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Delay in maternal transcript degradation in ovine embryos derived from low competence oocytes.

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Abbreviations

ACTB: Actin beta

ANOVA: analysis of variance

ATP1A1: ATPase Na+/K+ transporting subunit alpha 1

BSA: bovine serum albumin

CCNB1: cyclin B1

CDK1: cyclin dependent kinase 1 (also known as CDC2)

COC: oocyte and cumulus complex

DNMT1: DNA methyltransferase 1

DNMT3A: DNA methyltransferase 3A

DNMT3B: DNA methyltransferase 3B

DPPA3: developmental pluripotency-associated protein 3 (also known as PGC7 or STELLA)

EGA: embryonic genome activation

FSH: Follicle Stimulating Hormone

GDF9: growth differentiation factor 9

GV: germinal vesicle (GV)

IGF2R: insulin like growth factor 2 receptor

IVC: *in vitro* culture

IVF: *in vitro* fertilization

IVM: *in vitro* maturation

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LH: Luteinizing hormone

MELK: maternal embryonic leucine zipper kinase

MII: metaphase II oocyte

MZT: maternal to zygotic transition

NPM2: nucleoplasmin 2

OSS: oestrus sheep serum

PBS: phosphate-buffered saline

POU5F1: POU class 5 homeobox 1

RPL19: ribosomal protein L19

RT-PCR: real-time polymerase chain reaction (RT-PCR

SDHA: succinate dehydrogenase complex flavoprotein subunit A

SOF: synthetic oviductal fluid

YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

ZAR1: zygote arrest 1

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Abstract

Oocytes from prepubertal animals have a reduced ability to undergo embryo development and produce viable offspring. The present work used an ovine model consisting of oocytes derived from adult and prepubertal donors to assess the molecular status of oocytes and preimplantation embryos with different developmental competence. The lower potential of oocytes of young donors was confirmed in terms of *in vitro* developmental capabilities and kinetics. A panel of genes including maternal effect (*DPPA3, GDF9, NMP2, ZAR1)* and housekeeping genes (*ACTB, RPL19, SDHA, YWHAZ, ATP1A1)*, genes involved in DNA methylation *(DNMT1, DNMT3A, DNMT3B),* genomic imprinting *(IGF2R)*, pluripotency (*NANOG, POU5F1)* and cell cycle regulation *(CCNB1, CDK1, MELK*) was relatively quantified. Temporal analysis during oocyte maturation and preimplantation embryo development evidenced patterns associated with donor age. With a few gene-specific exceptions, the differential model showed a reduced transcript abundance in immature prepubertal oocytes that completely reversed trend after fertilization, when higher mRNA levels were consistently observed in early embryos, indicating a delay in maternal transcript degradation. We propose that the molecular shortage in the prepubertal oocyte may affect its developmental potential and impair the early pathways of maternal mRNA clearance in the embryo.

While confirming the different potential of oocytes derived from adult and prepubertal donors, our work showed for the first time a consistent delay in maternal transcript degradation in embryos derived from low competence oocytes that interestingly recalls the delayed developmental kinetics. Such abnormal transcript persistence may hinder further development and represents a novel perspective on the complexity of developmental competence.

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Introduction

The use of gametes derived from prepubertal animals in *in vitro* embryo production systems was initially triggered by the interest in improving livestock breeding programs, in terms of reduction of the generational interval and increase in reproductive efficiency (Khatir et al, 1996; Nicholas 1996; Morton 2008*)*. Gametes from prepubertal oocytes were indeed seen to be competent to undergo normal embryo development and produce viable offspring, albeit with lower rates (Armstrong et al, 1992; Revel et al, 1995; Armstrong et al, 1997; Ledda et al, 1999). The efforts in identifying the reasons for the reduced developmental competence not only improved the rates of embryo production from prepubertal donors (Earl et al, 1995; Ledda et al, 1999), but interestingly shed light into numerous aspects involved in oocyte potential. Morphological, cellular, biochemical and molecular studies confirmed the differential competence of gametes derived from adult and prepubertal donors in terms of resistance to cryopreservation (Leoni et al. 2006), metabolism and ultrastructure (O'Brien et al, 1996), transcript abundance (Leoni et al. 2007, Romar et al. 2011, Ledda et al. 2012, Bebbere et al, 2014), *in vitro* developmental competence and kinetics (Majerus et al, 1999; Leoni et al. 2006, 2015; Landry et al, 2016), mitochondrial distribution (Leoni et al. 2015) and methylation dynamics (Masala et al, 2017). Although the differential model has become a useful research tool independently on its breeding application, the limited competence of prepubertal oocytes is yet not fully understood.

The reduced developmental ability of prepubertal gametes is most likely due to several interdependent factors, which reflect the incomplete sexual maturity and related hormonal state of the animal. Oocytes retrieved from adult donors originate from the cyclic recruitment of ovarian follicles, when dynamics are under the control of a full hypothalamus-hypophysis-ovary axis. Conversely, oocytes collected from prepubertal donors derive from the initial recruitment (reviewed in McGee and Hsueh 2000), when the primordial follicles that leave their resting state and start to grow, develop to early antral stage, but physiologically proceed to atresia. That's because the levels of gonadrotrophins to which follicles are exposed in the prepubertal female are insufficient to promote further follicular growth. In accordance, ovarian stimulation using FSH of prepubertal donors was seen to improve the rate of blastocyst formation, underlining the importance of hormonal environment to insure oocyte ability to sustain development (Khatir et al, 1996).

