biological functions and potential upstream regulators. This attributed the probability of association between DE genes in the datasets and major biological functions affected. Also, IPA determines the upstream regulators of DE genes by referring to its database of previously known effects of different molecules (endogenous or exogenous) on target genes. Each upstream regulator has an overlap P value and an activation Z score. The activation Z score is an overall score based on the known effects (upregulation or downregulation) of a molecule on each of its target genes. An upstream regulator is thus attributed an activated (Z score >2), inhibited (Z score <−2) or uncertain state based on the observed changes in gene expression of known downstream targets. This analysis enabled

us to identify major upstream regulators as a consequence of follicular atresia.

OS treatment and granulosa cell collection

Samples were obtained according to a previously published study protocol of embryo production from Holstein Bos Taurus (Landry *et al.* 2016). Briefly, each animal was first treated with progesterone (Zoetis, Parsippany-Troy Hills, NJ, USA) in order to reduce the risk of spontaneous ovulation. All large follicles (superior or equal to 5 mm) were aspirated 36–48 h prior to administration of hormones. The OS program consisted of six injections of NIH Folltropin-V (Bioniche Animal Health, Belleville, ON, Canada) administered at 12-h intervals. According to animal age, body weight and/or based on previous stimulations, FSH was administered in five 30 mg, six 30 mg or six 40 mg doses of NIH Folltropin-V, followed by a coasting (no FSH) period of 30 or 43 h. Using transvaginal ultrasonography, follicular diameters were measured and cumulus-oocyte complexes (COCs) were collected by transvaginal puncture under epidural anesthesia (COOK Medical, Bloomington, IN, USA) using an 18-G needle and COOK aspiration unit (COOK Medical). Granulosa cells and COCs were collected in warm HEPES-buffered Tyrode's medium (TLH) containing Hepalean (10 IU/mL). After removing the oocytes and cumulus, granulosa cells were purified by centrifugation (1800 ***g*** RPM for 1 min) and the cell pellets were snap frozen on dry ice before RNA extraction. The OPU procedure was performed in a commercial IVF setting, namely at Boviteq (Saint-Hyacinthe, QC, Canada), a center specialized in bovine embryo transfer and other assisted reproduction technologies involved in research and development.

RNA extraction

Total RNA was extracted from granulosa cells from 32 heifers and 28 cows using the *RNeasy mini kit* (Qiagen), following the protocol recommended by the manufacturer. Total RNA integrity and concentration were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies) with the RNA NanoLab Chip (Agilent Technologies). All extracts were of good quality with RNA integrity numbers higher than 8.9.

Complementary DNA preparation and quantitative real-time polymerase chain reaction

To confirm the microarray analysis results, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on cDNA. Briefly, RNA (325 ng) from granulosa cells (all samples) was reverse-transcribed using a qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) with a mixture of oligo dT and random primers according to the manufacturer's recommendations. The primers used for qRT-PCR are listed in (Supplementary Table 1, see section on supplementary data given at the end of this article) and were designed using the IDT PrimerQuest tool (http://www.idtdna.com/primerquest/home/index) from sequences obtained using *Bos taurus* (taxid: 9913) reference RNA sequences (refseq_rna) and results from our meta-analysis. To confirm the specificity of each pair of primers, electrophoresis on a standard 1.2% agarose gel was performed for each amplified fragment. The PCR products were then purified with the QIAquick PCR purification kit (Qiagen), quantified using the NanoDrop ND-1000 and sequenced. The products were then used to create standard curves for quantification experiments, with dilutions ranging from 2×10^{-4} to 2×10^{-8} ng/nL. Real-time PCR was performed on a LightCycler 480 (Roche Diagnostics, Laval, QC, Canada) using SYBR incorporation in order to analyze gene expression stability in the two groups of granulosa cells (heifers with 30 h of coasting vs cows with 30 h of coasting; heifers with 43 h of coasting vs cows with 43 h of coasting). Each qRT-PCR reaction, in a final volume of 10 µL, contained the complementary DNA, 0.25 mM of each primer and 1X SYBR mixture (LightCycler 480 SYBR Green I Master, Roche Diagnostics). The PCR conditions used for all genes were as follows: denaturing for 10 min at 95°C; 50 PCR cycles (denaturing, 95°C for 10 s; annealing (Supplementary Table 2) for 10 s; extension, 72°C for 20 s), a melting curve (95°C) and a final cooling step at 40°C. Complementary DNA quantifications were performed using LightCycler 480 software version 1.5 (Roche Diagnostics) by comparison with the standard curves. PCR specificity was confirmed by melting-curve analysis provided by the LightCycler software. The data were normalized through geNORM, and the most stable reference genes were identified by the stepwise exclusion of the least stable gene and recalculating the M values. Four housekeeping genes, namely B-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), splicing factor 3 subunit 1 (*SF3A1*) and TATA-Box binding protein were found to be the most stable genes with *M* values ≤1.5 as recommended by the software to normalize our data. Normalized data were cleaned using the Rout method of outlier removal and were analyzed using unpaired *T* test in order to establish significance. Differences were considered to be statistically significant at the 95% confidence level ($P<0.05$). Values are presented as mean ± S.E.M. All statistical analyses were performed using Graphpad Prism, version 7.00 (GraphPad software).

