Expression of Maternal Transcripts During Bovine Oocyte In Vitro Maturation Is Affected by Donor Age

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Contents
The primary objective of this study was to compare expression of maternal transcripts in bovine oocyte populations with differential developmental competence: oocytes from prepubertal and pubertal animals; and oocytes from small (3–4 mm) and large (6–10 mm) follicles from pubertal animals. All transcripts were examined in oocytes prior to and after in vitro maturation (IVM). Genes were selected based on their known maternal effect in mouse (ZAR1, STELLA, HSF1, MATER/ NLRP5 and its paralogue NLRP9), or their identification as markers of oocyte maturation, either involved in redox metabolism (PRDX1, PRDX2) or meiotic progression (AURKA). Total or polyadenylated forms of the transcripts were followed by reverse transcription coupled to real-time PCR. Six polyadenylated transcripts were found significantly reduced after maturation irrespective of donor age or follicle diameter (p < 0.05). Within these six polyadenylated transcripts, ZAR1, NLRP9, HSF1, PRDX1 and PRDX2 were significantly reduced in oocytes from prepubertal animals compared to adult animals (p < 0.05). A younger age was also associated with lower abundance (total form) of PRDX2/ PRDX1 irrespective of maturation. Total HSF1, PRDX1 and polyadenylated NLRP9 showed a tendency (p values from 0.053 to 0.08) for a higher detection in oocytes from small follicles, thus encouraging further investigation of the follicle diameter model. However, at the present time, follicle size did not significantly affect expression of transcripts examined. In conclusion, this study demonstrates differences in the maternal store of RNA and its regulation during IVM which is dependent on donor age.

Introduction
Early embryonic failures are often related to poor oocyte quality because of the predominant role of maternal factors, at least until the embryo fully activates its own genome. In vitro experiments on the mammalian oocyte demonstrate the ability to accomplish nuclear competence and to sustain embryo development which is acquired successively during meiosis. Growth of the antral follicle and oocyte maturation are critical phases in generating a developmentally competent oocyte, thus the notion of cytoplasmic pre-maturation and maturation was developed; however, the underlying molecular events remain largely unknown (Bonnet et al. 2008; Ferreira et al. 2009). Transcription is very active in the growing oocyte, although the fully grown oocyte is essentially transcriptionally quiescent. Gene expression in early stage embryos relies mostly on post-transcriptional control of maternal transcripts accumulated during oocyte maturation. Therefore, suboptimal store of maternal RNA or inadequate regulation of their stability and polyadenylation are believed to be negatively impact subsequent embryo development.

Previous grouping of bovine oocyte populations based on their developmental competence (Blondin and Sirard 1995, Sun et al. 2008) makes this species a pertinent scientific model to address this hypothesis. Mature oocytes, collected by ovum pick-up shortly before ovulation, represent the optimal model of oocyte quality; up to 70% develop into blastocysts after in vitro fertilization (IVF), whereas only 30–40% of oocytes after in vitro maturation (IVM) (van de Leemput et al. 1999; Dielemann et al. 2002; Rizos et al. 2002; Humblot et al. 2005). Oocyte developmental competence appears to be acquired during antral follicular growth, as the proportion of competent oocytes increases with antral follicle diameter >3 mm (Pavlok et al. 1992; Lonergan et al. 1994; Blondin and Sirard 1995; Hagemann et al. 1999; Machatkova et al. 2004; Kauffold et al. 2005; Lequarre et al. 2005; Beker-van Woudenberg et al. 2006). Oocyte quality is also influenced by donor age: oocytes from prepubertal animals display a suboptimal developmental competence after IVF, parthenogenesis and nuclear transfer compared to oocytes from adult animals (Levesque and Sirard 1994; Revel et al. 1995; Damiani et al. 1996; Gandolfi et al. 1998; Khatir et al. 1998; Majerus et al. 2000; de Paz et al. 2001; Salamone et al. 2001; Kauffold et al. 2005). Biochemical and molecular parameters of these differential populations of oocytes have been investigated.