In metazoans, oocyte maturation, fertilization and early embryo development happen in the absence of *de novo* transcription; as a consequence, these events rely on the molecules that are stored during oocyte growth. Such

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maternal control lasts until activation of the nascent embryonic genome (EGA); by this time, the mRNAs of maternal origin are degraded and replaced by the molecules synthesized by the embryo. The molecular baggage of the oocyte is therefore crucial for the first developmental phases of the embryo and affects the activation of its genome as well. The accumulation of transcripts during oocyte growth occurs during follicle recruitment, it is thus likely affected by the different hormonal status encountered during initial or cyclic recruitment. In accordance, abundant literature sustains the cytoplasmic immaturity of prepubertal oocytes in terms of abundance of specific transcripts (Oropeza et al, 2004, Leoni et al, 2007; Bebbere et al, 2014; Bernal-Ulloa et al, 2016; Masala et al, 2017).

Aim of the present work was to assess the effect of oocyte donor age (adult or prepubertal) on the molecular status of oocytes and preimplantation embryos. An ovine model consisting of non-hormonally treated adult and prepubertal donors was used to analyze the temporal expression of a panel of seventeen genes involved in different cell functions. In parallel, oocyte differential quality was assessed in terms of *in vitro* developmental capabilities and kinetics.

Results

In vitro development of adult and prepubertal oocytes.

In Vitro Embryo Production

A total of 740 oocytes derived from prepubertal donors and 671 from adult ewes were employed to evaluate *in vitro* developmental competence. Maturation rate is similar in oocytes obtained from adult or prepubertal animals (Table 1). After fertilization, a significantly lower number of embryos cleaved ($P<0.01$) in the prepubertal group compared to the adult one with 60.4% prepubertal and 69.9% adult cleaved oocytes (Table 1). In addition, the kinetics of the first cleavage showed significant differences between the two groups, with a general delay in the timing of division in the prepubertal gametes (Table 1). Most oocytes derived from adult donors cleaved by 24 hrs post-fertilization, while the majority of prepubertal derived gametes required a longer time. The lower developmental competence of prepubertal oocytes was confirmed by the poorer development to blastocyst stage in terms of absolute rate (24.94% vs 59.73%, $P<0.01$) and developmental kinetics [1.67 % vs 17.45%, $P<0.01$ at 6 days post fertilization (d) and 12.8% vs 29.9%, $P<0.01$ at 7d; Table 2].

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RNA recovery in oocytes and pre-implantation embryos

The quantification of the exogenous *luciferase* mRNA performed by reverse transcription and Real Time PCR showed an RNA recovery rate of 52 ± 5 % (Mean ± SE) and no differences among samples.

Gene expression in oocytes and pre-implantation embryos deriving from adult and prepubertal donors

To investigate the relative expression of a panel of genes during oocyte maturation and embryo preimplantation development, oocytes and embryos were pooled in equal number (n=10 per pool). The relative quantification of all transcripts was performed after normalization against the exogenous *luciferase* mRNA levels and the equal number of oocytes and embryos (Su et al. 2007; Ohsugi et al. 2008; Evsikov and Evsikova 2009).

Messenger RNAs of all evaluated genes were detected in GV and MII oocytes and in embryos up to the 16C stage, independently of the donor age. In most cases, maximal transcript abundance was observed in the immature oocyte and decreased during embryo preimplantation development, with gene-specific patterns (Fig. 1-5). Conversely, *NANOG* mRNA level highest level was observed in embryos at the 8- and 16- cell stage (Fig 4).

In adult-derived oocytes most genes (*ACTB*, *DPPA3*, *GDF9*, *MELK*, *NPM2*, *POU5F1/OCT4, SDHA*, *RPL19*, *ZAR1* and *YWHAZ)* showed a significant reduction in mRNA abundance during oocyte maturation; conversely, only *NPM2* transcript decreased in prepubertal gametes (Panel A of figures 1-5). All transcripts showed a major drop around fertilization (mature oocytes vs two-cell embryos) in embryos derived from adult and prepubertal donors, with the exception of *YWHAZ, NANOG* and *CCNB1* in prepubertal embryos.

ACTB, RPL19, ATP1A1, DPPA3, NPM2, DNMT1 and *DNMT3B* mRNAs maintained a steady level in adult-derived embryos of 2-, 4-, 8- and 16-cells, while *CCNB1, SDHA, GDF9, IGF2R, MELK, YWHAZ and ZAR1* abundance was unvaried in 2-, 4- and 8- cell embryos, and significantly decreased in 16-cell stage embryos. *DNMT3A* transcript showed a significant drop in abundance in embryos at the 4C stage.

Conversely, embryos derived from prepubertal donors showed more variable patterns, with significant drops between 2- and 4- cell stage (*ZAR1*, *CDK1*), most often between 4- and 8- (*MELK*, *ZAR1*, *SDHA*, *ATP1A1, GDF9, CCNB1, CDK1, IGF2R, DNMT1* and *DNMT3B*), or 8- and 16- (*MELK*, *ZAR1*, *GDF9*, *CCNB1,*

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CDK1). In a few cases (*DPPA3*, *YWHAZ* and *RPL19),* the decrease during embryo development was so gradual that a significant decline was observed only between non-consecutive stages of embryo development (i.e. *DPPA3* in 2- vs 8- cell stages; Panel A of Figures 1-5).

Comparison between the expression patterns of adult vs prepubertal groups within each developmental stage (ie. Adult GV vs Prepubertal GV; Adult MII vs Prepubertal MII and so on; Panel B of figures 1-5) showed consistent differences which are summarized in Table 3. The analysis in oocytes revealed similar abundance for most mRNAs, with the exception of *ACTB*, *DPPA3*, *MELK*, *NANOG, POU5F1, RPL19* and *ZAR1* that show lower levels in GV prepubertal oocytes and *NPM2* transcript, which is more abundant in prepubertal MII oocytes (Panel B of figures 1-5 and Table 3). Conversely, most mRNAs show significant differences in embryos around the 2- and 4- cell stage [2 cell stage: *CDK1, DNMT3A, DPPA3, GDF9, IGF2R, MELK, NPM2, SDHA, POU5F, ZAR1;* 4 cell stage: *CCNB1*, *CDK1, DNMT3A, GDF9, MELK, POU5F1, RPL19, SDHA]*. No transcripts varied at the 8- or 16- cell stage. Overall, only four out of 17 genes (*DNMT1, DNMT3B, YWHAZ* and *ATP1A1*) did not differ in oocytes or embryos from donors of different age; most notably, all differentially expressed genes show lower abundance in oocytes, but higher levels in embryos derived from prepubertal donors (Panel B of figures 1-5 and Table 3).