Results

Using the vote counting method, we performed transcriptome meta-analyses of four independent microarray studies (five microarray datasets in total). Each individual array analyzed a different follicular status from the optimal period toward follicular atresia. The DE genes were proposed as potential biomarker candidates and were submitted to functional analysis within IPA and validation by real-time PCR.

In order to explore the effects of coasting time in young and older donors, data from 331 dairy cattle subjected to OS and coasting were randomly selected and divided into two groups based on age : 170 pre-pubertal heifers (ages from less than 10 months) and 161 post-pubertal heifers (ages between 12 and 18 months). There was no significant difference between the blastocyst rates of pre- and post-pubertal animals. However, the duration of

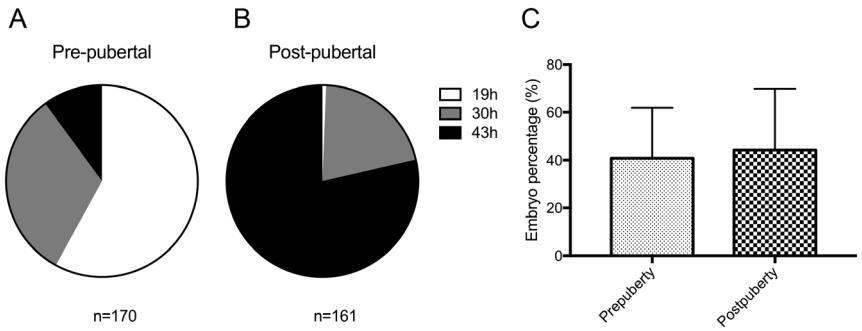

Figure 1 Relationship between oocyte donor age and coasting period length during ovarian stimulation regimens for (A) 170 pre-pubertal heifers and (B) 161 post-pubertal cows. For the same animals, (C) mean embryo percentages 7 days following *in vitro* fertilization of all oocytes matured.

coasting period required to achieve the same blastocyst yield in the two groups was significantly different (chi-square <0.0001) (Fig. 1).

Meta-analysis

Our hypothesis suggests that younger donors have a lower capability to sustain follicle growth during FSH withdrawal and this leads to follicular atresia. Thus, we compared the DE genes of a total of four microarrays that specifically compared granulosa cells from the optimal coasting period to granulosa cells from late differentiation, and granulosa cells from plateau follicles to granulosa cells from atretic follicle of 6–9 mm and >9 mm diameter. Using a vote counting meta-analysis in order to identify potential biomarkers, a total of 193 differentially expressed genes (DEGs) were significantly associated with late follicle differentiation and/or atresia in young donors (Supplementary Table 2). A summary of the 30 most DEGs are shown in Table 1.

Functional analysis

Using the datasets of modulated genes from the meta-analysis above, IPA revealed a significant association between a group of genes expressed differentially in granulosa cells and certain biological functions. The latter include cell death and survival, cellular development, connective tissues development and cellular growth and proliferation (Fig. 2). In addition, IPA generated statistically significant predictions for increased (*Z* score >2) or decreased (*Z* score <−2) activity of upstream transcriptional regulators that are the most activated or inhibited (Table 2A) and the most significant (Table 2B) in association with the average fold change of the DE genes from the meta-analysis.