A multi-parametric study on follicle diameter showed no significant differences at the oocyte level (Lequarre et al. 2005). In this study, oxygen, pyruvate uptake and lactate release were evaluated, and the corresponding follicle growth was not associated with distortion of neither the transcriptome nor the pattern of protein neosynthesis during IVM, although the transcriptome was analysed only in immature oocytes and onto a low density array of 1176 genes not including maternal effect genes. Thus, variation of a subset of developmentally important transcripts cannot be excluded. Prepubertal calf oocytes have been reported to differ from adult cow oocytes in several aspects: calf oocytes undergo nuclear maturation at a slower rate (Khatir et al. 1998), are smaller in diameter, metabolize glutamine and pyruvate at a lower rate, exhibit an earlier decline in protein synthesis (Gandolfi et al. 1998); and contain more microvilli on their cell surface, more endocytic vesicles and fewer mitochondria than cow oocytes (de Paz et al. 2001). Whether these features are related to developmental competence remains unknown.
The introduction of molecular biology has prompted the search for molecular markers of oocyte quality. Through the use of real-time PCR, transcript levels of selected target genes in oocytes were compared during in vitro vs in vivo maturation (Rizos et al. 2002; Jakobsen et al. 2006; Thelie et al. 2007), or in oocytes from growing follicles (Mourot et al. 2006; Racedo et al. 2008), or in oocytes selected by brilliant cresyl blue (Mota et al. 2009). Interestingly, two studies (Mourot et al. 2006; Thelie et al. 2007) reported a differential expression of the peroxiredoxin1 (PRDX1) transcript, whose protein product is involved in response to oxidative stress.

Maternal effect genes are obvious candidates as markers of developmental competence, as their aberrant expression would impair embryo development without abnormally altering oogenesis or folliculogenesis. Such genes have been mostly characterized in mice by knockout strategies. Oocytes from maternal effect gene-null mice are ovulated and fertilized, even though the resulting embryos did not develop beyond a few cell divisions (for reviews, see Zheng and Dean 2007, Sun et al. 2008). An example is heat-shock transcription factor 1 (HSF1) (Christians et al. 2000). Besides the bovine orthologues Zygote Arrest 1 (ZARI) (Uzbekova et al. 2006), STELLA [also Developmental Pluripotency Associated 3 (Dppa3)] (Thelie et al. 2007), Maternal Antigen that Embryos Require [MATER; also NACHT, leucine-rich-repeat and PYD containing 5 (NALP5)] (Pennetier et al. 2005, 2006) as well as its parologue NLRP9 (Dalbies-Tran et al. 2005) have been described as maternal effect genes.

Using real-time PCR, we have compared the expression of these developmentally important maternal effect genes in bovine oocyte populations with differential developmental competence: oocytes from 3 to 8 mm diameter follicles from prepubertal and pubertal animals; and oocytes from small (3–4 mm) and large (6–10 mm) follicles from pubertal animals. Oocytes from maternal effect gene-null mice are ovulated and fertilized, even though the resulting embryos did not develop beyond a few cell divisions (for reviews, see Zheng and Dean 2007, Sun et al. 2008). An example is heat-shock transcription factor 1 (HSF1) (Christians et al. 2000). Besides the bovine orthologues Zygote Arrest 1 (ZARI) (Uzbekova et al. 2006), STELLA [also Developmental Pluripotency Associated 3 (Dppa3)] (Thelie et al. 2007), Maternal Antigen that Embryos Require [MATER; also NACHT, leucine-rich-repeat and PYD containing 5 (NALP5)] (Pennetier et al. 2005, 2006) as well as its parologue NLRP9 (Dalbies-Tran et al. 2005) have been described as maternal effect genes.

**Materials and Methods**

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma (Sigma, Saint Quentin Fallavier, France).

**Oocyte collection and IVM**

Ovaries from 3 to 7 months old calves (displaying no sign of cyclic activity) and adult cows were obtained from a local slaughterhouse, and oocyte-cumulus complexes (OCC) were collected by aspiration from follicles approximately 3–8 mm in diameter. For follicle size study, follicles were dissected from cow ovaries, measured under the microscope using a graduated ocular, and separated into two groups based on their diameter, 3–4 mm (small) and 6–10 mm (large). The corresponding OCC were extracted, and those showing signs of atresia were discarded. Oocyte-cumulus complexes were washed several times in hepes-buffered TCM199. Ten OCC were submitted to mechanical treatment until separation of oocytes from surrounding somatic cells; absence of cumulus cells was checked visually under a microscope. Pools of 8–10 denuded immature oocytes were frozen in RNAlater solution (Ambion, Huntingdon, UK) at −80°C. The remaining OCC were allowed to undergo IVM for 22 h at 39°C in water-saturated air with 5% CO2; maturation medium was TCM199 supplemented as previously described (Donnay et al. 2004). Groups of OCC (calf vs cow) were incubated in maturation medium drops under mineral oil in four-well plates (Nunc, Roskilde, Denmark) at a ratio of 1 OCC/10 μl medium. After IVM, oocytes with an expanded cumulus were denuded as described, washed, and pools of 8–10 oocytes showing the extrusion of first polar body were frozen in RNAlater. The whole process was replicated four times with independent batches of ovaries for each model.