NANOG is the only analysed mRNA whose transcription is resumed by the nascent embryo genome at the 8- and 16- cell stage (Figure 4A and B). Both in adults and in prepubertal oocytes, *NANOG* abundance is steady during oocyte maturation. Conversely, it drops significantly around fertilization in adults, but not in prepubertal embryos (Figure 4A). While a significant higher abundance is observed in adult GV oocytes compared to prepubertal ones, no differences were observed between the two groups during further developmental stages (Figure 4B).

Gene expression in embryos at the blastocyst stage

As expected, the expression of MEGs *(ZAR1, NMP2, DPPA3, GDF9)* was not detected at the blastocyst stage. The other studied genes were expressed, but showed no difference in transcript abundance between adult or prepubertal derived blastocysts.

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Discussion

The most relevant finding of the present work is the delay in maternal transcript degradation observed in embryos derived from low competence oocytes before EGA. Such abnormally prolonged transcript persistence was never reported in early embryos and is most probably involved in the lower developmental competence of oocytes of derived from prepubertal donors.

The study was performed on a previously developed ovine model of differential developmental competence consisting of ovine oocytes derived from non-hormonally treated adult and prepubertal donors. Similar models were previously analyzed in terms of morphology, metabolism and molecular status (Leoni et al. 2006, 2007 and 2015; Romar et al. 2011; Ledda et al. 2012); in the present work, preliminarily to the molecular analysis, we confirmed the reduced developmental potential of prepubertal gametes in terms of *in vitro* developmental capabilities and kinetics. As expected, no differences were observed in terms of IVM, but both cleavage and blastocyst absolute rates and kinetics differed between the two groups of oocytes (Table 1 and 2), with prepubertal embryos achieving lower rates in a longer time. Such observations are in agreement with previous results obtained in several species (Leoni et al, 2006; Landry et al, 2016) and confirmed the lower potential of gametes derived from prepubertal donors.

The temporal expression analysis of the panel of genes showed specific patterns in oocytes and embryos derived from adult and prepubertal donors (Figures 1A-5A). The late EGA that characterizes large domestic animals compared to rodents (8-16 cell stage vs 1-2 cell stage) allowed to detail the expression patterns from fertilization to EGA, identifying the variations specific to the different developmental stages.

With a few gene-specific exceptions, analysis during maturation and early development in the adults evidenced some common features. First of all, relative quantification over oocyte maturation often showed a consistent drop in transcript abundance. As expected, such decrease was observed for all MEGs, with a drop of about 50% for *DPPA3*, *ZAR1* and *GDF9* and 89% for *NPM2* (Figure 2A). The same decrease was observed in all but one housekeeping genes (Figure 1A), in *MELK and POU5F1* (*OCT4*) (Panel A of figures 3 and 4). Interestingly, such significant decrement was never observed during maturation of prepubertal gametes. Comparison between adult and prepubertal oocytes showed a significant lower abundance for seven transcripts (*ACTB, RPL19, DPPA3, ZAR1, MELK, NANOG, POU5F1*) in the young-derived GVs (Panel B of figures 1-5; Table 3). This supports the hypothesis of a cytoplasmic immaturity of oocytes derived from young donors that

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may not be able to provide the proper amount of proteins needed for further development, as previously suggested (Leoni et al, 2007).

Analysis of adult-derived 2 cell embryos showed a second major decrease for all transcripts around fertilization, after which most mRNAs maintain a steady level and virtually disappear around the 8-16 cell stage (Panel A of figures 1-5). At this point of development, all maternal transcripts are replaced by mRNAs of embryonic origin. A significant decrease in transcript abundance was observed also in prepubertal derived 2 cell-embryos, but to a lesser extent, and the decrease during further development to 16C stage is more gradual (Panel A of figures 1-5). As a consequence, comparison between adult- and young-derived embryos consistently showed higher abundance of several mRNAs in prepubertal embryos at the 2C (11 out of 17 genes) and 4C stage (8 out of 17 genes; Table 3, panel B of figures 1-5), while no difference was observed at 8C and 16C. So less competent oocytes interestingly showed lower transcript abundance at the GV stage, but generated embryos with higher mRNA levels just prior to genome activation. Such reversed trend indicates an incomplete accumulation of mRNAs in the prepubertal oocyte, as earlier described, and a previously unreported persistence of maternal transcripts in embryos approaching EGA, which indicates a delay in degradation.

In all animals, the clearance of thousands mRNAs loaded into the egg by the mother is a key event in the transition from maternal control of development to control by the embryonic genome (Giraldez et al, 2006; Bushati et al, 2008; Bartel, 2009; Lund et al, 2009; Walzer and Lipshitz 2011); such elimination is indeed the first event of Maternal to- Zygotic Transition (MZT), which is followed by the beginning of embryonic genome transcription (Tadros and Lipshitz, 2009; Yartseva and Giraldez, 2015). Therefore, our observations of abnormal transcript persistence in prepubertal embryos before EGA indicates some kind of impairment in the mechanisms involved in MZT. Accordingly, embryos derived from young donors show lower development to blastocyst stage (Table 2); most probably, the ones that stop around EGA fail to achieve the dramatic cellular transitions that characterize MZT, including maternal transcript degradation.

