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Evaluation of epigenetic marks in human embryos derived from IVF and ICSI

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BACKGROUND: It has long been appreciated that environmental cues may trigger adaptive responses. Many of these responses are a result of changes in the epigenetic landscape influencing transcriptional states and consequently altering phenotypes. In the context of human reproductive health, the procedures necessary for assisted reproduction may result in altered phenotypes by primarily influencing DNA methylation. Among the well-documented effects of assisted reproduction technologies (ART), imprinted genes appear to be frequently altered, likely owing to their reliance on DNA methylation to impose parent-specific monoallelic expression. However, the generality of the potential deregulation of DNA methylation in ART-derived human embryos has not been evaluated.

METHODS: In this study, we evaluate the genome-wide DNA methylation together with chromatin organisation in human embryos derived by either IVF ($n = 89$) or ICSI ($n = 76$). DNA methylation was assessed using an antibody against 5-methyl-cytidine, and chromatin organisation by DNA staining.

RESULTS: Irrespective of the ART procedure, similar errors were observed in both groups and abnormal chromatin was positively correlated ($P < 0.001$) with inappropriate DNA methylation. Development up to the blastocyst stage was consistent with normal DNA methylation and chromatin organisation, reinforcing the importance of epigenetic regulation to form the early lineages of the blastocyst. Most importantly, we found no evidence that ICSI blastocysts were more severely affected than those derived by IVF.

CONCLUSIONS: We conclude that ICSI does not lead to an increased incidence of epigenetic errors (DNA methylation and chromatin) compared with IVF.

Key words: DNA methylation / human embryos / IVF / ICSI / epigenetics

Introduction

Among the rising frequencies of diseases in the developed world, the steady rise in infertility is among the most serious (de Mouzon *et al.*, 2009). Three decades on since the landmark birth of Louise Brown, as many as 1 in 60 live births occur as a consequence of assisted reproduction technologies (ART) with more than 3 million people born worldwide (22nd annual conference of the European Society of Human Reproduction and Embryology, www.eshre.com). Despite the importance of these procedures for the treatment of infertility, many questions remain unanswered concerning the immediate and long-term risks and the associated cost implications to health-care systems for these patients. Whether these procedures increase the potential for epigenetic errors, or epimutations, as a consequence of ART remains an important question to be answered (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003). In particular, the exposure to the environment through isolation, handling and culture of gametes necessary to achieve fertilisation *ex vivo*, may enhance

the possibility of detrimental epimutations (Fortier et al., 2008; Morgan et al., 2008; Rivera et al., 2008).

DNA methylation, histone modifications, replacement of histone variants in the nucleosome and chromatin structure have all been identified as epigenetic modifications, an instructional, overlying layer of information essential to interpret the underlying genetic code (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Germ cells, both oocytes and sperm are particularly sensitive to environmental changes leading to altered epigenotypes, in part as they ordinarily undergo epigenetic reprogramming in the course of normal gametogenesis (Sasaki and Matsui, 2008). Environmental changes can and do become translated into epigenetic alterations with particular importance associated with their ability to heritably perpetuate these marks and hence influence future generations (Whitelaw and Whitelaw, 2008). Importantly, these epimutations may lead to disease much later on in adult life (Barker, 2004; Jirtle and Skinner, 2007). In response to the growing demand for ART, there is a wide spread interest in the field of human reproductive health to assess whether the many and varied procedures, associated with the treatment of human infertility, are compatible with maintaining a life-long normal epigenotype.

Animal models have firmly established that manipulations of embryos, ranging from simple embryo culture to somatic nuclear transfer, may influence fetal growth and viability (Mann et al., 2004; Amor and Halliday, 2008). Many of these effects were found to have an epigenetic origin and frequently affected imprinted genes. Genomic imprinting is a non-Mendelian mode of inheritance that marks alleles for transcriptional activity based on their parent-of-origin. This necessitates that during gametogenesis, these imprints must be erased and re-established in accordance with the sex of the fetus. Maternal DNA methylation at imprinted loci is re-imposed gradually over the post-natal period of oocyte growth and maturation. Hence, maternal methylation imprints are particularly vulnerable to disturbances associated with superovulation, oocyte isolation and manipulation in culture, all procedures required for ART.

Several years ago, a series of reports triggered concern that children born as a result of ART were found to have increased frequencies of a number of diseases known to have an epigenetic aetiology (DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003; Moll et al., 2003; Halliday et al., 2004). Specifically, several reports alluded to the possibility that ICSI births were more likely to be associated with imprinting errors leading to disease syndromes (Cox et al., 2002; Orstavik et al., 2003). Retrospective studies of children diagnosed with these syndromes identified a consensus of epimutations associated with inappropriate methylation of maternal alleles at imprinted loci (Halliday et al., 2004; Lucifero et al., 2004a, b; Niemitz and Feinberg, 2004). These errors were assumed to have arisen in response to the environmental changes triggered by ovarian hyperstimulation, a frequent consequence of superovulation during ART procedures resulting in the sequestration of immature oocytes, leading to a failure to establish appropriate imprinting during later stages of oogenesis (Halliday et al., 2004; Lucifero et al., 2004a, b; Niemitz and Feinberg, 2004; Gomes et al., 2009).