Validation of meta-analysis gene expression

Confirmation of the microarray and meta-analysis results was obtained in the form of real-time qPCR data for transcripts selected on the basis of differential expression in the poor pre-pubertal donors and on the basis of the meta-analysis. Six candidate biomarkers were selected according to the difference in their expression levels compared to the optimal condition and their relevance in follicular atresia: Mitochondria-localized glutamic acid-rich protein (*MGARP*) also known as the ovary-specific acidic protein (*OSAP*), Gasdermin B (*GSDMB*), creatine kinase brain isoform (*CKB*), potassium channel tetramerization domain containing 8 (*KCTD8*), cysteine-rich secretory protein LCCL domain containing 2 (*CRISPLD2*) and inhibin A (*INHA*) (Fig. 3). The expression

Table 1 The top 15 most downregulated genes and the top 15 most upregulated genes in the meta-analysis of granulosa cells are shown with their average fold change and *P* value. A complete list of DEGs is available in Supplementary Table 2.

Gene symbol	Average FC	Average *P* value	Gene symbol	Average FC	Average *P* value
CALB2	−3.80	0.0287743	SH3RF1	2.37	0.0212858
NMB	−3.17	0.0083525	FOLR2	2.38	0.0395062
INHA	−2.90	0.0223478	CLCA2	2.40	0.0393079
SUSD4	−2.62	0.0214671	CKB	2.41	0.0235166
GSTA5	−2.51	0.0298568	DEFB4	2.41	0.0096596
TBC1D8	−2.39	0.0154918	GSDMB	2.46	0.0261716
GRB14	−2.36	0.0277227	ETS2	2.52	0.0166111
OSAP	−2.34	0.0205689	CD5L	2.74	0.0355574
ECRG4	−2.27	0.0171964	SWAP70	2.77	0.0571574
NAP1L5	−2.22	0.0092056	IL1A	2.96	0.0040150
CITED1	−2.21	0.0052008	CTSS	3.26	0.0236380
TOX2	−2.20	0.0268395	PLAT	3.60	0.0300998
LRRC17	−2.19	0.0158701	TRIB1	3.70	0.0242082
CRISPLD2	−2.12	0.0547113	DEFB5	3.76	0.0253889
FDFT1	−2.06	0.0070817	FABP4	4.02	0.0304273

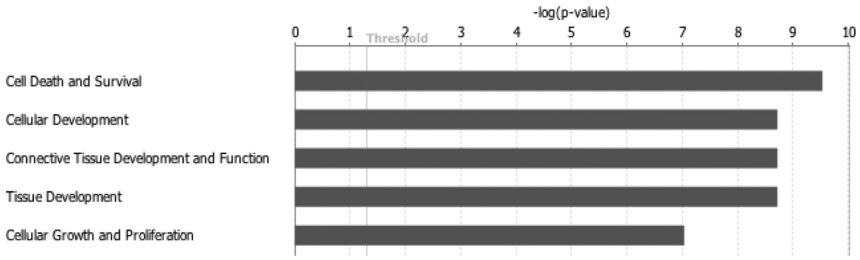

Figure 2 The five most significant biological functions from the meta-analysis datasets of the differentially expressed genes (DEGs) in granulosa cells associated with follicle late differentiation and atresia. The threshold line represents the significant *P* value.

patterns of all candidate genes were consistent with the meta-analysis results.

Discussion

The results of this study showed that pre-pubertal donors have different follicular dynamics following FSH withdrawal and reach follicular atresia sooner than post-pubertal donors. With the accumulation of transcriptomic data from microarray experiments, it was possible to use meta-analysis to further investigate the transcriptional regulation of genes across multiple studies and obtain biologically significant information (Tseng *et al.* 2012). Using the vote counting method for meta-analysis of DEGs (Rikke *et al.* 2015), we compared granulosa cells from optimal follicles to granulosa cells from late differentiated and atretic follicles in superstimulated cows.