Studies in Drosophila showed that some pathways of maternal mRNA clearance are inherited in the oocyte cytoplasm, while others are synthesized de novo by the embryo, highlighting a combined action of both the maternal cytoplasm and zygotic nucleus to the MZT (Bashirullah et al, 1999). The presence of two main modes of mRNA degradation, maternal and zygotic, was confirmed in mouse embryos, where a subset of

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maternal transcripts is degraded soon after fertilization, while others show later decreases around EGA at the two-cell stage (Hamatani et al, 2004). Two waves of transcript destabilization were observed also in human embryos (Dobson et al, 2004; Zhang et al, 2009). As maternal and zygotic degradation activities appear to be a general feature of the metazoan MZT, the existence of similar mechanisms in sheep may be hypothesized. In this species EGA occurs around the 8 cell stage, so we may postulate that the first waves of transcript degradation observed around oocyte maturation and fertilization are controlled by products of maternal origin, while the final phases of transcript clearance that occur as EGA approaches depend on the proper activation of the embryonic genome. This hypothesis would ascribe the higher mRNA abundance observed in 2C and 4C prepubertal embryos to impairment of the first degradation activity, which depends on molecules of maternal origin. Accordingly, prepubertal oocytes at the GV stage show lower transcript abundance, which may impair its developmental competence. Oocytes with a proper transcriptome will in fact have better chances to achieve fertilization, early embryo development and MZT (including maternal transcript clearance) and to complete preimplantation and further development (Table 2). Conversely, oocytes carrying an inadequate transcriptome may have impaired pathways of maternal mRNA clearance that prevent proper EGA and further development.

In the present work, the analysis of gene expression was performed on pools of ten oocytes/embryos, so the observed patterns describe the mean abundance of potentially very different individual mRNA levels. It is likely that each prepubertal pool comprises single oocytes/embryos with transcript levels similar to the adults (prepubertal oocytes/embryos with better quality that will proceed with development) together with ones with altered abundance (prepubertal oocytes/embryos with lower quality that are more likely to arrest at some point of preimplantation development). Therefore, we propose that embryos with significant delay in transcript degradation are likely to arrest during *in vitro* development, because they fail to proceed to MZT and EGA. The observation of similar transcript level at 8-cell stage suggests that such arrest may occur at 2- and 4- cell stage, so that only embryos with higher competence (and proper transcript degradation) would proceed to the 8- cell stage. Accordingly, expression patterns at the 16 cell and blastocyst stage support the hypothesis that embryos able to activate their genome do not show altered transcript abundance on the basis of oocyte donor origin. The similar expression in adults and prepubertals of the only mRNA of embryonic origin, *NANOG,* further sustains such theory (Figure 4A and B). In other words, while pools of oocytes and very early embryos include competent and not competent elements (many of which will not proceed to EGA and reach the

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blastocyst stage), the selection that occurs during *in vitro* development allows only more competent embryos to proceed with development, especially as EGA approaches. Accordingly, embryos that do not reach the blastocyst stage often stop around the time of EGA during *in vitro* culture (Memili and First, 1999).

Although it is accepted that maternal transcript degradation around EGA is critical for embryo development, the exact functions of this important mechanism are not clear (Tadros and Lipshitz 2009). Removal of maternal mRNAs might be necessary to avoid abnormal mRNA dosage in the embryo. The persistence of maternal mRNAs after EGA would indeed result in altered transcript dosage, as observed in often lethal chromosomal aneuploidies (Lindsley et al, 1972). Alternatively, elimination of specific maternal transcripts per se could be essential for early development and be instructive or permissive (Tadros and Lipshitz 2009). In the former case, the gradual decrease in maternal mRNA levels in early embryos exerts a specific function, for instance modulating the action of the relative encoded protein. Conversely, permissive maternal transcript elimination would allow patterned embryonic transcripts to exert their influence. For example, ubiquitously distributed maternal mRNAs could be eliminated to achieve a a spatially and temporally patterned transcription of their embryonic counterparts. Finally, clearance may be aimed to remove transcripts that are crucial during oogenesis (e.g. to protect the oocyte) but which are no longer needed in the embryo (Zeng et al, 2004; Pan et al, 2005). These hypotheses are not mutually exclusive and most probably all underlie the importance of transcript clearance in early embryos. Although further research is needed to clarify the specific effects of impaired maternal mRNA degradation during MZT, the evidence achieved so far clearly indicates detrimental effects for embryo further development.

The present work has some limitations. Firstly, the paucity of material did not allow the quantification of the RNA used for gene expression analysis. This opens up to different interpretations of the over abundance of specific transcripts, such as stabilization of single RNAs on a background of general RNA degradation. Another concern is that the study addressed only mRNA expression, while relative proteins were not examined. Transcript levels do not necessarily represent the exact protein abundance, as gene expression is controlled at multiple levels. Therefore it is not clear whether the increased transcript levels in prepubertal embryos result in altered protein abundance, or if only transcript clearance is affected**.** Further research is needed to clarify such issues.

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In conclusion, our work confirmed that oocytes derived from adult or prepubertal donors have different *in vitro* developmental capabilities and kinetics. We showed a cytoplasmic insufficiency of prepubertal GV oocytes in terms of transcript abundance and for the first time a consistent delay in maternal transcript degradation in prepubertal early embryos. We propose that the molecular shortage in the oocyte may affect its developmental potential and impair the early pathways of maternal mRNA clearance in the embryo. We suggest that such abnormal maternal transcript persistence may hinder further development and contribute with a novel perspective to the complexity of developmental competence.

