Heat stress effects on the cumulus cells surrounding the bovine oocyte during maturation: altered matrix metalloproteinase 9 and progesterone production

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Abstract

When the effects of heat stress are detrimental during maturation, cumulus cells are intimately associated with the oocyte. To determine the extent to which heat stress affects these cells, in this study, transcriptome profiles of the cumulus that surrounded control and heat-stressed oocytes (41 °C during the first 12 h only and then shifted back to 38.5 °C) during *in vitro* maturation (IVM) were compared using Affymetrix bovine microarrays. The comparison of cumulus-derived profiles revealed a number of transcripts whose levels were increased ($n=11$) or decreased ($n=13$) $\geq$ twofold after heat stress exposure ($P<0.01$), sufficient to reduce the development of blastocysts by 46.4%. In a separate study, quantitative PCR (qPCR) was used to confirm heat-induced differences in the relative abundances of the transcripts of five different genes (caveolin 1, matrix metalloproteinase 9, FSH receptor, Indian hedgehog homolog, and inducible nitric oxide synthase). Heat stress exposure resulted in >1.7 -fold decrease in the protein levels of latent matrix metalloproteinase 9 (proMMP9). Heat-induced reductions in transcript levels were noted at 6 h IVM with reductions in proMMP9 protein levels at 18 h IVM ($P=0.0002$). Independent of temperature, proMMP9 levels at 24 h IVM were positively correlated with the development rate of blastocysts ($R^2=0.36$; $P=0.002$). The production of progesterone increased during maturation; heat-induced increases were evident by 12 h IVM ($P=0.002$). Both MMP9 and progesterone are associated with the developmental competence of the oocyte; thus, it seems plausible for some of the negative consequences of heat stress on the cumulus–oocyte complex to be mediated through heat-induced perturbations occurring in the surrounding cumulus.

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Introduction

Hyperthermia-induced reductions in fertility in bovine species are related to i) alterations in the maternal environment (i.e. endocrine-mediated or other; reviewed by Rensis & Scaramuzzi (2003)) and ii) direct negative effects of elevated temperatures on the oocyte and early embryo (Edwards & Hansen 1996, 1997, Edwards *et al.* 2001). Marked changes occurring in the nucleus and ooplasm during maturation explain why maturing oocytes are susceptible to the direct effects of heat stress (Edwards & Hansen 1996, Roth & Hansen 2004, Edwards *et al.* 2005, Schrock *et al.* 2007).

When the effects of heat stress are most detrimental to the competence of maturing oocytes to develop to the blastocyst stage after fertilization (i.e. during the first 10–12 h of maturation (Putney *et al.* 1989, Edwards & Hansen 1996)), the surrounding cumulus cells are intimately associated with the oocyte. To this end,

numerous cytoplasmic projections extend from the cumulus cells through the zona pellucida penetrating the oolemma sufficiently to form gap junctional complexes (Hyttel *et al.* 1986a, 1986b). Gap junctional complexes between the cumulus and oolemma effectively permit a bidirectional exchange of small metabolic substrates (e.g. choline) and second messengers (e.g. cAMP), whereas the transfer of larger molecules (e.g. KIT ligand) may occur through secretion and receptor-mediated endocytosis (reviewed by Kidder & Vanderhyden (2010)). Even though gap junctional complexes break down during maturation (Hyttel *et al.* 1986a, 1986b), the surrounding cumulus cells encase the oocyte in an extracellular matrix, which plays a key role in ovulation and subsequent fertilization (reviewed by Russell & Robker (2007)).

The presence of cumulus cells is essential for progression to metaphase II and acquisition of embryonic competence. For instance, the removal of cumulus before

maturation impairs the progression to metaphase II and development of blastocysts (Zhang *et al.* 1995, Geshi *et al.* 2000). Culture of denuded bovine oocytes in the presence of a dispersed cumulus or a monolayer is not sufficient to restore the developmental competence (Zhang *et al.* 1995, Luciano *et al.* 2005). Rather, improved competence of denuded oocytes to develop into blastocysts after fertilization has been shown to be dependent on the co-presence of intact cumulus–oocyte complexes at the beginning and through the end of maturation and IVF, suggesting the importance of cumulus-derived secreted factors (Luciano *et al.* 2005).

Unlike the oocyte, cumulus cells are transcriptionally active (Regassa *et al.* 2011) and are capable of undergoing a heat shock response (Payton *et al.* 2011). In porcine species, the effects of heat stress on maturation rates only occurred when cumuli were present (Yuan *et al.* 2008). Prolonged heat stress exposure in bovine species (2.5 °C above the normal body temperature for 24 h) reduced the production of hyaluronan and impaired the expansion of cumulus (Lenz *et al.* 1983). In the mouse, heat stress exposure (1.5 °C above the normal body temperature for 6 h) after reaching maturity (i.e. metaphase II stage) accelerated the aging of oocytes, presumably by increasing the production of an aging-promoting factor by cumulus cells (Qiao *et al.* 2008).

Mindful that cumulus cells are affected by heat stress and essential for the acquisition of embryonic competence after fertilization, we hypothesized that some of the deleterious effects of heat stress on the cumulus–oocyte complex reducing the development of blastocysts may be mediated through heat-induced perturbations occurring in the surrounding cumulus. To determine the extent to which this may be occurring, transcriptome profiles of the cumulus that surrounded the control and heat-stressed oocytes (heat stress applied at 41 °C during the first 12 h only and then shifted back to 38.5 °C) during *in vitro* maturation (IVM) were compared using Affymetrix bovine microarrays. Performing IVF on subsets of cumulus–oocyte complexes allowed assessment of the extent to which cumulus-derived changes related to changes in the competence of the oocytes enveloped therein allowed them to develop to the blastocyst stage. The obtained results prompted a closer examination of heat-induced perturbations in the production of matrix metalloproteinase 9 (MMP9) and progesterone by the control and heat-stressed cumulus–oocyte complexes during maturation.