Interestingly, embryo rates from pre-pubertal animals, younger than 8 months of age, increased significantly (from 30% to 40% blastocysts) over the last few years (Landry *et al.* 2016, 2017 and this paper), and this is a reflection of the empirical adaptation of coasting time. We previously demonstrated that oocytes originating

Table 2 Upstream regulator analysis on the datasets from the granulosa cell's meta-analysis.

Upstream regulator	Molecule type	Predicted activation state	Activation *Z* score	*P* Value of overlap
(A) Most activated				
NFKB	Complex	Activated	2.183	*1.45E-01*
CXCL12	Cytokine	Activated	2.000	2.59E-02
STAT1	Transcription regulator		1.982	*1.33E-01*
IL1A	Cytokine		1.965	4.73E-02
IL6	Cytokine		1.942	*6.06E-02*
IL1B	Cytokine		1.913	7.56E-04
CSF2	Cytokine		1.912	5.46E-03
CREB1	Transcription regulator		1.890	1.41E-02
APP	Other		1.769	2.24E-04
TLR4	Transmembrane receptor		1.756	5.49E-03
TNF	Cytokine		1.629	6.61E-05
IFNG	Cytokine		1.616	1.78E-03
AHR	Ligand-dependent NR		−1.741	1.40E-02
Progesterone	Chemical-endogenous		−1.757	2.78E-03
PSEN1	Peptidase		−1.969	7.53E-03
(B) Most significant				
β-Estradiol	Chemical-endogenous mammalian		0.993	9.24E-07
VEGF	Group		−0.786	3.05E-05
TNF	Cytokine		1.629	6.61E-05
TP53	Transcription regulator		0.623	8.87E-05
HRAS	Enzyme			1.25E-04
APP	Other		1.769	2.24E-04
ACKR1	g-Protein coupled receptor			2.56E-04
VEGFA	Growth factor		0.818	3.13E-04
HIC1	Transcription regulator		0	4.07E-04
IL10RA	Transmembrane receptor		−0.788	4.07E-04
ESR1	Ligand-dependent nuclear receptor		0.612	4.34E-04
FSH	Complex			5.27E-04
HNF1B	Transcription regulator		−0.152	6.01E-04
LH	Complex			7.51E-04
IL1B	Cytokine		1.913	7.56E-04

(A) Most activated and (B) most significant upstream regulators. For each upstream regulator, molecule type, predicted activation state, *Z* score and statistical significance (*P* value of overlap between the dataset and the genes that are regulated by the upstream regulator) are shown in the table. Italics indicates non-significant.

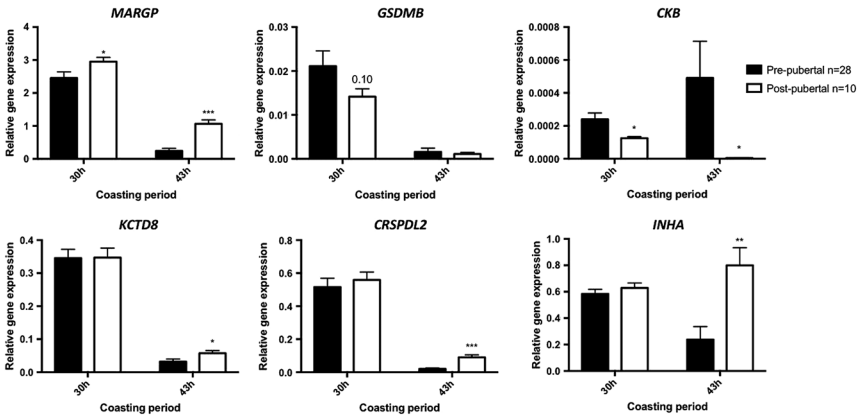

Figure 3 Gene expression levels of six selected biomarkers were used to assess the atresia level at 30 h and 43 h of coasting in pre- and post-pubertal bovine granulosa cells. RT-qPCR values are mean relative expression with standard error of the mean compared to the reference groups (black bar). Significant differences were calculated using unpaired *T* test (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