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Materials and Methods

All chemicals were purchased from Sigma Chemical CO. (St. Louis, MO, USA) unless otherwise stated**.**

Oocyte Recovery and *In Vitro* Maturation

Oocytes were recovered from the ovaries of pre-pubertal (30– 40 days of age, body weight 6–10 kg) and adult (4–5 years of age, body weight 35–40 kg) Sarda sheep, collected at a local slaughterhouse and transported to the laboratory within 1 h in Dulbecco's phosphate-buffered saline (PBS) with antibiotics. After washing in fresh medium, ovaries were sliced using a micro□blade and the follicle content was released in TCM□199 medium (with Earle's salts bicarbonate) supplemented with 25 mmol HEPES, 0.1 g/L penicillin, 0.1 g/L streptomycin and 0.1% (w/v) polyvinyl alcohol.

Cumulus oocyte complexes (COCs) derived from both prepubertal and adult donors, showing several intact cumulus cell layers and a compact cytoplasm, were selected and matured *in vitro* (IVM) in TCM 199 supplemented with 10% heat treated oestrus sheep serum (OSS), 0.1 IU/ml FSH and 0.1 IU/ml LH (Pergonal, Serono Italy) and 100 μM cysteamine.

Then, 30–35 COCs were cultured for 24 h in 5% CO_2 in air at 38.5°C in four□well Petri dishes (Nunclon; Nalge Nunc, Roskilde, 100 Denmark) with 600 μL maturation medium, layered with 300 μL mineral oil. After IVM, oocytes underwent cumulus and corona cells removal by gentle pipetting and only oocytes presenting compact cytoplasm and the first polar body were selected.

In vitro fertilization and embryo development

Frozen-thawed spermatozoa of one single ram of proven fertility (Sarda breed, 4 years old, body weight 55 kg) were used for all *in vitro* embryo production experiments. Two straws were thawed per IVF and spermatozoa were selected by swim-up technique (1×10^6 spermatozoa * mL). IVM oocytes were incubated in synthetic oviductal fluid (SOF) medium (Tervit et al*,* 1972) with 2% OSS, 1 μg/mL heparin and 1 μg/ mL hypotaurine for 22 h at 38.5°C in an atmosphere of 5% CO2 and 5% O_2 in N_2 in four-well Petri dishes (Nunclon, Nalge Nunc). Thereafter, presumptive zygotes were transferred and cultured for 8 days in four-well Petri dishes containing SOF plus essential and non-essential amino acids at oviductal concentrations (Walker et al, 1996)

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+0.4% bovine serum albumin (BSA) under mineral oil, in a maximum humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C (Bogliolo et al. 2011).

At 24 and 30 hr post-fertilization the number of cleaved oocytes, showing two distinct blastomeres, was recorded.

The cleaved oocytes were transferred to a culture medium composed of SOF medium supplemented with 4 mg/ml BSA and essential and non essential amino acids at oviductal concentration (Walker et al, 1996) and kept in maximum humidified atmosphere at 38.5°C, 5% O_2, and 5% CO_2 up to the blastocyst stage.

Gene expression analysis

Details on gene expression analysis by real-time PCR are described according to the MIQE guidelines (Bustin et al, 2009).

Oocyte and embryo collection for gene expression analysis

The RNA samples were isolated from oocytes and embryos obtained during several IVM, IVF and IVC sessions, as follows:

- pools of denuded germinal vesicles (GV) and IVM metaphase II (MII) oocytes derived from prepubertal (P) or adult (A) donors (n=5 pools of 10 oocytes per stage and experimental group).
- *in vitro* produced (IVP) embryos at two- (2C), four- (4C), eight- (8C), 16- (16C) cell stage derived from P or A donors (n=3 pools of 10 embryos per stage and experimental group)
- IVP blastocysts derived from P or A donors (n=3 pools of 7 blastocysts per experimental group)

Oocytes and embryos were denuded via gentle pipetting in order to completely remove any somatic cell and added to 30 µl RLT buffer (RNeasy Micro Kit, Qiagen, Hilden, Germany), snap frozen in liquid nitrogen and stored at – 80°C until RNA isolation.

RNA Isolation and reverse transcription

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Total RNA was isolated from the pools of oocytes or embryos with the RNeasy Micro Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Five pg of luciferase mRNA (Promega) were added to each group prior to RNA extraction to account for RNA loss during the isolation process.

During the procedure, RNA was treated with DNase I to exclude any potential genomic DNA contamination. Isolated RNA was eluted in 15 µL RNase-free water and 13.5 µL immediately used for reverse transcription–polymerase chain reaction (RT-PCR).

Reverse-transcription was performed in a final volume of 20 µL, consisting of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 1 mM dNTPs, 2.5 µM random hexamer primers, 20 U RNase OUT and 100 U SuperScript III RT (all purchased at Invitrogen Corporation, Carlsbad, CA). The reaction tubes were incubated at 25°C for 10 min, then at 42°C for 1 h and finally at 70°C for 15 min to inactivate the reaction. One tube without RNA and one with RNA, but without reverse transcriptase, were analysed as negative controls. To quantify the mRNA recovery rate, 5 pg of luciferase mRNA (not subjected to RNA isolation) was subjected to cDNA synthesis as well and subsequently quantified by Real Time PCR.

Real time-polymerase chain reaction

Primers for all analysed genes were designed to be intron-spanning and are listed in Table 4.

Relative quantification of transcripts was performed by real-time polymerase chain reaction (RT-PCR) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR was performed in a 15 µL reaction volume containing 7.5 µL 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of each primer and cDNA equivalent to 0.25 oocytes or embryos.

The PCR protocol consisted in two incubation steps (50°C for 5 min and 95°C for 2 min), followed by 40 cycles of amplification program [95°C for 15 seconds (s) and gene-specific annealing temperature (see Table 4) for 30 s], a melting curve programme (65–95°C, starting fluorescence acquisition at 65°C and taking measurements at 10-s intervals until the temperature reached 95°C) and finally a cooling step to 4°C. Fluorescence data were acquired during the annealing step.

To minimise handling variation, all samples to be compared were run on the same plate using a PCR master mix containing all reaction components apart from the sample.

