



DNA methylation dynamics during oocyte and embryo development and its association with environmental induced alterations

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Abstract

Epigenetic mechanisms are fundamental to successful gametogenesis and development. The fertilized egg undergoes global DNA demethylation to facilitate remodelling from two differentiated gamete-specific states to a pluripotent embryonic state. Maintenance of appropriate levels of DNA methylation during preimplantation development is essential to embryo viability. Recent advances in epigenetic research have highlighted the susceptibility of foetal epigenetic programming to maternal health and nutritional status, particularly, at the time of conception. There is much evidence that maternal stress impacts on ovarian function, leading to compromised oocytes presented for fertilization in a suboptimal environment. Similarly, declining fertility has become a substantive issue in western countries, where it is primarily associated with high mean ages at childbearing. Thus the use of assisted reproduction technologies (ART) interventions to overcome low fertility is increasing steadily across the globe. In addition, the use of prolonged in vitro culture following the removal and storage of oocytes and/or ovarian tissue in advance of cancer treatment, or to circumvent ovarian aging, is increasing rapidly. ART is associated with compromised pre and post -natal outcomes, including premature birth, low birth weight, congenital abnormalities and elevated risk of epigenetic disorders. There is extensive evidence from studies in cattle that embryos produced by conventional ART protocols are susceptible to errors in epigenetic programming. The present review discusses the impact of intrinsic physiological status and external environments on oocyte and embryo DNA methylation with regard to data available from mouse, human and bovine models.

Keywords: epigenetic, oocyte, embryo, mammals.

Introduction

Genomic imprinting is an epigenetic process, which enables parent-of-origin expression of a cohort of mammalian genes (Fowden *et al.*, 2006). The correct dosage of imprinted gene expression has been shown to be critically important to embryonic growth, development, placental function and postnatal behaviour and metabolism (Reik and Walter, 2001; Davies *et al.*, 2005; Smith *et al.*, 2006). Parent-of-origin expression of imprinted genes is facilitated through asymmetrical epigenetic marks on either the maternal or paternal allele. Generally, imprinted genes are arranged in clusters containing differentially marked, CpG rich domains, known as differentially methylated regions (DMRs); the most extensively studied of these marks is DNA methylation

(Lucifero *et al.* 2004). Mammalian DNA methylation patterns required for genomic imprinting are subject to periods of dynamic reprogramming during development and are established at different developmental time points, depending on whether they are transmitted through the male or the female germline. Paternal DMRs acquire their methylation marks prior to birth in the prospermatogonia (Davis *et al.*, 2000; Li *et al.*, 2004); while DNA methylation marks at maternal DMRs are established postnatally in the growing oocyte (Hiura *et al.*, 2006). Epigenetic reprogramming involves DNA methylation erasure and re-establishment at two points in the life cycle, firstly after fertilization in the zygote, and secondly in primordial germ cells (PGCs), which are the direct progenitors of sperm or oocyte. Following fertilization, global DNA demethylation occurs in the zygote to facilitate remodelling from two distinct differentiated gamete-specific states to a pluripotent embryonic state (Hales *et al.*, 2011; Torres-Padilla and Ciosk, 2013). The epigenetic signature inherited from the gametes (excepting the parental imprints) is erased and developmental totipotency is restored. In the PGCs, parental imprints are erased and de novo establishment of new methylation landscapes that are different between male and female germlines restore germline developmental potential (Seisenberger *et al.*, 2013). There is extensive evidence in cattle, human and laboratory model species that intrinsic physiological status and external environments, such as assisted reproductive technologies (ART), can dramatically alter the epigenetic landscape of gamete, embryonic, foetal and adult tissues, leading to impaired function/ adverse health outcomes in adult life. The mechanisms and implications with regard to data available from mouse, human and bovine models are reviewed.

DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos

DNA methylation erasure

Recent studies have started to elucidate how global demethylation in the zygote and PGCs is orchestrated. It appears that active demethylation occurs on the paternal genome in one-cell embryos (Maher and Reik, 2000; Oswald *et al.*, 2000) and passive demethylation occurs on the maternal genome from the two-cell until the blastocyst -stage (Reik *et al.*, 2001). It would appear that the major wave of genome-wide demethylation occurs between fertilization and the two-cell stage in humans with only subtle changes in DNA methylation levels occurring thereafter (Guo *et al.*, 2014). In mouse, the most important period of demethylation occurs at the zygote stage, followed by gradual demethylation until the blastocyst stage (Smith *et al.*, 2012). In both human and mouse, demethylation of the paternal genome occurs much faster than that of the maternal genome (Guo *et al.*, 2014; Smith *et al.*, 2014). Several models for active DNA demethylation have been proposed (Morgan *et al.*, 2005; Wu and Zhang, 2010): Active demethylation mediated by the ten-eleven-translocation (TET) family member, Tet3, appears to