**Isolation of RNA, reverse-transcription and real-time PCR**

For each experimental model (donor age, follicle size), RNA from four biological replicates for the four populations was isolated in parallel. One picogram of luciferase mRNA (Promega, Charbonnières-les-Bains, France) per oocyte was added as an exogenous standard. There is so far a lack of consensus on the choice of an internal standard in the specific model of oocyte maturation, as during the process many housekeeping transcripts undergo degradation and/or deadenylation. Therefore, normalization to an exogenous RNA has been widely applied in the field (Bettegowda et al. 2006; Pisani et al. 2008; Vigneault et al. 2009). Total DNAse-treated RNA was extracted using the PicoPure ARN Isolation Kit (Alphelys, Plaisir, France) following the manufacturer’s instructions. It was divided into two equal parts, and reverse transcription was performed using either oligo(dT)15 primers (Promega) or random hexamers (Promega) at 42°C for 50 min using SuperScript-II reverse transcriptase (Invitrogen, Cergy-Pontoise, France) followed by 15 min inactivation at 70°C. Target cDNA were then quantified by real-time PCR using iQ SYBR green supermix (Bio-Rad, Marnes la Coquette, France) in a MyCycler system (Bio-Rad) using a cDNA amount equivalent to 0.2 oocyte and specific primers for each gene (Table 1) as previously published (Thelie et al. 2007). Forty PCR cycles (95°C for 30 s, 60°C for 30 s, 72°C for 20 s) were followed by acquisition of the melting curve. For each sample, the median value of PCR triplicates was considered and, the data (deduced from a standard curve, as was the PCR efficiency in the 85–105% range) was normalized to the median value for exogenous luciferase. Then, for each population the average of four biological replicates was calculated. Finally, for the ‘animal age’ and ‘follicle diameter’ differential models, expression was reported.
relative to immature adult oocytes and immature oocytes from large follicles, respectively. Indeed, as the two differential models compared in this article have been analysed in two sets of independent experiments, we cannot compare expression between the two models, only between the different populations within one differential model.

Statistical analysis

Data are presented as the mean ± SEM. Data were analyzed by one-way ANOVA with oocyte maturational stage, age of oocyte donor (calf and cow) and size of follicles (small and large) as fixed factors. When ANOVA revealed a significant effect, values were compared by the post hoc Tukey test. A p value < 0.05 was taken to denote statistical significance.

Results

Using real-time PCR, we have analyzed expression of several target transcripts in oocytes collected from calves and cows, and from cow small (3–4 mm) and large (6–10 mm) follicles, as well as their regulation during IVM. We have used a double approach to analyze the transcripts abundance or total form (based on oligo(dT)-primed RT) and their polyA-tail regulation during IVM. Using real-time PCR, we have analyzed expression of several target transcripts in oocytes from calves and cows, and from cow small (3–4 mm) and large (6–10 mm) follicles, as well as their regulation during IVM.

Effect of oocyte donor age

First, we analyzed the effect of donor age on transcript level before or after IVM (Fig. 1). We noticed a broader variability among the biological replicates of prepubertal immature oocytes. For all genes, similar profiles of variability among the biological replicates of prepubertal or adult animals. There was no significant change in abundance (total form) after IVM. However, after IVM the polyadenylated form of MATER, ZAR1, NLRP9, HSF1, PRDX1 and PRDX2 decreased in both age groups, while neither AURKA nor STELLA variants displayed significant change.

An effect of oocyte donor age was evidenced in several instances. Abundance of PRDX transcripts was higher in oocytes from adult animals than prepubertal animals, both before and after maturation for PRDX2 whereas only in mature oocytes for PRDX1. Also polyadenylated transcripts for ZAR1, NLRP9, HSF1, PRDX1 and PRDX2 decreased to a lower level in calf oocytes when compared to cow, with the strongest difference for PRDX2 (ratio of 50%).