Materials and methods

Transcriptome profiles of the cumulus surrounding the control and heat-stressed oocytes

Intact cumulus–oocyte complexes underwent IVM at 38.5 °C (control) or 41 °C (heat stress applied during the first 12 h only and then shifted back to 38.5 °C) as shown in Fig. 1. After 24 h

Figure 1 Experimental design and endpoints for evaluating the transcriptome profiles of the cumulus surrounding the control and heat-stressed oocytes. Intact cumulus–oocyte complexes (COCs) underwent IVM at 38.5 °C (control) or 41 °C (heat stress applied during the first 12 h only and then shifted back to 38.5 °C). After a total of 24 h of IVM (h IVM), the surrounding cumulus was harvested; pelleted cells were maintained separately based on the treatment and lysed in RNA extraction buffer. Thereafter, RNA was isolated for the microarray analysis. Before performing IVF on other subsets of control and heat-stressed cumulus–oocyte complexes, the conditioned maturation medium was collected and stored at –20 °C. The development of blastocysts was assessed 8 days after IVF. This experiment was replicated eight different times using 16 microarray chips in total.

of IVM (h IVM), the surrounding cumulus was harvested by vortexing cumulus–oocyte complexes for 4 min in a 0.3% hyaluronidase HEPES-buffered medium. Pelleted cumulus cells originating from 60 cumulus–oocyte complexes, devoid of any denuded oocytes and maintained separately according to the treatment, were lysed in an RNA extraction buffer (Molecular Devices, Sunnyvale, CA, USA) and stored at –80 °C. Conditioned maturation medium resulting from the culture of control and heat-stressed cumulus–oocyte complexes (35–50 per 500 µl medium) was stored at –20 °C. Other subsets of control and heat-stressed cumulus–oocyte complexes (35–50 per 500 µl IVM medium) underwent IVF using Percoll-prepared frozen–thawed semen (pool of two bulls; 500 000 motile sperm/ml) and were allowed to develop to the blastocyst stage as described by Edwards *et al.* (2009) to document the extent to which heat stress reduced the developmental competence of the oocyte enveloped within the cumulus cells. The development of blastocysts was assessed 8 days after IVF.

To provide a more precise test of the hypothesis (i.e. some of the deleterious effects of heat stress on the cumulus–oocyte

complex reducing the development of blastocysts may be mediated through heat-induced perturbations occurring in the surrounding cumulus), RNA isolation was limited to cumulus samples from those experimental replicates ($n=8$) whereby heat stress exposure during oocyte maturation reduced the development of blastocysts by $\geq 20\%$ (PicoPure RNA isolation kit, Molecular Devices). After TURBO DNase treatment (Applied Biosystems/Ambion, Austin, TX, USA), DNA-free total RNA (25 ng) was amplified (Ovation RNA Amplification System V2 kit, NuGEN Technologies, Inc., San Carlos, CA, USA). The resulting cDNA was hybridized to GeneChip Bovine Genome Arrays (Affymetrix, Inc., Santa Clara, CA, USA) and scanned using the GeneChip Scanner 3000 7G with Auto-Loader and the Affymetrix GeneChip Command Console.

Cumulus–oocyte complexes were harvested from antral follicles (~ 3 to 10 mm) as described previously (Payton *et al.* 2011) from abattoir-derived ovaries between the months of January and May and October through December (Brown Packing Company, Gaffney, SC, USA). This experiment was replicated using eight different batches of cumulus–oocyte complexes collected on different occasions (16 total microarray chips) utilizing a total of 2279 cumulus–oocyte complexes (960 were utilized for transcriptome profiling and 1139 for developmental controls).

Relative abundance of certain transcripts (CAV1, MMP9, FSHR, IHH, and NOS2) in the cumulus surrounding the control and heat-stressed oocytes using quantitative PCR (qPCR)

To validate some of the microarray results, the relative abundance of five transcripts identified as differentially expressed in the cumulus surrounding the control and heat-stressed oocytes was examined using cumulus-derived cDNA from a different experimental effort described by Payton *et al.*

(2011). In that study, intact cumulus–oocyte complexes were randomly allotted to culture at 38.5 °C for 0 (i.e. soon after the removal of follicles, but before placement into the IVM medium), 12, or 24 h IVM. Other subsets of intact cumulus–oocyte complexes were cultured at 41.0 °C for 12 h IVM and removed for analysis or cultured for another 12 h after switching from 41.0 to 38.5 °C. At the designated time periods, the surrounding cumulus was harvested by vortexing intact control and heat-stressed cumulus–oocyte complexes (60 per treatment group). The pelleted cumulus cells, devoid of any denuded oocytes, were maintained separately according to the origin of treatment and lysed in RNA extraction buffer for eventual RNA isolation and RT as described by Payton *et al.* (2011). Other subsets of control and heat-stressed cumulus–oocyte complexes underwent IVF (~ 50 per 500 μ l medium) using Percoll-prepared frozen–thawed semen and were allowed to develop to the blastocyst stage to document the extent to which heat stress reduced the developmental competence of the oocyte enveloped within the cumulus cells. The development of blastocysts was assessed 8 days after IVF.

To evaluate the relative abundance of caveolin 1 (CAV1), MMP9, FSH receptor (FSHR), Indian hedgehog homolog (IHH), and nitric oxide synthase 2 (NOS2) transcripts, primer sets were designed using the FastPCR software (ver 4.0.27; Kalendar *et al.* 2009) based on the 5' and 3' ends of transcripts when feasible (Table 1). The efficiencies of primer sets were between 90 and 110%. To ensure specificity, PCR products were sequenced. Each qPCR contained 100 pg of total RNA and was subjected to 50 °C (2 min), 95 °C (10 min), followed by 40 cycles of denaturation for 15 s (95 °C), annealing for 30 s (see Table 1 for temperatures), and extension for 30 s (72 °C), followed by the construction of a dissociation curve. Data were normalized to a calibrator sample using the $\Delta\Delta C_t$ method and correction with GFP as an exogenous control for loading (7300 System SDS RQ

Table 1 Sequences of primers and annealing conditions used for quantitative PCR analyses.

Gene symbol	Entrez number	Amplicon location (bp)	Primer sets	Annealing temperature (°C)	Primer concentration (nM)
CAV1	281040	80–217	5'-CGTAGACTCAGAGGGACATCTC 3'-ACCAGATCTCCTTGGTGTGC	55	200
FSHR	281172	194–357	5'-CTCCCAGGGATGCAGTCGA 3'-TGTAAGTTGGCAGGTTGGA	55	400
		2115–2245	5'-ACACACTTATCCCCCTAAGAC 3'-AGTGGAAGTGCTCTGTCAGC	55	200
IHH	522714	57–157	5'-CCAACTACAATCCAGACATCATCTTC 3'-GATGCCAGCGAGTTCAGGC	60	200
		1432–1552	5'-CCCACTCCTCGGGACACATACG 3'-CCCACTCCTCGGGACACATACG	60	400
NOS2	282876	307–425	5'-AAGGCCAAAGGGGATCTCTC 3'-TGAGGTAGAAGCTCGTCTGG	55	200
		3578–3699	5'-CCCCTCTCCTTCCCTCTCAAACC 3'-GAGGCCAGTTTCTCCACCAC	55	600
MMP2 ^a	282872	1729–2132	5'-ACGAAGACCCACAGGAGGAG 3'-CAGCAATCGGATTTGATGC	56	400
MMP9 ^b	282871	482–554	5'-TGACGGCCCCGAAGCT 3'-GGATCGAAGGGATACCCATCT	56	400
18S rRNA ^c	493779	980–1079	5'-AAGACGGACAGAGCGAAAG 3'-GGTCGGAACACGACGTATCT	60	200
GFP ^c	–	–	5'-CAACTTCAAGACCCGCCACA 3'-TCTGGTAAAAGGACAGGGCCA	–	800

Referenced primer sets: ^aPortela *et al.* (2009), ^bWeber *et al.* (2006), and ^cPayton *et al.* (2011).