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be important for both (Guo *et al.*, 2014; Smith *et al.*, 2014), while Base Excision Repair (BER) pathway may also be important (Xu and Walsh, 2014). Maintenance of appropriate levels of DNA methylation during preimplantation embryo development is essential for the viability of embryos (Barton *et al.*, 2001). As well as the marked allele being resistant to demethylation, equally important appears to be the ability of the unmarked allele to resist de novo methylation following implantation (Proudhon *et al.*, 2012; Rutledge *et al.*, 2014).

PGCs arise in the epiblast of the developing embryo (Ginsburg *et al.*, 1990), where they inherit the somatic epigenetic traits that are present in the cells of the epiblast at this time, including significant levels of global DNA methylation (Seki *et al.*, 2005; Ohinata *et al.*, 2009). Therefore, PGCs need to reprogramme from a somatic profile into that of germ cells that will give rise to the gametes, with the capacity to form the totipotent zygote in the next generation. DNA methylation is globally erased during migration of PGCs towards the genital ridge. Epigenetic reprogramming in PGCs requires remodelling of the chromatin structure, global changes in the transcriptional landscape and the resetting of imprint DNA methylation marks. It is essential for the development of the next generation that the parental imprints are erased in PGCs and that new imprints are established that reflect the gender of the embryo. These imprints are then maintained in the gametes derived from the PGCs and will contribute to the epigenome of the future zygote (Seisenberger *et al.*, 2013).

The mechanisms involved in PGC demethylation have been difficult to elucidate, due to the inaccessible nature of PGCs. Up until recently, studies in mouse focused on the period between E11.5 and E13.5 (Reik *et al.*, 2001; Hajkova *et al.*, 2002). The short duration of this period, specifically the low number of cell cycles, lead to the consensus that DNA demethylation in PGCs was an active process. The protein activation-induced deaminase (*Aid*), has been conclusively shown to be involved in global DNA de-methylation in PGCs, whereas TDG, BER and the TET proteins have been proposed without definitive proof (Seisenberger *et al.*, 2013). There is some evidence to support the possibility that DNA methylation erasure is initiated earlier, at the time of the down regulation of the DNA methylation machinery transcripts (Seki *et al.*, 2005; Kurimoto *et al.*, 2008; Guibert *et al.*, 2012). If the period of global erasure is extended back to this stage, then passive demethylation also becomes a possibility.

Reprogramming of DNA Methylation

During spermatogenesis and oogenesis, epigenetic modifications are established which are required for normal embryonic progression (Bourc'his *et al.*, 2001; Rideout *et al.*, 2001; O'Doherty *et al.*, 2012). Reprogramming the maternal genome, through appropriate DNA methylation of differentially methylated regions (DMRs), is central in regulating genomic imprinting (Li *et al.*, 1993), a process in which a small cohort of genes are exclusively expressed from a single allele, according to parent-of-origin (Preece and Moore, 2000). Imprinted gene DMRs are actively established during mammalian gametogenesis (Reik *et al.*, 2001), the vast majority of which acquire DNA methylation from the maternal germline during the oocyte growth phase (Ueda *et al.*, 1992; Lucifero *et al.*, 2002, 2004; Hiura *et al.*, 2006). During postnatal mouse oogenesis, imprinting establishment occurs asynchronously at different imprinted genes during the transition from primordial to antral follicle stages (Lucifero *et al.*, 2004; Hiura *et al.*, 2006). The DMRs tested in human and bovine oocytes have shown the expected pattern of methylation (Geuns *et al.*, 2003, 2007; El Hajj *et al.*, 2011; Heinzmann *et al.*, 2011; O'Doherty *et al.*, 2012; Urrego *et al.*,