Effect of follicle size

As observed previously, during maturation most polyadenylated forms (MATER, ZAR1, NLRP9, HSF1, PRDX1, PRDX2) fell close to or below half their initial level (Fig. 2), independently of follicle size; again no difference was observed for AURKA or STELLA transcripts. There was no significant effect of follicle diameter on any transcript, although total form of PRDX1 in mature oocytes (p = 0.08), HSF1 in immature oocytes (p = 0.06) and polyadenylated NLRP9 transcript in immature oocytes (p = 0.053) tended to be higher in oocytes from small follicles.

Discussion

relies mostly on RNA accumulated in the egg during oogenesis so it has been hypothesized that developmental competence may be mirrored by the oocyte transcriptome. Here, we investigate whether this difference was correlated to the expression and regulation of transcripts from known maternal effect genes and markers of oocyte maturation.

**Regulation during IVM**

We have described the distinct evolution of total and polyadenylated forms of several oocyte transcripts during IVM including HSF1 which is studied in bovine for the first time. Maturation did not significantly affect the abundance of these nine maternal transcripts but the polyadenylated form of NLRP9 decreased by approximately 40% after IVM, PRDX1 by 70%, and MATER, ZAR1 and PRDX2 by 75–85%. The qualitative profiles (decrease or non-significant variation) we observed and the average ratios of polyadenylated transcripts in mature oocytes (relative to immature) are coincident to previous data with a different maturation medium (Thelie et al. 2007), suggesting that these transcripts are unaffected by the absence of serum. The present study confirms the profiles (decrease or non-significant variation) obtained for this set of genes. This consistency indicates the robustness of our approach, and has prompted us to apply the same methodology to compare quantitative data obtained from oocyte populations with differential developmental competence.

**Effect of follicle diameter**

We did not evidence a significant effect of follicle diameter on detection of this panel of transcripts in immature oocytes nor in their regulation during IVM. This was not unexpected, as transcription is already reduced during the corresponding phase, i.e. when the follicle grows from 3 mm to approximately 10 mm. In a medium-scale transcriptomic approach targeting approximately 1000 genes, oocytes from 3 to 4 mm follicles vs over 5 mm follicles displayed little difference (Lequarre et al. 2005). This study provides evidence that, in this respect, maternal-effect genes do not display a distinctive behaviour when compared to the majority of other genes. Heat-shock transcription factor 1 tended to be slightly more abundant in immature oocytes from smaller follicles, but in the absence of data in the

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**Fig. 1. Variation of transcripts during in vitro maturation of oocytes collected from adult (a) or prepubertal (p) animals. IO and MO refer to immature and in vitro-matured oocytes, respectively. Variation of total forms (grey bars) or polyadenylated forms (black bars). The ratio to the mean value in immature adult oocytes is represented (mean ± SEM). For each gene and form, *indicates a significant effect of maturation, and # a significant effect of animal age (p < 0.05).**

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literature, any interpretation would be very speculative. Among non-maternal-effect genes, our data for AURKA are in agreement with previous analysis revealing no variation throughout antral follicle growth from below 3 mm to over 8 mm (Mourot et al. 2006). Peroxiredoxin1, which tended to be more abundant in mature oocytes originating from smaller follicles, will be discussed in the next paragraph.

Effect of animal age

In this study, we noticed a marked variability among biological replicates of prepubertal immature oocytes that has also been reported when evaluating the content of polyadenylated transcripts in calf and cow oocytes (Lequarre et al. 2004). In our study, several polyadenylated transcripts displayed statistically significant differences. This is in agreement with a report of similar amounts of polyadenylated RNA in cow and calf immature oocytes (53 ± 4 vs 57 ± 8 pg, mean ± SEM), and a similar drop after IVM (to 25 ± 3 and 32 ± 7 pg, mean ± SEM) (Lequarre et al. 2004). Our data also corroborate available proteome analyses: protein patterns were mostly conserved in cow and calf oocytes, whether looking at constitutive or neo-synthesized proteins, but several proteins were detected preferentially or specifically in cow or calf oocytes (Gandolfi et al. 1998; Khatir et al. 1998).