Prompted by these reports, we undertook a study to evaluate the possibility that two common forms of ART, IVF and ICSI were associated with epigenetic errors primarily related to changes in global DNA methylation as revealed using an antibody to 5-methyl-cytosine (5MeC). Antibody approaches to epigenetic marks, especially DNA methylation, afford the possibility of revealing architectural features of the nuclear organisation not otherwise achievable by conventional molecular techniques, in addition to supplying valuable data on individual embryos.

Our results do not allow us to distinguish between the possibilities that infertility per se, rather than ART procedures, may play an important part in predisposition to epimutations that lead to diseases of an epigenetic basis. Interestingly, embryos developing to the blastocyst stage had an ostensibly normal epigenotype irrespective of the procedure used to derive them. We find that while differences in the underlying causes of infertility may vary, comparisons of classes of abnormalities observed among arrested embryos, and the severity of these abnormality, did not differ irrespective of the elected procedure. We conclude that ICSI does not lead to an increased incidence of epigenetic errors.

Materials and Methods

In order to obtain the maximum amount of information from this study, we chose to use an immunofluorescence approach that permitted investigation of individual embryos and individual patients undergoing cycles of ART. Embryos were donated by couples after informed consent and became available for inclusion in the study on Day 2 or Day 3 upon completion of the treatment cycle. Embryos were cultured in G1 medium (Vitrolife, Sweden) until Day 3 at 37°C in a humidified atmosphere of 7.5% CO_2 in air. Embryos were then cultured in G2 medium until Day 6 after which they were fixed in 4% paraformaldehyde (PFA) for 15 min and stored in phosphate-buffered saline (PBS) 0.05% Tween20 (PBT) at 4°C until further use. Embryos that were not at blastocyst stage on Day 6 were considered arrested. Approximately, equal numbers of non-progressing human embryos were evaluated arising from either IVF ($n = 75$) or ICSI ($n = 63$) procedures. Embryos that had reached the blastocyst stage were analysed separately (IVF, $n = 14$; ICSI, $n = 13$). The research was licensed by the HFEA and was approved by the Local Research Ethics Committee.

DNA methylation staining

Immunostaining was performed as previously described (Santos et al., 2002). Briefly, fixed embryos were permeabilised in 0.2% Triton X-100 in PBS for 30 min and incubated with 4N HCl containing 0.1% Triton X-100 for 10 min; the embryos were blocked overnight in 1% BSA PBT (BS). Staining was achieved with 1:500 anti-5MeC (Eurogentec: MMS-900P-B) in BS at room temperature (RT) for 1 h and detected with a secondary antibody coupled to AF594 (Invitrogen-Molecular Probes, at 1:500). DNA was stained with the intercalating dye YOYO-1™ iodide (green) (Invitrogen-Molecular Probes) at 100 nM for 10 min.

Multiple labelling of blastocysts

We have developed a sequential protocol to detect a combination of multiple epigenetic marks in early embryos. Here, we modify this protocol to include pluripotency factors in conjunction with DNA methylation. Immunostaining was performed as previously described (Santos et al., 2005). Briefly, after permeabilisation in 0.2% Triton X-100 in PBS for 30 min and blocking overnight in BS, the embryos were incubated with 1:20 anti-NANOG (R&D Systems: AF1997) in BS at RT for 1 h and detected with secondary antibodies coupled to AF568 (Invitrogen-Molecular Probes, at 1:500). Following extensive washing in BS, the embryos were post-fixed

in 2% PFA for 10 min after which the staining of DNA methylation was completed as described above using an AF488 secondary antibody. DNA was stained with 5 μg/ml 4′,6-Diamidino-2-phenylindole for 10 min.

Microscopy and image analysis

(1) Observations were made using an Olympus BX41 epifluorescence microscope. Images were recorded digitally with separate filter sets for YOYO-1™ and Alexa Fluor 594 using analySIS 3.1 (SIS GmbH). Greyscale images were pseudo-coloured and merged using Adobe Photoshop CS2. For three-colour images, the acquisition was performed with a Zeiss LSM 510 Meta confocal microscope equipped with a 'Plan-Apochromat' 40x DIC oil-immersion objective. Serial optical sections were collected (minimum 700 × 700 pixel size, z-step 0.46 μm). Selected images were assembled and pseudo-coloured using Adobe Photoshop CS2.

Statistical analysis

Pearson's exact test was used for comparison (Microsoft Office Excel 2007). All *P*-values are two-tailed and $P < 0.05$ was considered significant.