2014). However, there appear to be differences in the mechanisms and timings of imprint establishment among mammals, (see review by (Hanna and Kelsey, 2014)). For example, the oocyte growth phase in humans and cattle is quite protracted, lasting months (Fair, 2003; Fair *et al.*, 1997). Acquisition of the maternal imprints commences in the final phase of oocyte growth and the individual imprints appear to be established in a size-specific manner (Geuns *et al.*, 2003, 2007; Arima and Wake, 2006; Sato *et al.*, 2007; O'Doherty *et al.*, 2012). In bovine, for example, acquisition of the maternal imprints commences when oocytes reach a diameter of 110 to 120 μm ; *SNRPN* and *MEST* are fully methylated by 120 μm , whereas *IGF2R*, *PEG10* and *PLAGL* were only partially methylated at this size (O'Doherty *et al.*, 2012). Recent studies have established that gene transcription determines the characteristic DNA methylation landscape of the mature murine oocyte. Indeed it was determined that transcription events could account for 85-90% of DNA methylation established in the oocyte, including methylated CpG islands (CGIs) and imprinted DMRs (Veselovska *et al.*, 2015). However, a small number of expressed genes escape DNA methylation, as well as a small number of CGIs within active transcription units. Indeed, it suggests that gene expression perturbations during oocyte follicular growth could result in alterations in DNA methylation in mature gametes, including at CGIs. Since a fraction of the oocyte DNA methylome is maintained to some extent in pre-implantation embryos just before the embryonic onset of de novo methylation, environmentally induced changes in gene expression in female germ cells could lead to alterations in the epigenome of the next generation, with possible transgenerational effects (Borgel *et al.*, 2010; Smallwood *et al.*, 2011). Thus, physiological or external factors that disrupt the transcription environment during oocyte follicular growth could result in alterations in DNA methylation in mature gametes.

Genome-wide studies in human gametes and embryos have revealed many similar mechanisms of global DNA methylation reprogramming between humans and mice (Guo *et al.*, 2014; Smith *et al.*, 2014). Imprinting marks must be maintained during the sensitive oocyte-to-embryo transition. As this stage of development is under maternal control, the role of maternal effect genes in genomic imprint regulation is of interest. Several key maternal effect proteins that protect imprinted methylation sites during preimplantation development have been identified and include: PGC7 (also known as STELLA or DPPA3): In mice, PGC7 was shown to maintain imprints at a subset of loci, including *Peg1*, *Peg3*, and *Peg10* domains and the paternally imprinted *H19-Igf2* (H19DMR) and *Rasgrf1* domains (Nakamura *et al.*, 2007); zinc finger protein 57 (ZFP57), which is required for the methylation of the *Snrpn* domain in mouse oocytes and for the maintenance of DNA methylation after fertilisation at *Dlk1*, *Peg1*, *Peg3* and *Nnat* domains (Li *et al.*, 2008); tripartite motif-containing 28 protein (TRIM28; also known as KAP1/TIF1b), TRIM28 may regulate epigenetic stability in mouse oocytes and embryos by protecting against passive demethylation (Messerschmidt *et al.*, 2012) and finally, DNMT1 (Hirasawa *et al.*, 2008). Two DNMT1 isoforms are present in mature oocytes and preimplantation embryos: oocyte-specific (DNMT1o) and somatic (DNMT1s) isoforms. DNMT1o accumulates during oocyte growth (Bao *et al.*, 2000), and is the abundant form expressed in oocytes and preimplantation embryos (Mertineit *et al.*, 1998). There are five known family members of the DNMTs (DNMT1, DNMT2, DNMT3A, DNMT3B AND DNMT3L), which are believed to be responsible for establishing and maintaining methylation patterns (Ooi *et al.*, 2009). Gene targeting studies in mice have demonstrated that the methyltransferases DNMT3A, DNMT3B and DNMT1 are indispensable for



embryonic survival (Li *et al.*, 1992; Okano *et al.*, 1999). These findings are likely to apply also in cattle, as we have demonstrated the presence of DNMT3A, DNMT3B and DNMT3L in bovine oocytes during the critical period of DNA methylation imprint acquisition (O'Doherty *et al.*, 2012). Although not addressed here, it must be recognized that additional epigenetic modifications that are established in the oocyte, such as histone post-transcriptional modifications that signal active and silent chromatin and regulatory elements, also impact on decisions in the early embryo and any alterations or failures could lead to impaired epigenetic quality of the oocyte that might impact on early embryonic development and longer-term health.

Epigenetic effect of maternal physiological status on embryo and foetal-oocyte programming and adult offspring health

The protracted nature of oocyte growth in bovine and humans could result in repeated exposure of oocytes to environmental challenges, resulting in an accumulation of insults over the lifetime of the female. For example, it is likely that most females will experience alterations to their diet and nutritional status, development of metabolic and/or infectious disease, exposure to environmental toxins and stress and in some cases administration of assisted reproductive therapies during their lives. Since an important fraction of the oocyte DNA methylome is maintained during preimplantation embryogenesis, environmentally-induced changes in gene expression in oocytes could lead to alterations in the epigenome of the next generation.