Study Software, ver 1.3; Applied Biosystems). The calibrator for the *FSHR*, *IHH*, and *NOS2* transcripts was set to the 0-h IVM cumulus sample within each experimental replicate. *CAV1* and *MMP9* transcripts were not detected in the 0-h IVM cumulus samples, thus the calibrator was set to the 12-h IVM and 38.5 °C cumulus for these samples. Cumulus-derived cDNA was obtained from the cumulus of cumulus–oocyte complexes collected on nine different occasions using a total of 3696 cumulus–oocyte complexes (2640 were utilized for qPCR and 1056 for developmental controls).

Production of MMPs (MMP9 and MMP2) by the control and heat-stressed cumulus–oocyte complexes

The levels of proMMP9 and MMP2 were first examined in the conditioned maturation medium harvested at 24 h IVM and originating after the culture of intact control and heat-stressed cumulus–oocyte complexes (35–50 per 500 µl IVM medium), whereby the cumuli were utilized for transcriptome profiling (Fig. 1). To examine the repeatability of heat-induced reductions in proMMP9 levels observed in the conditioned maturation medium at 24 h IVM, an additional study was conducted. To this end, the conditioned maturation medium was collected after the culture of control and heat-stressed cumulus–oocyte complexes for 24 h IVM (38 to 60 per 500 µl IVM medium). This study was replicated on 12 different occasions (i.e. days with two different conditioned medium samples per treatment group) using a total of 2335 cumulus–oocyte complexes.

The relative levels of proMMP9 and MMP2 were assessed using gelatin zymography as described by Toth & Fridman (2001) with the following modifications. After assessing protein concentration (FluoroProfile protein quantification kit; Sigma Chemical Co.), the conditioned maturation medium was diluted with the Tris–glycine–SDS sample buffer (final concentration 1 µg/µl). The samples were incubated at room temperature for 10 min before loading 20 µg onto 7.5% polyacrylamide–0.1% (w/v) gelatin gels. The gels were run for ~2.5 h at 125 V and then developed for 18 h at 37 °C. Dried Coomassie-stained gels were scanned at 600 dpi resolution, and the resulting images were analyzed using ImageJ (ver 1.42q; Rasband 1997–2012) as described by Leber & Balkwill (1997). To discriminate between latent and active forms of MMPs, unconditioned and conditioned maturation media were subjected to treatment with 1 mM *p*-aminophenylmercuric acetate (APMA) for 1 h at 37 °C before gelatin zymography. Latent forms of recombinant human matrix metalloproteinase 2 (proMMP2) and matrix metalloproteinase 9 (proMMP9) enzymes (AnaSpec, San Jose, CA, USA) were used as standards.

Production of MMPs (MMP9 and MMP2) and progesterone during IVM of cumulus–oocyte complexes at 38.5 or 41.0 °C

Intact cumulus–oocyte complexes underwent IVM for 24 h at 38.5 °C (control) or 41.0 °C (heat stress applied during the first 12 h only and then shifted back to 38.5 °C). Subsets (35–40 per treatment group) were removed from culture at 0, 6, 12, 18,

and 24 h IVM and lysed in an extraction buffer (RNeasy Mini Kit; Qiagen) before storage at –80 °C. The associated conditioned maturation medium was collected, maintained separately based on treatment and h IVM, and stored at –20 °C. Total RNA was isolated and treated with TURBO DNase (Ambion) as per the manufacturer's instructions. Before qPCR, total RNA from each sample was reverse transcribed in the absence of MMLV enzyme and subjected to qPCR using 18S rRNA primers to ensure the lack of genomic DNA contamination. Genomic DNA-free total RNA (250 ng) was spiked with *GFP* cRNA (250 pg) before adding oligo d(T) primers (500 ng; Promega Corporation), 200 units of MMLV reverse transcriptase (Promega), 500 nM dNTPs (Thermo Fisher Scientific, Waltham, MA, USA), and 25 units SUPERase In (Ambion) in 1 × MMLV buffer (25 µl final volume). RT was performed at 37 °C for 1 h followed by heat inactivation at 94 °C for 10 min. The relative abundance of *MMP9* and *MMP2* transcripts was assessed using qPCR as described above; the 6-h IVM cumulus sample, within each replicate, was set as the calibrator. The levels of secreted MMP9 and MMP2 were evaluated in the conditioned maturation medium using gelatin zymography as described above. The amounts of progesterone were measured using a solid-phase RIA (Seals *et al.* 1998); the sensitivity of the assay was 0.02 ng/ml, and the inter- and intra-assay coefficients of variation were 7.6 and 6.0% respectively. This study was replicated on four different occasions (two different conditioned medium pools per treatment group per day) using a total of 2349 cumulus–oocyte complexes.

Statistical analyses

Microarrays were assessed through standard quality control measures for Affymetrix arrays. All quality control measures (preprocessing and analyses) were performed using the statistical computing language R (R Development Core Team, 2012, version 2.15.1) and Bioconductor (Gentleman *et al.* 2004). The intensities of the transcripts were estimated from the probe level data in the log2 scale using three normalization methods: variance stabilization normalization (VSN) (Huber *et al.* 2002), robust multi-array average (RMA) (Irizarry *et al.* 2003), and GCRMA (Wu *et al.* 2004). Data were filtered to remove any probes called marginal or absent on all the arrays and the Affymetrix control probes. Differential expression was tested for each normalization method using Limma (ver 3.14.1; Smyth 2004). Only probes having a *P* value < 0.01 and a fold change of at least 2 and called present for at least half of the arrays were considered significant. The intersection of the lists produced by each normalization method was used to generate the list of differentially expressed transcripts. This approach increases the stringency of the study, improves robustness to experimental noise, and reduces the false discovery rate (FDR); thus, no FDR correction method for multiple testing is necessary. Annotation of the probes was performed using an R annotation package derived from *Bos taurus* build 6.0. Overrepresented gene ontology (GO) terms were identified (*P* ≤ 0.05; based on the number of significant transcripts vs the number expected of the total number of annotated transcripts within each GO term). Similarly,

Table 2 List of transcripts differently expressed in the cumulus after 24 h of *in vitro* maturation of cumulus–oocyte complexes at 38.5 vs 41.0 °C (heat stress applied during the first 12 h only and then shifted back to 38.5 °C) as detected by the microarray analysis.