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The PCR products were analysed by generating a melting curve to check the specificity and identity of the amplification product. For each primer pair, the efficiency of the PCR reaction was determined by building a standard curve with serial dilutions of a known amount of template, covering at least 3 orders of magnitude, so that the calibration curve's linear interval included the interval above and below the abundance of the targets. Only primers achieving an efficiency of reaction between 90 and 110 % (3.6 > slope > 3.1) and a coefficient of determination $r^2 > 0.99$ were used for the analysis.

The sizes of the RT-PCR products were further confirmed by gel electrophoresis on a 2% agarose gel stained with Sybr Safe (Invitrogen) and visualised by exposure to blue light. The PCR products were sequenced (Model 3130 xl Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) after purification with MinElute PCR purification kit (Qiagen) and sequence identities were confirmed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

The relative quantification of the transcripts was performed after normalization against the exogenous *luciferase* mRNA levels and the number of oocytes and embryos (Su et al, 2007; Ohsugi et al, 2008; Evsikov and Evsikova 2009). Real-time RT-PCR data are presented as ΔCq, mean ± SEM. The relative amount of the target mRNA of each specific sample was obtained after subtraction of the calibrator expression level (ΔCq sample = Cq sample – Cq calibrator), where the calibrator was the sample showing the highest abundance (in most cases a pool of oocytes at the GV stage).

Statistical analysis

Gene expression data were analysed using the MINITAB Release 12.1 software package (Minitab Inc., State College, PA, USA). After testing for normality and equal variance using the Kolmogorov–Smirnov and Levene's tests respectively, transcript data were analysed with general linear model analysis of variance (ANOVA), followed by Tukey's *post hoc* comparisons when *P* values were significant.

Differences in maturation, fertilization, cleavage and blastocyst rates between prepubertal and adult oocytes were determined by the Chi-square test.

Differences were considered significant when $P < 0.05$.

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The authors have no competing interest to declare.

Figure legends

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	Oocytes	Matured	Cleaved (in hours post-fertilization)		
			24 hr	30 hr	Total
Prepubertal	740	697 (94.2%)	160 (38.1%) [a]	261 (61.9%) [a]	421 (60.4%) [a]
Adult	671	642 (95.7%)	341 (76.2%) [b]	106 (23.7%) [b]	447 (69.6%) [b]

Table 1. IVM and cleavage rates of prepubertal and adult oocytes.
Maturation rate is calculated on the initial number of oocytes. Total cleavage rates are calculated on the number of mature oocytes. Cleavage rates at 24 and 30 hr are calculated on total cleaved embryos. Different letters within each column indicate a significant difference (P<0.05). Hr: hours post-fertilization.

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	Cleaved embryos	Blastocysts			Total Blastocysts
		6d	7d	8d	
Prepubertal	421	7 (1.67%) [a]	54 (12.8%) [a]	44 (10.4%) [a]	105 (24.94%) [a]
Adult	447	78 (17.45%) [b]	134 (29.9%) [b]	55 (12.3%) [a]	267 (59.73%) [b]

Table 2. Blastocyst rates of prepubertal and adult oocytes.
Blastocyst rates are calculated on the number of cleaved embryos. Different letters within each column indicate a significant difference ($P<0.01$).
d= days post fertilization.

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	GV	MII	2C	4C	8C	16C	BL
Housekeeping genes							
ACTB	A>P						
ATP1A1							
RPL19	A>P		P>A*	P>A			
SDHA			P>A	P>A			
YWHAZ							
Maternal Effect Genes							
DPPA3	A>P		P>A**				
GDF9			P>A	P>A			
NPM2		P>A	P>A				
ZAR1	A>P		P>A				
Genes involved in cell cycle regulation							
CCNB1				P>A***			
CDK1			P>A	P>A			
MELK	A>P		P>A*	P>A			
Genes involved in pluripotency							
NANOG	A>P						
POU5F1	A>P*		P>A	P>A			
Genes involved in epigenetic mechanisms							
DNMT1							
DNMT3A			P>A	P>A			
DNMT3B							
IGF2R			P>A				

Table 3. Transcripts with different abundance by stage ($P < 0.05$).
A= Adult, P=Prepubertal. Grey and orange backgrounds indicate significant higher abundance in adult- and prepubertal- derived gametes/embryos, respectively.
* $P = 0.054$; ** $P = 0.052$; *** $P = 0.083$