There is substantial evidence that the maternal environment pre-conception and during embryonic and foetal development, including inadequate nutrition and over-nutrition, influences the development and future health of the individual (Barker *et al.*, 1989). The condition, health status and age of the oocyte donor (mother) can affect the quality of the oocyte and the health of the resulting offspring (Ashworth *et al.*, 2009). This is especially relevant to the fertility of European women, where the average age at which women deliver their first child has increased to almost 30 years (<http://www.unpopulation.org>; 2014, United Nations Population Division Department of Economic and Social Affairs). The mechanisms behind these effects are unknown, but most likely alteration of gene expression in the oocyte and surrounding follicle cells, is instrumental. Studies in mice (Dahlhoff *et al.*, 2014) and cattle (O'Doherty *et al.*, 2014), have shown that DNA methylation in oocytes is modified by maternal diet or physiology. Postpartum lactating dairy cows are frequently pathogenically and metabolically challenged due to preferential partitioning of energy to milk production, leading to poor reproductive performance within their critical re-breeding window. The adverse metabolic environment induced by lactation alters the metabolomic, steroidogenic and transcriptomic profile of ovarian follicles during their development in postpartum lactating cows compared to non-lactating heifers (Bender *et al.*, 2010; Walsh *et al.*, 2012a, b). Metabolic differences include higher concentrations of saturated and lower concentrations of poly unsaturated -fatty acids and altered amino acid profiles in follicular fluid from lactating cows compared to those from dry heifers (Bender *et al.*, 2010). Steroidogenesis is also compromised; dominant follicle estradiol and progesterone synthesis is reduced during differentiation and luteinization, respectively (Walsh *et al.*, 2012b). At the level of the transcriptome the expression profiles of transcripts associated with steroid biosynthesis (Walsh *et al.*, 2012b), immune cell function and chemotaxis (Walsh *et al.*, 2012b) are also different. We have analysed the

methylation status of a several candidate imprinted DMRs in fully-grown oocytes from postpartum cows. The resulting data revealed hypomethylation at the DMRs of a number of maternally methylated imprinted genes (*PEG3*, *PLAGL1* and *SNRPN*), in oocytes recovered from postpartum cows during the period of most acute negative energy balance (NEB) and greatest metabolic stress. The contribution of an inappropriate follicular fluid fatty acid profile to the aberrant methylation status was investigated *in vitro*. The findings confirmed the sensitivity of *PLAG1* to the follicular environment (O'Doherty *et al.*, 2014). The most famous comparative situation in humans is the Dutch winter famine cohort; specifically, individuals conceived during famine presented hypomethylation of the *IGF2*-DMR compared with their non-exposed siblings, almost sixty years after exposure (Heijmans *et al.*, 2008). Furthermore, genome-wide analysis of DMRs in the cohort detected a number of differentially methylated CpG regions, usually at regulatory regions (Ravelli *et al.*, 1998; Oger *et al.*, 2014). Moreover, the findings of an additional study indicated that the nutrition-dependent changes in methylation were gene- or tissue-restricted (Veenendaal *et al.*, 2012). While NEB is a highly important issue for high yielding postpartum dairy cows, most Western societies do not expect to ever experience a scenario like the Dutch winter famine; obesity and high glycemic diets are possibly the most significant issues facing western women. Obesity induces multiple changes in the ovary, such as leptin resistance, lipotoxicity and local inflammation, all of which could have an impact on processes involved in establishment of the oocyte's normal epigenetic programme. There is much concern that maternal obesity and associated complications during early embryo life leave a "nutritional imprint" with long-term effects on the promotion of obesity and related conditions in adulthood (Ravelli *et al.*, 1998). Data from animal models fed high fat diets clearly endorses the validity of these concerns. Consumption of a high fat (HF) diet during gestation (35 - 60% of calories from fat) is associated with offspring obesity, hypertension, abnormal cholesterol metabolism and cardiovascular disease, for review see Seki *et al.*, 2012). Several rodent models have been employed to investigate the epigenetic changes induced by a maternal HF diet. Results from a selection of models demonstrate that a HF diet during pregnancy can induce epigenetic modification, including changes in DNMT expression (Liu *et al.*, 2011; Zhou and Pan, 2011), altered DNA methylation of genes involved in obesity, and energy homeostasis (Martínez *et al.*, 2014) hypomethylation of the *Me4r* gene, which plays a role in body weight regulation in mice (Gong *et al.*, 2010); and altered transcription and methylation status of *Igf-2* in fetal livers from dams fed HF diets (van Straten *et al.*, 2010). Obviously studies on human tissues are more restrictive, however, recent studies employing genome-wide epigenetic analysis also revealed the potential for widespread DNA methylation variation in foetal tissues exposed to maternal gestational diabetes (Finer *et al.*, 2015).