Gene name	Symbol	Entrez number	Affymetrix probeset ID	Average fold change ^a
Increased in heat-stressed cumulus				
Fetal and adult testis expressed 1	<i>FATE1</i>	281153	Bt.5914.1.S1	3.78
Wingless-type MMTV integration site family, member 2B	<i>WNT2B</i>	445420	Bt.27254.1.A1	3.05
Vanin 1	<i>VNN1</i>	526704	Bt.28243.1.S1	3.03
			Bt.28243.2.S1	2.64
Aarachidonate lipoxygenase, epidermal	<i>ALOX12E</i>	787450	Bt.8917.1.S1	2.91
Orosomucoid 1	<i>ORM1</i>	497200	Bt.2490.1.A1	2.77
Actin, α -1, skeletal muscle	<i>ACTA1</i>	281592	Bt.8435.1.S1	2.70
Nitric oxide synthase 2, inducible	<i>NOS2</i>	282876	Bt.8479.1.A1	2.67
Indian hedgehog homolog (<i>Drosophila</i>)	<i>IHH</i>	522714	Bt.22104.1.S1	2.66
Fibromodulin	<i>FMOD</i>	281168	Bt.3797.1.S1	2.64
FSH receptor	<i>FSHR</i>	281172	Bt.4919.1.S1	2.53
Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	<i>CHST7</i>	540357	Bt.13912.1.S1	2.43
Decreased in heat-stressed cumulus				
Not assigned		767920	Bt.2577.1.S1	−2.17
Trophoblast Kunitz domain protein 1-like	<i>LOC618696</i>	618696	Bt.26365.1.A1	−2.30
Nerve growth factor (β -polypeptide)	<i>NGF</i>	281350	Bt.12757.1.S1	−2.41
ADP-ribosylation factor-like 15	<i>ISL1</i>	614040	Bt.28168.1.A1	−2.54
Galanin prepropeptide	<i>GAL</i>	280799	Bt.176.1.S1	−2.56
Neurotrimin	<i>NTM</i>	534414	Bt.24379.1.A1	−2.62
Hypothesized transcript	<i>EMR3</i>	526347	Bt.21220.1.S1	−2.63
Caveolin 1, caveolae protein, 22 kDa	<i>CAV1</i>	281040	Bt.2230.1.S1	−2.64
Potassium voltage-gated channel, Shab-related subfamily, member 2	<i>KCNB2</i>	535990	Bt.26994.1.A1	−2.66
Purkinje cell protein 4 like 1	<i>PCP4L1</i>	100126444	Bt.5420.1.A1	−2.81
Chemokine (C-X-C motif) ligand 2	<i>CXCL2</i>	281212	Bt.611.1.S2	−2.90
Neuropeptide VF precursor	<i>NPVF</i>	281451	Bt.7042.1.S1	−2.94
Matrix metalloproteinase 9 (gelatinase B)	<i>MMP9</i>	282871	Bt.4714.1.S1	−3.22

^aAveraged from values obtained using three normalization methods where *P* value <0.01 and differences in relative abundance were at least twofold.

differentially abundant transcripts were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway analysis (Kanehisa *et al.* 2006).

Other data were analyzed as a randomized block design with fixed effects of maturation temperature and hours of IVM when appropriate, blocking on replicate (i.e. day of oocyte collection), using binomial or normal distributions as appropriate and generalized linear mixed models (PROC GLIMMIX; SAS 9.2, SAS Inst., Inc., Cary, NC, USA). Treatment differences were determined using protected least significant differences and reported as least squares means $\pm$ S.E.M. using the inverse link option. Regression of MMP9 levels explaining the development of blastocysts was assessed after blocking on replicate, and R-square was calculated from likelihoods (PROC GLIMMIX) as proposed by Nagelkerke (1991).

Results

Transcriptome profiles of the cumulus surrounding the control and heat-stressed oocytes

The comparison of the cumulus-derived transcriptome profiles after IVM of intact cumulus–oocyte complexes at 38.5 or 41 °C (heat stress during the first 12 h only and then shifted back to 38.5 °C for a total of 24 h;

experimental schematic shown in Fig. 1) revealed a number of transcripts whose levels were increased ($n=11$) or decreased ($n=13$; see Table 2) by at least twofold after heat stress exposure ($P<0.01$). Of the 24 transcripts, 23 were annotated. GO enrichment analysis was used to examine which molecular functions, biological processes, or cellular components may differ in the cumulus originating from the control and heat-stressed cumulus–oocyte complexes (Table 3). Only one molecular function was overrepresented from the list of differentially expressed transcripts, whereas several biological processes and cellular components were found to be enriched. Biological pathway analysis (KEGG) suggested a possible overrepresentation of the Hedgehog signaling pathway in the cumulus surrounding the heat-stressed oocytes (*IHH* and *WNT2B* were two significant transcripts of the 20 annotated ones; $P=0.002$).

Changes occurring at the transcriptome level in the cumulus surrounding the oocytes while heat stressed during maturation were coincident with a 46.4% reduction in the development of blastocysts (27.4 vs 15.7%; S.E.M.=1.8%; $P=0.004$ for the control and heat-stressed oocytes respectively) in the subsets of cumulus–oocyte complexes undergoing IVF.

Table 3 Top gene ontology terms identified as overrepresented in cumulus derived from the cumulus–oocyte complexes matured after 24 h at 38.5 vs 41.0 °C (heat stress applied during the first 12 h only and then shifted back to 38.5 °C).