Abundance (total RNA) was unaffected by animal age, except for the PRDX transcripts, which will be discussed later. When considering the polyadenylated forms, none of the selected transcripts displayed a significant difference between cow and calf immature oocytes. Indeed, the genes are not included in the partially available list of age-affected transcripts evidenced by a cDNA array approach (Patel et al. 2007). Lower detection (by semi-quantitative RT-PCR) of several transcripts in germinal vesicle stage oocytes from prepubertal vs adult ewes has also been reported (Leoni et al. 2007). On the other hand, among those polyadenylated transcripts undergoing a major drop during IVM, most decreased even further down in calf oocytes (ZAR1, NLRP9, HSF1, PRDX1, PRDX2). Although the following interpretation is a long shot, it could reveal a more active metabolism and more efficient deadenylation in calf oocytes. Thus, the control of maternal transcripts polyA tail would be deregulated in calf oocytes, affecting their developmental competence. Supporting this hypothesis, several transcripts from predicted developmentally incompetent oocytes

Fig. 2. Variation of maternal effect transcripts during in vitro maturation of cow oocytes collected from large (l) and small (s) follicles. IO and MO refer to immature and in vitro-matured oocytes, respectively. Variation of total forms (grey bars) or polyadenylated forms (black bars). The ratio to the mean value of immature oocytes from large follicle is represented (mean ± SEM). For each gene and form, *indicates a significant effect of maturation (p < 0.05). There was no significant effect of follicle diameter.
displayed shorter polyA tails when compared to competent oocytes, although this was observed not only in in vitro-matured oocytes but also in immature oocytes (Brevini-Gandolfi et al. 1999).

PRDX: markers of oocyte developmental competence?
The influence of oxidative stress onto oocyte maturation and embryonic development (Guerin et al. 2001; Ferreira et al. 2009) has led to investigate expression of the peroxiredoxin genes in gametes or embryos. In bovine, the transcripts are detected in oocytes (Dalbies-Tran and Mermillod 2003, Leyens et al. 2004a), and PRDX1 and PRDX2 transcripts were identified as markers of oocyte maturation, as their polyadenylated form exhibited a major decrease during the process (Dalbies-Tran and Mermillod 2003; Thelie et al. 2009). PRDX2 and PRDX6 proteins were reported to be degraded and synthesized, respectively, during IVM (Leyens et al. 2004b; Bhojwani et al. 2006). Peroxiredoxin1 abundance was differentially regulated during in vitro and in vivo maturation, degraded more severely in the latter case producing oocytes of optimal quality (Thelie et al. 2007). Here, there was an effect of animal age on the total and polyadenylated forms of PRDX1, with a lower detection in calf oocytes, i.e. less competent oocytes. In our second model, PRDX1 tended (p = 0.08) to be more abundant in mature oocytes originating from smaller follicles, that are less competent. However, Mourot et al. (2006) observed a higher level of PRDX1 in immature oocytes from follicles over 8 mm. Despite of distinct follicle classes being analysed in the two studies (3–4 mm and > 6 mm in our case vs < 3 mm, 3–5 mm, 5–8 mm and > 8 mm), it seems unlikely that this would result into observing opposite effects. The discrepancy might rather originate from targeting different variants or normalizing to an internal standard as normalizing implies that expression data are reported relative to an experimental group which is arbitrary decided by the own researcher. Overall, there does not appear to be a simple correlation between PRDX1 expression and developmental potential. Besides PRDX1, other PRDX transcripts were reported to vary between oocyte populations with uneven developmental potential. PRDX5 transcript was over-represented in immature calf oocytes (Patel et al. 2007), an opposite ratio than observed here for PRDX1 and PRDX2. Finally, PRDX6 transcript was decreased in cumulus-free matured oocytes, an alternative model of poor quality (Leyens et al. 2004b). Altogether, various PRDX transcripts appear to be affected in several models of developmental competence (in vivo maturation, animal age, follicle diameter, absence of cumulus cells). Their deregulation likely reflects/causes general alteration of the redox metabolism in oocytes of low developmental potential. However, their expression level is not directly correlated with oocyte quality and a precise range of suitable expression level needs to be defined before they can be used as predictive markers of embryo development.

In conclusion, this study reveals subtle variations in the maternal store of RNA and its regulation over IVM depending on donor age; this supports the hypothesis of a suboptimal molecular maturation of oocytes from prepubertal animals underlying poor developmental competence. It encourages further investigation of the follicle diameter model. Finally, it reinforces the peroxiredoxin genes as candidate markers of developmental potential.

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Conflict of Interest
None of the authors have any conflict of interest to declare.

Authors contributions
RR, RDT and PM contributed to research design, acquisition and interpretation of data. TDS, PP, CP, AT, MEDA participated in the acquisition of data. RR and TDS analysed data. RR and RDT participated in the writing, drafting and critical reviewing of the manuscript.

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