Extra-ovarian factors affecting oocyte development: The effect of Assisted Reproductive Technologies (ART) Research:

Assisted reproduction technologies are now responsible for the birth of tens of thousands of children yearly and up to 2% of all births in some countries (2004). A growing number of human and animal model studies have indicated the likely presence of postnatal consequences of ART and infertility, some associated with epigenetic alterations in conceptuses and placentae (Walter and Paulsen, 2003; Nelissen *et al.*, 2014; Whitelaw *et al.*, 2014). Ovarian



hyperstimulation has been associated with alterations in global DNA methylation levels in two-cell mouse embryos and reduced pre-implantation development in vitro (Shi and Haaf, 2002). The majority of mouse studies have found no effects on imprinting establishment, although several reports indicate that ovarian stimulation might interfere with the capacity to maintain imprinting during pre-implantation development, for review see Anckaert and Fair, 2015). In the human population, there have been reports of an increased risk for certain imprinting disorders following ART (Huntriss and Picton, 2008). These involved cases of Beckwith-Wiedemann Syndrome, Silver-Russell Syndrome and Angelman Syndrome. In addition to epigenetic defects localized to specific genes, alterations have also been observed at additional loci (Rossignol *et al.*, 2006), suggesting a more global epigenetic disruption after assisted reproduction in humans. These concerns are reinforced by the detection of changes in both the epigenome and transcriptome in physiologically normal ART offspring (Batcheller *et al.*, 2011).

There is much endeavor to expand the range of ARTs offered to patients to include ovarian cortical cryopreservation and subsequent retransplantation or in vitro culture to patients facing ovarian toxic chemotherapy. While there has been some progress (Laronda *et al.*, 2014), currently, there is no data available to measure the epigenetic impact of such treatments on the cultured oocyte or resulting embryo. The findings from studies in mice suggest that the oocyte imprint establishment during in-vitro follicle culture conditions progresses normally (Anckaert *et al.*, 2009, 2013; Hiura *et al.*, 2006; Lucifero *et al.*, 2002, 2004; Trapphoff *et al.*, 2010) and candidate imprints were not perturbed in two-cell embryos obtained after IVF of oocytes derived from the same in-vitro follicle system (El Hajj *et al.*, 2011), however, in vitro culture of the resulting embryos was associated with loss of imprinting for several genes in fetal and placental tissues (Mann *et al.*, 2004; Rivera *et al.*, 2008). This might indicate a different susceptibility of the in vitro grown and matured oocyte compared to the embryo, to culture-induced effects. Furthermore, care needs to be taken when extrapolating mouse data to support ART interventions in humans, as discussed above, the protracted nature of oocyte growth and the extended residency of the oocyte in the human ovary, may result in increased opportunities or sensitivity to aberrant epigenetic programming.

In cattle, research into developmental epigenetics has primarily focused on cloned bovine embryos, due to the high degree of pregnancy loss following transfer of embryos produced by somatic cell nuclear transfer (SCNT; Chavatte-Palmer *et al.*, 2012). Nevertheless, evidence does exist to indicate that embryos produced by more conventional ART protocols are also susceptible to erroneous epigenetic programming (Urrego *et al.*, 2014, for review). Global methylation profiling of bovine embryos revealed remarkable differences in the DNA methylation profile of blastocysts depending on developmental stages completed under in vitro culture condition. Furthermore, DNA methylation patterns of CpG islands and repetitive elements were also affected in the blastocysts depending on the stage of the embryo subjected to in vitro culture (Salilew-Wondim *et al.*, 2015). Moreover, the data from cattle is supported by the extensive body of work describing differences at the gross morphological, ultrastructural, physiological, chromosomal, transcriptional and metabolic levels in in vitro-derived embryos compared with their *in vivo*-derived counterparts (reviewed in Lonergan and Fair 2008; Anckaert and Fair, 2015).

Conclusion

The regulation of oocyte and embryo development is

a highly orchestrated, multi-regulatory process. DNA methylation is an important epigenetic regulatory mechanism. The prolonged growth phase and residency within the ovary endured by oocytes from larger mammals means that multiple opportunities for exposure to potential environmental hazard exists. Therefore it is important that in so far as possible, optimal physiological and external environments are achieved, through dietary management, health care and benign ex vivo assisted reproduction interventions.

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