Accession no.	Terms	No. of genes annotated	No. of genes expected	No. of genes significant ^a		P value ^b
Molecular function						
GO:0008083	Growth factor activity	74	0.15	+2	−0	0.009
Biological process						
GO:0006952	Defense response	144	0.32	+1	−3	0.0002
GO:0006809	Nitric oxide biosynthetic process	21	0.05	+1	−1	0.001
GO:0007218	Neuropeptide signaling pathway	27	0.06	+2	−0	0.002
GO:0001666	Response to hypoxia	28	0.06	+1	−1	0.002
GO:0045596	Negative regulation of cell differentiation	52	0.12	+1	−1	0.006
GO:0048856	Anatomical structure development	724	1.62	+4	−2	0.003
GO:0030001	Metal ion transport	165	0.37	+2	−0	0.051
GO:0002376	Immune system process	345	0.77	+1	−2	0.038
GO:0045597	Positive regulation of cell differentiation	56	0.13	+1	−1	0.007
GO:0006807	Nitrogen compound metabolic process	196	0.44	+1	−2	0.008
Cellular component						
GO:0031225	Anchored to membrane	30	0.07	+1	−1	0.002
GO:0048471	Perinuclear region of cytoplasm	37	0.09	+1	−1	0.003
GO:0005887	Integral to plasma membrane	75	0.18	+2	−0	0.013
GO:0005576	Extracellular region	611	1.46	+5	−2	0.0003
GO:0005578	Proteinaceous extracellular matrix	129	0.31	+1	−1	0.037
GO:0005615	Extracellular space	139	0.33	+1	−1	0.043
GO:0000139	Golgi membrane	151	0.36	+1	−1	0.049
GO:0005938	Cell cortex	52	0.12	+1	−1	0.007

^aNumber of genes with a positive fold change (+) and a negative fold change (−); i.e. expression was increased or decreased in heat-stressed cumulus respectively. ^bBased upon the number of significant genes vs the number expected of the total number of annotated bovine genes within each gene ontology term.

Relative abundance of certain transcripts (*CAV1*, *MMP9*, *FSHR*, *IHH*, and *NOS2*) in the cumulus surrounding the control and heat-stressed oocytes using qPCR

To determine the repeatability of some of the heat-induced changes detected using microarrays, the relative abundance of *CAV1*, *MMP9*, *FSHR*, *IHH*, and inducible *NOS2* transcripts was examined in the cumulus originating from cumulus–oocyte complexes cultured at 38.5 °C for 0 (i.e. soon after the removal of follicles, but before placement into the IVM medium; non-matured), 12, or 24 h IVM. Other subsets of intact cumulus–oocyte complexes were cultured at 41.0 °C for 12 h IVM and removed for analysis or cultured for another 12 h after switching from 41 to 38.5 °C.

CAV1 and *MMP9* transcripts were not detectable in cumulus RNA originating from the non-matured cumulus–oocyte complexes. Although similar at 12 h IVM, the relative abundance of *CAV1* transcripts after 24 h IVM was 1.5-fold less in the cumulus originating from the heat-stressed oocytes ($P=0.037$; Fig. 2A). Exposure to 41.0 °C reduced *MMP9* transcript levels by ~2.5-fold at 12 and 24 h IVM ($P<0.0001$; Fig. 2B). Primer sets targeting both the 3' (Fig. 2C, D, and E) and 5' (Fig. 2, inset panels C, D, and E) regions of the *FSHR*, *IHH*, and *NOS2* genes were derived for evaluating the relative abundance of the full-length mRNA. Relative quantities of these transcripts were highest at the onset of maturation (Fig. 2C, D, and E). While heat stress exposure had no effect on the relative abundance of *FSHR* transcripts at 12 h IVM, relative abundance after

24 h IVM was approximately twofold higher in the cumulus originating from the heat-stressed oocytes ($P=0.005$; Fig. 2C). In contrast, heat-related differences in the abundance of *IHH* transcripts were only detected at 12 h IVM (Fig. 2D). The abundance of *NOS2* transcripts was higher in the cumulus originating from the heat-stressed oocytes at 12 and 24 h IVM (Fig. 2E).

The aforementioned changes occurring at the transcript level in the cumulus surrounding the oocytes while heat stressed during maturation were coincident with a 30.9% reduction in the development of blastocysts (30.7 vs 21.2%; S.E.M.=2.1%; $P=0.01$ for the control and heat-stressed oocytes respectively) in the subsets of cumulus–oocyte complexes undergoing IVF.

Production of MMPs (*MMP9* and *MMP2*) by the control and heat-stressed cumulus–oocyte complexes

Because higher *MMP9* levels have been associated with the developmental competence of oocytes (Robert *et al.* 2001), implantation and pregnancy rates (Lee *et al.* 2005, Horka *et al.* 2012), and fertility (Dubois *et al.* 2000), their abundance in the conditioned maturation medium (generated as a part of the transcriptome profiling efforts depicted in Fig. 1) observed after 24 h IVM in the control and heat-stressed oocytes was examined. Heat stress exposure during the first 12 h of maturation resulted in a 1.8-fold decrease in the levels of latent *MMP9* at 24 h IVM (~105 kDa; pro*MMP9*); active *MMP2* (~65 kDa; *MMP2*) was not affected (Fig. 3).

Figure 2 Relative abundance of the caveolin 1 (*CAV1*), matrix metalloproteinase 9 (*MMP9*), FSH receptor (*FSHR*), Indian hedgehog homolog (*IHH*), and inducible nitric oxide synthase (*NOS2*) transcripts in the cumulus surrounding the control and heat-stressed oocytes using qPCR. Transcripts were examined in cumulus originating from the cumulus–oocyte complexes cultured at 38.5 °C for 0 (i.e. soon after the removal of follicles, but before placement into the IVM medium; non-matured), 12, or 24 h IVM. Other subsets of intact cumulus–oocyte complexes were cultured at 41.0 °C for 12 h IVM and removed for analysis or cultured for another 12 h after switching from 41.0 to 38.5 °C (41.0/38.5 °C). The relative abundance of *CAV1* (A) and *MMP9* (B) mRNAs were determined after calibration to 12-h IVM and 38.5 °C cumulus sample as neither were detectable (ND) at 0 h IVM. Calibrator sample for calculating the relative abundance of *FSHR* (C), *IHH* (D) and *NOS2* (E) transcripts was the 0-h IVM cumulus sample. The insets in panels C, D, and E depict data utilizing primers for the 3' end of the *FSHR*, *IHH*, and *NOS2* genes respectively. Bars (least squares means $\pm$ s.e.m.) with different letters denote significance for the main effects of maturation temperature (Temp.) and/or time (h IVM) or interaction of maturation temperature and time (Temp. × h IVM).

The repeatability of the aforementioned findings was confirmed in a separate additional study using the conditioned maturation medium obtained after the maturation of the intact control and heat-stressed cumulus–oocyte complexes for 24 h. To this end, heat stress exposure during the first 12 h only resulted in a

1.7-fold reduction in proMMP9 levels (331 vs 194 densitometric units per cumulus–oocyte complex matured at 38.5 and 41.0 °C respectively, s.e.m.=29; $P=0.0008$) without any effect on MMP2 levels (231 vs 256 densitometric units per cumulus–oocyte complex matured at 38.5 and 41.0 °C respectively, s.e.m.=24;

Figure 3 Heat-induced decreases in matrix metalloproteinase (MMP) levels in the conditioned medium collected after the maturation of cumulus–oocyte complexes for 24 h IVM as depicted in Fig. 1. (A) Gelatin zymogram of representative samples: C – control (38.5 °C); H – heat stress (41.0 °C for the first 12 h IVM and then shifted back to 38.5 °C); M – marker, relative molecular weight standards; proMMP9 – latent form of MMP9; and MMP2 – the active form of MMP2. (B) Comparison of the band intensities of proMMP9 and the active form of MMP2 after adjusting for the number of cumulus–oocyte complexes (COCs) per well. *Bars (least squares means $\pm$ S.E.M.) statistically different within a MMP comparison.