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Gene	GenBank accession n.	Primers	Annealing temperature	Size (bps)
ACTB	NM_001009784	F: 5' TTCCTGGGTATGGATCCTG 3' R: 5' GGTGATCTCCTTCTGCATCC 3'	60° C	162
ATP1A1	X02813	F: 5' GCTGACTTGGTCATCTGCAA 3' R: 5' CATTCCAGGGCAGTAGGAAA 3'	58° C	129
CCNB1	L26548	F: 5' CAGTGTATGACAGGTAATGC 3' R: 5' CGTAGTCCAGCATAGTTAGT 3'	56° C	134
CDK1	NM_001142508	F: 5' TCCTGGTCAGTTCATGGATTC 3' R: 5' CTGTGGAGAACTCTTCTAGAG 3'	56° C	96
DNMT1	NM_001009473	F: 5' CAGCTCTCGTACATCCACAG 3' R: 5' AATCTCGCGTAGTCTTGGTC 3'	60° C	158
DNMT3A	XM_015094252	F: 5' GTGATGATTGATGCCAAAGA 3' R: 5' GGTCCTCACTTTGCTGAACT 3'	60° C	165
DNMT3B	XM_012189044	F: 5' ATTGCAACAGGGTACTTGGT 3' R: 5' ATATTTGATGTTGCCCTCGT 3'	60° C	122
DPPA3	XM_004006906	F: 5' GAGTGAGCGGAGATACAGGA 3' R: 5' CTTCGCACTCTTGATCGAAT 3'	58° C	135
GDF9	AF078545	F: 5' CAGACGCCACCTCTACAACA 3' R: 5' CAGGAAAGGGAAAAGAAATGG 3'	58° C	198
IGF2R	AF353513	F: 5' CATTACTTCGAGTGGAGGAC 3' R: 5' ATCAAGACCAGCGGTGCTTA 3'	60° C	137
luciferase	AF093685	F: 5' GCTGGGCGTTAATCAGAGAG 3' R: 5' GTGTTCGTCTTCGTCCCAGT 3'	58° C	151
MELK	XM_004004250	F: 5' TGCCTTGAAGAACCTGAGAC 3' R: 5' GCTGTGCACATAAGCAACTG 3'	60° C	220
NPM2	XM_004004208	F: 5' GGAGGAAGATGATGATGACG 3' R: 5' CATGGTTCTTCCTTGGTCAC 3'	60° C	208
POU5F1	NM_174580	F: 5' GAGGAGTCCCAGGACATCAA 3' R: 5' CCGCAGCTTACACATGTTCT 3'	56° C	204
RPL19	XM_004012837	F: 5' CAACTCCCGCCAGCAGAT 3' R: 5' CCGGGAATGGACAGTCACA 3'	56 ° C	127
SDHA	XM_004017097	F: 5' CATCCACTACATGACGGAGCA 3' R: 5' ATCTTGCCATCTTCAGTTCTGCTA 3'	60 ° C	125
YWHAZ	NM_001267887	F: 5' TGTAGGAGCCCGTAGGTCATCT 3' R: 5' TTCTCTCTGTATTCTCGAGCCATCT 3'	60 ° C	168
ZAR1	HM037367	F: 5' CACTGCAAGGACTGCAATATC 3' R: 5' CAGGTGATATCCTCCACTC 3'	60° C	137

Table 4. Primers used for Real-Time PCR experiments.

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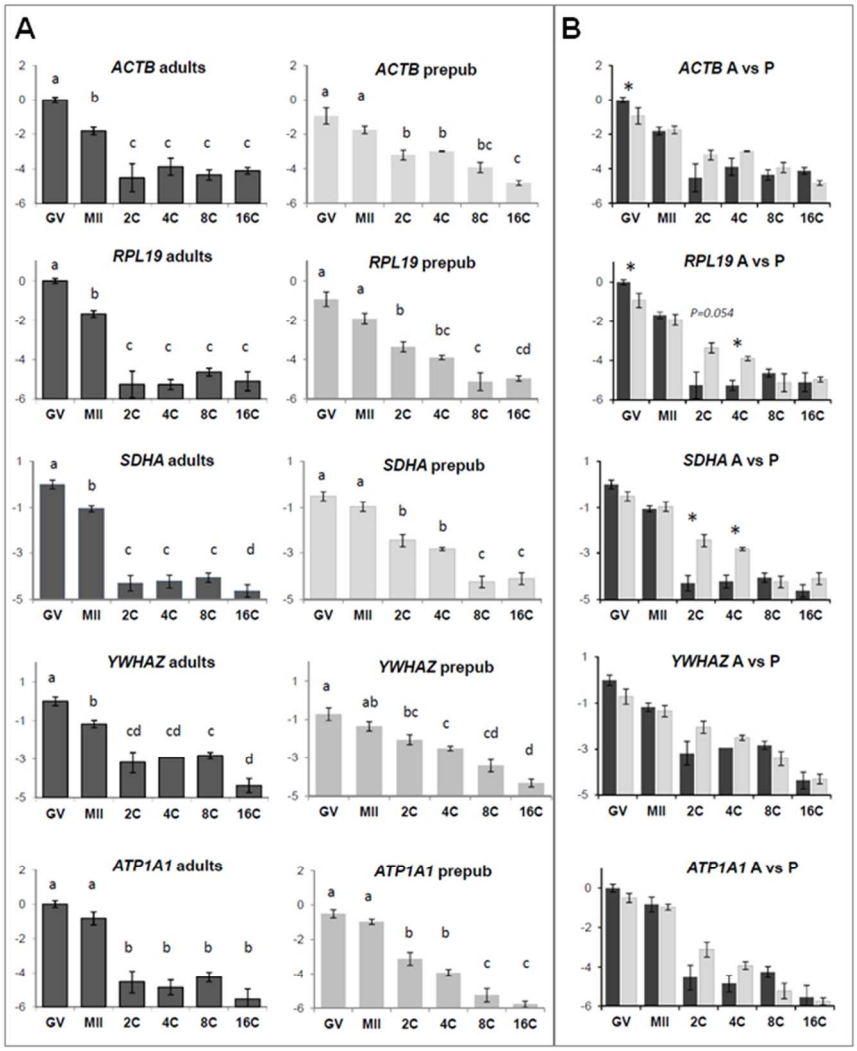

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190x254mm (96 x 96 DPI)