from heifers younger than 10 months of age are less competent compared to oocytes from sexually mature cows (Landry *et al.* 2016). Before the use of coasting, many studies demonstrated that bovine embryos could be produced successfully *in vitro* using oocytes from sexually immature animals (Majerus *et al.* 1999, Palma *et al.* 2001) but with a significant reduction in embryos yields (Revel *et al.* 1995, Presicce *et al.* 1997, Khatir *et al.* 1998). In the present study, the embryo yield was similar between pre- and post-pubertal cows. However, the coasting period was significantly different between the two groups, suggesting that a shorter period of FSH withdrawal in pre-pubertal animal results in different follicle dynamics and a better embryo outcome. Our meta-analysis may explain the differences as we found DEGs between optimal and late differentiation and atretic follicles that characterize the follicle status in pre- and post-pubertal animals.

Gene analysis

In this study, the genes were analyzed in a standard contrast between pre- and post-pubertal oocyte donors. In this context, depending on the selected biomarkers, they can be associated with optimal timing or late differentiation and atresia. The mitochondria-localized glutamic acid-rich protein (MGARP), also known as the OSAP, is upregulated in dominant follicles (Liu *et al.* 2009). Although the detailed regulatory mechanisms are not yet fully understood, it is known that MGARP is a mitochondrial transmembrane protein that operates during steroidogenesis (Matsumoto *et al.* 2009). It was proposed that MGARP functions as a transporter for the precursors of steroidogenesis and/or helps the newly synthesized steroids exit mitochondria (Zhou *et al.* 2011). Moreover, its expression increased in the same proportion as sex hormone levels (Zhou *et al.* 2011). This association of *MGARP* with steroidogenesis is consistent with our data demonstrating its downregulation in atretic follicles and its association with lower progesterone production as predicted by IPA.

The gene encoding for cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2) is also regulated by progesterone as its expression was significantly increased following progesterone injection in mice (Sriraman *et al.* 2010, Yoo *et al.* 2014). Moreover, the suppression of CRISPLD2 was associated with the downregulation of some important extracellular matrix genes (Zhang *et al.* 2016), which suggests a potential role in the regulation of the ECM enzymes and proteins following the increase in progesterone. Interestingly, CRISPLD2 also inhibited inflammation by the modulation of interleukin IL1-B and IL6 (Zhang *et al.* 2016). The lack of *CRISPLD2* expression contributes to the increase in inflammation and potentially the increase in ROS since atresia is an inflammatory process of cellular death. This suggests that after the same time of coasting, younger donors are more advanced in atresia signaling compared to mature cows.

The inhibins (inhibin A and inhibin B) are glycoprotein hormones from the transforming growth factor B (TGF-B) family. Inhibin A (INHA) is mainly secreted by differentiated granulosa cells under LH during the preovulatory phase (Welt *et al.* 1999, Welt & Schneyer 2001) while inhibin B (INHB) is secreted by developing granulosa cells (Broekmans *et al.* 2009). The expression of *INHA* was detected in granulosa cells from dominant follicles and in the corpus luteum, but not in small antral follicles (Schwall *et al.* 1990). Thus, the level of INHA depends on gonadotropin stimulation and on the stage of follicle development (Welt & Schneyer 2001). The expression of *INHA* decreased faster (at 43 h of coasting) in young animals, supporting the hypothesis of a potential lack of LH action. Taken together, these results suggest that in younger donors, the absence of progesterone, potentially due to the lack of LH signaling, downregulate essential genes that control steroidogenesis and inflammation, leading to the promotion of cellular death and atresia.

Younger donors also expressed multiple atresia biomarkers. The creatine kinase (CK), brain isoform (CKB) is a fundamental enzyme for cellular energy due