$P=0.0008$) without any effect on MMP2 levels (231 vs 256 densitometric units per cumulus–oocyte complex matured at 38.5 and 41.0 °C respectively, S.E.M.=24; $P=0.217$). In this experimental effort, heat-induced reductions in proMMP9 levels were coincident with a 19.4% reduction in the development of blastocysts (26.3 vs 21.2% for the control and heat-stressed groups respectively; S.E.M.=1.7%; $P<0.03$) in the subsets of cumulus–oocyte complexes undergoing IVF.

Data from transcriptome profiling effort and the additional study conducted to examine the repeatability of heat-induced reductions in proMMP9 levels were combined to examine the relationship of proMMP9 levels in the maturation medium at 24 h IVM with the development rate of blastocysts after oocytes enveloped in the affected cumulus underwent IVF. In this dataset, the development rate of blastocysts ranged from 7 to 45%. Independent of maturation temperature, the development of blastocysts was positively associated with proMMP9 levels in the conditioned maturation medium at 24 h IVM (Fig. 4; $R^2=0.36$; $P=0.002$).

The initial identities of MMP9 and MMP2 were determined by comparison with the molecular weight standards in the adjacent lane (molecular weight macro; ImageJ). Bands were confirmed to be those of gelatinases as the presence of 20 mM EDTA during zymogram development eliminated the digestion of gelatin (data not shown). Subsequent confirmation of band identities was done by comparing the conditioned maturation medium with recombinant human standards of proMMP9 and proMMP2 in the absence or presence of gelatinase activator, APMA. Treatment of the conditioned maturation medium with APMA resulted in a 105 kDa band, identified as that of proMMP9, being activated to an ~87 kDa form without a similar alteration being observed for the 65 kDa band identified as that belonging to MMP2 (Fig. 5). Low-molecular-weight forms observed for recombinant standards and unconditioned maturation medium were probably due to the absence of tissue inhibitors of metalloproteinases (Fig. 5; Toth & Fridman 2001). The majority of MMP2 may have originated from fetal bovine serum in the maturation medium, as its presence was detected in the non-conditioned supplemented medium (Fig. 5), yet it was absent in the M199 base medium (data not shown).

Production of MMPs (MMP9 and MMP2) and progesterone during IVM of cumulus–oocyte complexes at 38.5 or 41.0 °C

MMP9 transcripts were not detected in the non-matured cumulus–oocyte complexes (Fig. 6). Although heat-induced reductions in transcript levels were first observed at 6 h IVM ($P<0.0001$; Fig. 6A), reductions in protein levels (proMMP9) were not observed until

Figure 4 Percentage blastocyst development after the fertilization of control (open circles) and heat-stressed (filled circles) cumulus–oocyte complexes plotted against the levels of latent matrix metalloproteinase 9 (proMMP9) in the conditioned maturation medium after 24 h IVM. Control oocytes were cultured at 38.5 °C, whereas those experiencing heat stress were cultured at 41.0 °C for the first 12 h IVM and then shifted to 38.5 °C.

Figure 5 Gelatin zymogram loaded with recombinant matrix metalloproteinase 9 (latent form, proMMP9), conditioned maturation medium (CMM), recombinant proMMP2, and non-conditioned maturation medium (OMM) without (–) and with (+) *p*-aminophenylmercuric acetate (APMA) pretreatment. The latent forms of metalloproteinases (105 kDa for proMMP9 and 72 kDa for proMMP2) cleave into not only active forms (87 kDa for proMMP9 and 62 kDa for proMMP2) but also intermediate forms (68 kDa for MMP2) and low-molecular-mass forms (77 and 71 kDa for MMP9 and 56 kDa for MMP2) probably due to the absence of tissue inhibitors of metalloproteinases (Toth & Fridman 2001). M – marker lane of relative molecular weight standards used to estimate the size of bands.

18 h IVM ($P=0.0002$; Fig. 6C). MMP2 transcripts were not detected at any of the time points examined (Fig. 6B); protein levels remained constant throughout maturation and were not affected by heat stress exposure (Fig. 6D). The production of progesterone increased during IVM ($P=0.024$); heat-induced increases were evident at 12 h IVM ($P=0.002$; Fig. 7). Results showing

heat-induced increases in the production of progesterone were obtained again on using the conditioned maturation medium originating from the microarray study. The amounts of progesterone produced per each control and heat-stressed cumulus–oocyte complex were 529 and 636 pg respectively ($P=0.0015$; S.E.M. = 36). Progesterone was not detected in the non-conditioned maturation medium.

Discussion

In an effort to identify processes perturbed in the cumulus enveloping the maturing oocyte during heat stress, biological themes were extracted from a list of differentially expressed transcripts by functional annotation in conjunction with GO and pathway analyses. Although a number of functions were determined to be overrepresented, a few of the GO terms were represented by more than two genes. As a consequence, focus was placed on individual transcripts found to be differentially expressed.

To this end, findings described herein provide novel insights into the molecular and cellular mechanism(s) whereby heat stress exposure during maturation directly affects the intimately associated cumulus cells that envelop the maturing oocyte. Heat-induced reductions in the development of blastocysts after

Figure 6 Production of matrix metalloproteinases (MMP9 and MMP2) during IVM of cumulus–oocyte complexes at 38.5 or 41.0 °C. Cumulus–oocyte complexes underwent IVM for 24 h at 38.5 °C (control) or 41.0 °C (heat stress applied during the first 12 h only and then shifted back to 38.5 °C). Subsets were removed from culture at 0, 6, 12, 18, and 24 h IVM. The associated conditioned maturation medium was collected and maintained separately based on treatment and h IVM. (A) Relative abundance of MMP9 mRNA as determined by qPCR; samples were calibrated to the 6-h and 38.5 °C COC sample as mRNA for MMP9 was not detectable (ND) at 0 h IVM. Bars (least squares means $\pm$ S.E.M.) with different letters denote significance for the main effect of maturation temperature (Temp.). (B) Presence of MMP2 transcripts in total RNA was not detectable (ND) by qPCR at any time or IVM temperature. (C) Levels of proMMP9 enzyme in the conditioned medium per gelatin zymography at 6, 12, 18, and 24 h of maturation. Bars with different letters denote a significant IVM temperature $\times$ hours of maturation interaction (Temp. $\times$ h IVM). (D) Levels of MMP2 enzyme in the CMM were similar regardless of the increased time or temperature of maturation. NA, not applicable.