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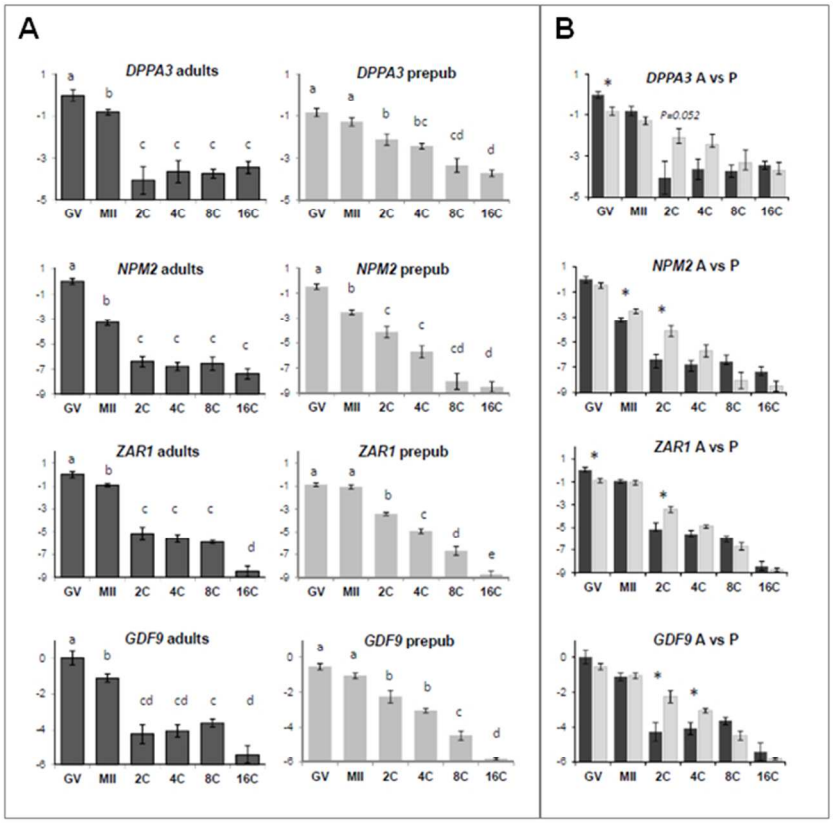

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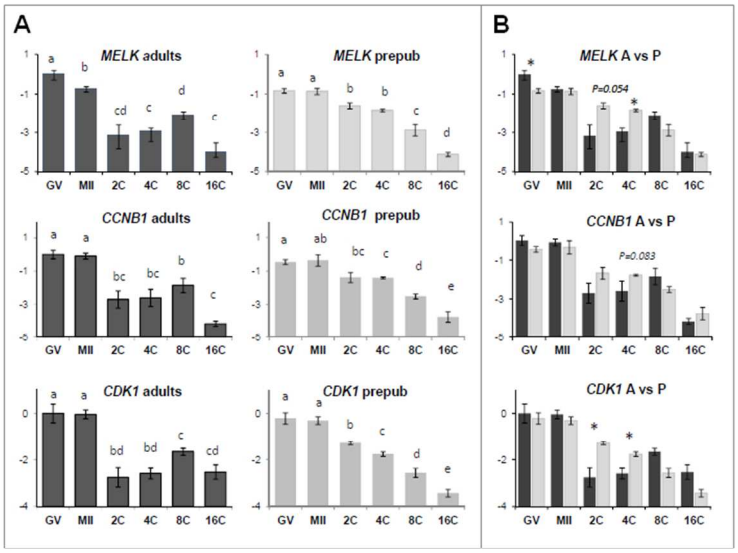

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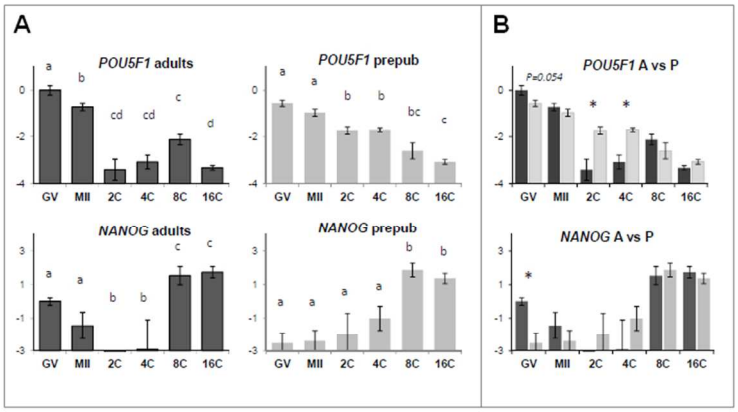

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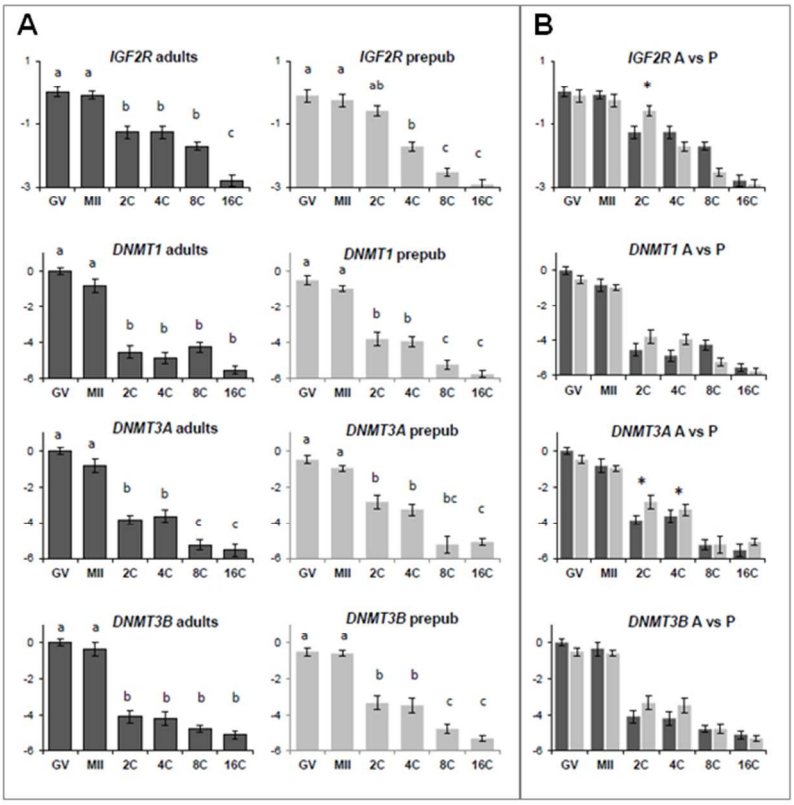

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