to its mechanism of storing, buffering and transporting high-energy phosphates from the mitochondria to places that require energy (Zervou *et al.* 2017). The apoptosis process can be ATP dependent or not, but most commonly, follicular atresia occurs by caspase activation, an ATP-dependent process (Tsujimoto 1997). Based on the high demand of ATP for atresia and the fundamental role of CKB in the maintenance of cellular energy, we can suggest that CKB levels are expected to be increased in atretic follicles. Moreover, a recent study demonstrated higher expression of *CKB* in pre-pubertal donors compared to post-pubertal cows (Landry *et al.* 2017). Similarly, the potassium channel tetramerization domain 8 (KCTD8) gene is part of a voltage-gated potassium channel that affects the permeation of potassium (K+) through the channel pore (Paus *et al.* 2012). A reduction of intracellular levels of K+ may be one of the permissive signals for apoptosis (Yu 2003). Low intracellular K+ decreased the DNA-binding activity of anti-apoptotic transcription factors and increased the binding activity of the pro-apoptotic transcription factors (p53 and forkhead) (Yang *et al.* 2006). Several classes of potassium channels are expressed in granulosa cells (Mason *et al.* 2002, Traut *et al.* 2009), and it was previously suggested that the loss of potassium in oocytes and granulosa cells may be involved in initiating the cascade of events leading to their demise (Perez *et al.* 2000). Suppressing the potassium efflux in whole cells prevents the activation of the nuclease and caspase activity whereas enhancing the efflux of K+ activates those apoptotic enzymes (Hughes and Cidlowski, 1999). This suggests that a lower level of KCTD8 would reduce the intracellular level of K+ by modulating its permeability in granulosa cells and may lead the follicle to atresia in pre-pubertal donors. On the other hand, it is possible that a high concentration of KCTD8 prevents atresia in older animals by conserving normal level of K+ in granulosa cells.

Gasdermin B (GSDMB) is one of the four members of the Gasdermin family and plays a role in the regulation of epithelial proliferation by acting as a tumor suppressor (Hergueta-Redondo *et al.* 2014). Interestingly, GSDMB is a regulator of TGF-B1 expression, which has dual functions with a role in granulosa cell proliferation, differentiation and apoptosis (Kale *et al.* 2013). We suggest that GSDMB is an important mediator of cellular death in granulosa cells and its expression increases apoptotic signals and TGFB to move the follicle toward atresia.

Functional analysis

We also performed functional analysis of the DE gene dataset to describe the biological functions and the upstream regulators associated with the follicle demise. One of the most important factors in folliculogenesis is the balance between cell survival and cell death. It comes to no surprise that biological functions such as cell death and survival, cellular development, growth and proliferation were modulated during granulosa cell late differentiation and atresia. Indeed, apoptosis signaling plays a critical role in the maintenance of the ovarian function in order to remove most follicles that are not suited for ovulation. The first sign of atresia is the initiation of apoptosis in mural granulosa cells, which involves both intrinsic and extrinsic signaling (Carou *et al.* 2017). A complete description of apoptotic pathways in granulosa cells is not available yet, but a spectrum of pro- and anti-apoptosis molecules are finely regulated by hormones such as gonadotropins, growth factors, cytokines and estrogens (Matsuda *et al.* 2012). Interestingly, the upstream regulator analysis revealed some potential modulators of atresia.

The initiation of follicular atresia is orchestrated by the depletion of key survival-promoting factors and/or by specific death ligand molecules (Matsuda-Minehata *et al.* 2006). In granulosa cells, estradiol is produced by the aromatase under FSH and has various actions such as promoting folliculogenesis and inhibiting cell apoptosis (see review Rosenfeld *et al.* 2001). We believe that the decline in FSH and estradiol before the LH surge is a trigger for the initiation of follicle differentiation (Sirard *et al.* 2006). Thus, the follicle produces cytokines in order to increase inflammation and vascularization, which are activated similarly in early apoptosis (Hatzirodos *et al.* 2014). If the LH receptor is expressed in the dominant follicle, the LH surge will trigger differentiation and ovulation. On the other hand, the absence of LHR and/or LH signaling will push the follicle toward atresia following a progressive depletion of survival factors