Figure 7 Progesterone production during IVM of cumulus-oocyte complexes at 38.5 or 41.0 °C. Cumulus-oocyte complexes underwent IVM for 24 h at 38.5 °C (control) or 41.0 °C (heat stress applied up to the first 12 h only and then shifted back to 38.5 °C). The conditioned maturation medium was collected and maintained separately based on treatment and h IVM. Bars (least squares means $\pm$ S.E.M.) with different letters denote a significant IVM temperature $\times$ h IVM interaction.

fertilization were coincident with changes in the relative abundance of several developmentally important cumulus-derived transcripts. Validation experiments confirmed heat-induced reductions in the MMP9 transcript and protein levels. Heat-induced increases in the production of progesterone attest to the notion that the cumulus cells originating from the heat-stressed oocytes are functionally different from their non-heat-stressed counterparts. Because of the importance of these two cumulus-derived components (Regassa *et al.* 2011) in the developmental competence of the oocyte (Dubois *et al.* 2000, Silva & Knight 2000, Robert *et al.* 2001, Yamashita *et al.* 2003, Lee *et al.* 2005, Aparicio *et al.* 2011), collectively these findings support the notion for some of the negative consequences of heat stress on the cumulus-oocyte complex are mediated through the cumulus.

During IVM, MMP9 is expressed exclusively by the cumulus surrounding the oocyte (Regassa *et al.* 2011). Our study shows a strong relationship between proMMP9 levels and embryo development occurring after fertilization of the cumulus-oocyte complexes. Thus, heat-induced reductions in MMP9 levels may provide a plausible mechanism to explain why some of the hyperthermia-related reductions in the developmental competence of maturing oocytes occur. This transcript has been identified previously as a marker of bovine oocyte competence because its presence was only detected in the granulosa of follicles yielding oocytes competent to undergo development into embryos after fertilization (Robert *et al.* 2001). Lee *et al.* (2005) concluded that high levels of proMMP9 in preovulatory follicular fluid were requisite for successful implantation and pregnancy in human IVF. The absence

of MMP9 is detrimental to fertility as deficient mice have smaller litter sizes and increased percentages of infertile breeding pairs (Dubois *et al.* 2000).

MMP9 is secreted as a 92 kDa glycosylated pro-matloenzyme, which transforms from a latent (proMMP9) to an active form through a cascade of proteolytic steps involving other MMPs (e.g. MMP2, MMP3, MMP7, and MMP13), urokinase type (uPA), and tissue type plasminogen activators (tPA; reviewed by Van den Steen *et al.* (2002)). Active MMP9 may cleave various substrates (e.g. myelin basic protein, growth factors, interleukins, interleukin receptors, and protease inhibitors; reviewed by Van den Steen *et al.* (2002)). Interaction with the tissue inhibitors of matrix metalloproteinase 1 (TIMP1) regulates activity (reviewed by Van den Steen *et al.* (2002)). Using different approaches (i.e. microarray, qPCR, and zymography) on numerous different occasions with different batches of oocytes, it was found that heat stress consistently reduced transcript (2.2-fold (qPCR) to 3.2-fold (microarray)) and proMMP9 (1.7- to 2-fold) levels. Inability to detect the active MMP9 enzyme may be due to a short half-life (Demestre *et al.* 2005). This does not preclude a functional role for proMMP9 as active MMP9 has rarely been observed in conditioned media even when activity is evident (Fridman *et al.* 2003). Heat stress exposure reduced the transcript levels of PLA2, MMP1, and MMP19 by 1.7-, 1.6-, and 1.8-fold respectively; the abundance of MMP23B, PLAT, TIMP1, and TIMP2 transcripts was not altered (data not shown). Cumulus RNA did not have detectable levels of MMP2 transcripts, which is consistent with a study reporting presence in theca cells only (Liu *et al.* 1998).

The lack of available studies documenting the importance of MMPs during oocyte maturation poses a challenge for explaining the biological significance of heat-induced reductions in proMMP9 levels. In other cell types, MMPs regulate the bioavailability of various growth factors and cytokines (reviewed by Sternlicht & Werb (2001)). The active form of MMP9 in keratinocytes associates with hyaluronan-CD44 (hyaluronic acid receptor) at the cell surface, cleaving transforming growth factor- β into an active form (Yu & Stamenkovic 2000), which influences meiotic maturation in porcine (Coskun & Lin 1994) and murine (Feng *et al.* 1988, Tsafiri *et al.* 1989) oocytes. Notably, each of these components is present in the bovine cumulus-oocyte complex (this study, Furnus *et al.* 2003, Gilchrist *et al.* 2003, Schoenfelder & Einspanier 2003, Regassa *et al.* 2011). Depending on the extent to which function is similar in other cell types, heat-induced reductions in cumulus-derived MMP9 levels may alter the balance of factors favoring stimulatory ligands for meiotic maturation.

While functional significance remains speculative, lower than expected MMP9 levels observed at 24 h IVM may be a consequence of heat-induced increases in cumulus-derived progesterone production. In two

different studies utilizing cumulus–oocyte complexes collected on numerous occasions, heat stress exposure increased the production of progesterone. Microarray data showed concomitant heat-induced increases in the cumulus-derived transcripts of *CYP11A1* (1.6-fold change in the cytochrome P450 side-chain cleavage enzyme responsible for the conversion of cholesterol into pregnenolone; Payne & Hales (2004)) and *HSD3B1* (1.8-fold change in 3 β -hydroxysteroid dehydrogenase, which catalyzes the conversion of pregnenolone into progesterone; Payne & Hales (2004)). Others have shown that progesterone suppresses the secretion of MMP9 in cultured cervical fibroblasts (Imada *et al.* 1997) and first-trimester placental cells (Shimonovitz *et al.* 1998). Using human endometrial explants, Marbaix *et al.* (1992) showed that physiological concentrations of progesterone almost completely abolished the secretion of MMP9.

Cumulus cells secrete less estradiol and more progesterone during oocyte maturation (Schoenfelder *et al.* 2003, Tosca *et al.* 2007). Changes in functionality are probably attributable to a reversion to a mural granulosa cell state (Chaffin *et al.* 2012). In this context, heat-induced increases in cumulus-derived progesterone, while a novel finding of our study, are consistent with data reported by Wolfenson *et al.* (1997) showing an increased production of progesterone by granulosa cells originating from the follicles of heat-stressed cows. Hyperthermia-related consequences were probably due to the direct effects of elevated temperatures as Bridges *et al.* (2005) showed that the cumulative secretion of progesterone by follicle wall pieces in a medium alone was higher when cultured at 41.0 °C than at 39 or 37 °C.