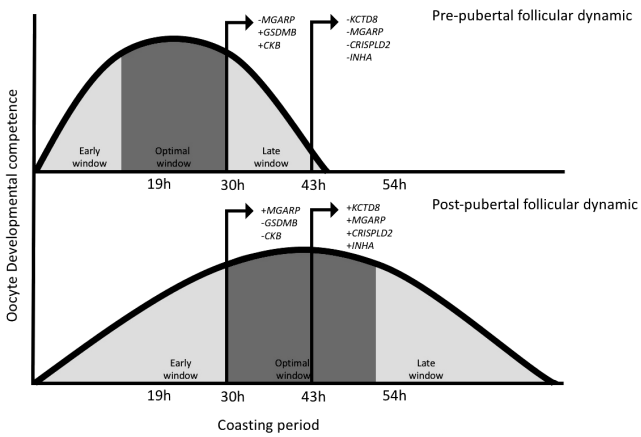

Figure 4 Schematization of follicle dynamics following coasting in pre- and post-pubertal donors. The oocyte developmental competence is represented by the black curve across the coasting period. Each specific phase of follicle dynamics is described inside the curve as follows: early, optimal and late windows of oocyte developmental competence. The arrows represent the expression of the selected biomarkers, a plus or negative (+/−) sign represents an increase or decrease in gene expression compared to the other group for the same period of coasting.

initiated by FSH, estradiol and/or basal LH. Follicle atresia was increased in cows treated with an antagonist to GnRH (Cetrotide) that removes basal LH during FSH withdrawal (Nivet *et al.* 2017), suggesting a crucial role of basal LH in follicle survival. It is interesting to notice that early signs of atresia have a positive effect on oocyte developmental competence (Sirard *et al.* 1999, Vassena *et al.* 2003), which is characteristic of the first step in the initiation of follicle differentiation following FSH decline or withdrawal (Sirard *et al.* 2006).

Follicular atresia is also induced by death ligand-receptors such as the tumor necrosis factor (TNF) signaling (Matsuda *et al.* 2012, Yamamoto *et al.* 2015). In a recent meta-analysis, we determined that TNF is an important regulator of cell differentiation not only associated with optimal oocyte developmental competence but also associated with atresia in follicle persistence (Landry *et al.* 2018). Indeed, TNF is a multifunctional pro-inflammatory cytokine that mediates a wide range of biological functions by binding to two specific receptors (type I and II). Type I receptor contains an intracellular death domain and type II induces gene expression, cell survival, growth and differentiation (Sakumoto *et al.* 2003). TNF plays a broad role during the preovulatory period as it induced ovulation in perfused rat ovaries and the effect was further increased by the addition of LH (Brännström *et al.* 1995). On the other hand, TNF expression was increased to promote the demise of unruptured follicles (Yamamoto *et al.* 2015), necrosis being the result of an inflammatory reaction (Yang *et al.* 2015). However, inflammation also plays a role during folliculogenesis as it is well accepted that mammalian ovulation is comparable to an inflammatory reaction (Jabbour *et al.* 2009). In this study, we showed that atresia involves multiple cytokines inducing inflammation. The follicle persistence without LH surge may cause an inflammatory cascade through the non-canonical pathway of NF-kB, which stimulates the production of pro-inflammatory cytokines such as IL-1B, IL6, INFG and angiogenesis factors (Machelon & Nome 1999, Bhattacharyya *et al.* 2010, Santulli *et al.* 2015, Yang *et al.* 2015) that modulate follicular regression.

Conclusion

The FSH decline before the LH surge plays a crucial role during oocyte developmental competence acquisition, which can be reproduced artificially by FSH withdrawal. However, we showed that pre- and post-pubertal cows have different dynamics following coasting (Fig. 4) and younger donors are less capable of sustaining follicular survival, most likely due to their lack in LH signaling. Thus, it is recommended that FSH withdrawal be shorter in younger donors in order to achieve higher blastocyst rates. Moreover, we provided a set of new biomarkers that will help to characterize the follicular status of atresia in bovine granulosa cells. Those biomarkers will provide new insight in the evaluation of OS regimens.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0186.

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.

Funding

The Natural Sciences and Engineering Research Council of Canada – Collaborative Research and Development (NSERC-CRD grant no. RDCPJ461697-13) provided funding for this study. D A Landry received studentship support from NSERC and REDIH (CIHR Training program in Reproduction, Early Development, and Impact on Health). L R-P received a studentship support from ELAP (Emerging Leader in the Americas Program).

Acknowledgement

The authors would like to thank Boviteq for allowing the use of granulosa cells and data from their heifers and cows.

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Received 10 April 2018
First decision 8 May 2018
Revised manuscript received 25 May 2018
Accepted 15 June 2018