Exposure to higher-than-‘expected’ progesterone levels from the surrounding cumulus during heat stress may not be without a negative consequence on subsequent embryo development. In porcine oocytes, a higher level of progesterone production by cumulus cells accelerated the breakdown of germinal vesicles (Yamashita *et al.* 2003). Furthermore, increasing amounts of progesterone in the maturation medium enhanced the meiotic progression of bovine oocytes (Sirotkin 1992). Although the effects of progesterone levels observed after heat stress exposure on meiotic maturation were not determined in this study, findings in two different species document the effects of heat stress exposure during maturation on hastening developmentally important processes. For instance, Baumgartner & Chrisman (1981) reported a higher percentage of murine oocytes having a bicellular classification (i.e. oocytes contained two cells, with one presumed to be the first polar body) after *in vivo* heat stress exposure. This is consistent with results reported by Kim *et al.* (2002) suggesting that a short-term heat shock permissive of meiotic maturation in murine oocytes accelerated the breakdown of germinal vesicles. After direct application of heat stress

the same as that used in our study, Edwards *et al.* (2005) demonstrated that more bovine oocytes progressed to metaphase I by 8 h IVM, metaphase II by 18 h IVM, and completed cortical granule translocation to the oolemma by 24 h IVM compared with those not exposed to heat stress. Rispoli *et al.* (2011) showed that heat-stressed oocytes similar to older MII oocytes were activated more readily than younger MII oocytes. Schrock *et al.* (2007) demonstrated that earlier fertilization improved the developmental competence of heat-stressed oocytes.

Siqueira *et al.* (2012) showed that progesterone induces meiotic maturation in bovine species in a dose-dependent manner. When cultured with follicular hemisections, progression to MII was enhanced using progesterone concentrations ≤ 100 ng; at concentrations ≥ 1000 ng, meiotic progression was less than optimal. Depending on the extent to which heat-induced increases in the production of progesterone affect meiotic progression, it would be interesting to evaluate progesterone levels in instances where the consequences of heat stress are severe enough to inhibit meiotic progression (Roth & Hansen 2005, Maya-Soriano *et al.* 2012, Nabenishi *et al.* 2012).

In two different studies reported herein, the relative abundance of the transcripts of *NOS2* (the inducible form of nitric oxide synthase) was higher in cumulus originating from the heat-stressed oocytes at 24 h IVM (2.3-fold (qPCR) to 2.7-fold (microarray)). The results reported by Matta *et al.* (2009) showing that the inhibition of *NOS2* during oocyte maturation reduced cumulus expansion, meiotic progression after germinal vesicle breakdown, cortical granule migration, and embryo development after IVF warrant further investigation. Depending on the extent to which transcript levels correspond to the presence of functional protein, it is plausible for heat-induced increases to explain some of the heat-induced hastening of meiotic maturation noted previously.

The Hedgehog signaling pathway has previously been shown to play a role in the development of theca and the expansion of cumuli in mice (Wijgerde *et al.* 2005, Ren *et al.* 2009). The Indian Hedgehog ligand after binding to the Patched receptor stimulates intracellular signaling to alter the transcription of downstream targets including the WNT family members (reviewed by Cohen (2003)). The abundance of *IHH* transcripts decreased during oocyte maturation (this study, Hernandez-Gonzalez *et al.* 2006, Ren *et al.* 2009, Regassa *et al.* 2011). Cumulus originating from the heat-stressed oocytes had higher amounts after 12 h IVM (4.7-fold, qPCR) or 24 h IVM (2.7-fold, microarray). Because heat stress exposure also resulted in a threefold increase in *WNT2B* transcript levels, it is plausible for heat-induced differences to be meaningful. In murine oocytes, elevated Hedgehog signaling resulted in a disorganized expansion of cumulus (Ren *et al.* 2009).

The effects of heat stress exposure on cumulus–oocyte complexes are dependent on severity and duration (Edwards & Hansen 1996, Payton *et al.* 2004). Prolonged heat stress exposure reduces the expansion of cumulus by reducing the production of hyaluronic acid (Lenz *et al.* 1983). In the context of our model, however, the expansion of cumulus after exposure during the first 12 h IVM is more often than not indistinguishable in heat-stressed vs non-heat-stressed controls at 24 h IVM. The expansion of cumulus is mediated, in large part, by FSH (Eppig 1979a, 1979b). The use of microarray and qPCR showed that while *FSHR* transcript levels were decreased (2.5-fold and 2.1-fold respectively) during maturation (this study, Nuttinck *et al.* 2004, Hernandez-Gonzalez *et al.* 2006, Regassa *et al.* 2011, Salhab *et al.* 2011), cumulus originating from the heat-stressed oocytes had higher amounts at 24 h IVM.

Caveolin 1, a scaffolding protein of lipid raft domains on the plasma membrane, has been shown to regulate signal transduction (Ceresa & Schmid 2000) and to co-immunoprecipitate with FSHR (McKenize & Cohen 2009). Heat-induced reductions in the abundance of *CAV1* transcripts (1.7-fold (qPCR) to 2.6-fold (microarray)) may be indicative of impaired signal transduction of FSHR. Although interesting, inferences related to the significance of these and other transcript-based findings are limited, as Nuttinck *et al.* (2004) reported that changes in *FSHR* transcript levels during maturation are not always accompanied by changes in protein levels.

Nonetheless, heat-induced perturbations evident in the cumulus cells that envelop the maturing oocyte involving different biological processes, cellular components, and molecular functions are intriguing as the presence of cumulus is required for the acquisition of developmental competence. Because cumulus cells are transcriptionally active, it was not surprising that heat stress exposure invoked a defense response (*CXCL2*, *VNN1*, *NOS2*, and *ORM1*) similar to that which occurs in response to an injury. Hypoxia-related responses (*CAV1* and *NOS2*) have been shown to activate the heat shock response in other cell types (Benjamin *et al.* 1990). Cumulus cells are no exception, as Payton *et al.* (2011) have previously shown that heat stress exposure similar to that utilized in our study elicited a heat shock response. In the context of the studies described, herein heat stress exposure affected growth factor activity (*CXCL2* and *NGF*). When coupled with changes in transcript levels associated with the extracellular region (*MMP9*, *NPVF*, *CXCL2*, *GAL*, *NGF*, *FMOD*, and *ORM*), these findings are suggestive of heat-induced alterations in the paracrine signaling microenvironment enveloping the maturing oocyte. Depending on the extent to which this occurs, it is certainly plausible for consequences to possibly affect the maturing oocyte enveloped within.

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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