Results

IVF and ICSI-generated human embryos from the zygote through to the expanded blastocyst stage were fixed and prepared for DNA methylation evaluation using the 5MeC antibody. To visualise nuclear organisation and hence the status of chromatin, embryos were stained with YOYO1™, a sequence non-specific cyanine dye that quantitatively stains DNA.

In order to score embryos, a guideline for normal levels of DNA methylation and its organisation needed to be established. This guideline was informed by comparison to other mammalian systems and extensive experience in the mouse, where both active paternal and passive demethylation have been established (Monk *et al.*, 1987; Dean *et al.*, 2001; Young and Beaujean, 2004). The consensus for normal DNA methylation and hence the epigenotype considered to be appropriate for samples in this study is presented in Fig. 1A. Human pre-implantation stage embryos appear to undergo limited passive DNA demethylation from the 2-cell up to the 8–16 cell stage or in the fifth cell cycle at which time *de novo* DNA methylation appears to occur. Our observations are in close agreement with previously published results (Fulka *et al.*, 2004), and as such suggest they represent a good approximation of a normal DNA methylation profile.

Interestingly, fully expanded blastocysts (Fig. 1As–v) are asymmetric in terms of DNA methylation with a hypomethylated trophectoderm and a hypermethylated inner cell mass (ICM). This asymmetric pattern of DNA methylation is quantitatively different between species (Fig. 1B) but qualitatively conserved (Fig. 1Ba and b). Overall, human blastocysts more closely resemble bovine (Fig. 1Ba) and ovine blastocysts (Young and Beaujean, 2004) than mouse embryos (Dean *et al.*, 2001 and Fig. 1Bb, this manuscript). Thereafter throughout the study, this panel (Fig. 1A) was used as a guideline to assess the extent of correct DNA methylation on a stage-specific basis.

Approximately, equal numbers of IVF and ICSI embryos were evaluated and double scored for chromatin organisation and DNA methylation signals. For the purpose of the analysis, samples were separated into arrested and non-arrested embryos in keeping with the appropriate stage for 6 days of culture. In this study, these non-arrested embryos should have reached the blastocyst stage at this time. These data are reported in Table I. The spectrum of epigenetic abnormalities, including chromatin and DNA methylation mis-regulation, in embryos that had not reached the expected stage by Day 6 are presented in Fig. 2. These include profound abnormalities of organisation and structure of the nucleus including inappropriately high DNA content, multi-nucleate blastomeres and apoptotic nuclei leading to mosaic patterns of chromatin mis-regulation within a single embryo (Fig. 2A). Examples of normal and hypermethylated nuclei are depicted for IVF and ICSI-derived embryos (Fig. 2B). Given the paucity of the samples obtained from both procedures, it was not possible to establish meaningful frequencies of the classes of abnormalities individually. Additionally, many embryos suffered from multiple errors although they were scored only once. Examples illustrating the quantitative and qualitative elements of inappropriate DNA methylation have been included together with genome-wide chromatin irregularities in embryos generated by either IVF or ICSI. These abnormalities suggest cell cycle arrest as a recurring problem in the case of multinucleate blastomeres and point to an early origin, both shortly after fertilisation (Supplementary Material, Fig. S1) or later on during cleavage divisions (Fig. 2A).

Irrespective of the ART procedure, for arrested embryos, the data obeyed a bimodal distribution with an apparent equal probability of being either normal or abnormal in both registers. Very few embryos had only DNA methylation errors (Fig. 3: IVF = 13.19% versus ICSI = 9.64%) or only chromatin errors (Fig. 3: IVF = 5.49% versus ICSI = 7.23%). Statistical evaluation of the distribution for arrested embryos arising from IVF and ICSI scored for epigenetic abnormalities (DNA methylation, chromatin organisation or both) indicated no significant difference was observed as a consequence of the procedure per se (Table I, $P > 0.5$). Moreover, these analyses suggested that there were an extremely high correlation between normal quantitative features of chromatin and DNA methylation (Table II, $P < 0.001$).This observation suggests that there may well be an underlying mechanistic linkage between normal DNA methylation and the associated chromatin organisation that dictates nuclear architecture. Specific nucleosomal histone modifications have been implicated together with DNA methylation to reinforce repressive chromatin environments widely throughout the genome (Tamaru and Selker, 2001; Fuks *et al.*, 2003; Tamaru *et al.*, 2003; Jackson *et al.*, 2004).

In order to maximise the potential information from materials donated to research, embryos were handled on a patient by patient basis. Theoretically, this information might connect the epigenetic 'fitness' of the embryo with pregnancy outcomes in addition to the primary focus of the study. As data had been collected in a patient-specific manner, potential trends, connecting normal epigenotypes, assessing gross epigenetic features and pregnancy outcomes might be evident. Although normal epigenotypes were identified within this study, no correlation was supported to positive pregnancy outcomes irrespective of the ART procedure (Supplementary Material, Table SII).

Developmental milestones and epigenotype

Although clear conclusions could be drawn in comparisons of arrested embryos derived from IVF and ICSI procedures (namely that no

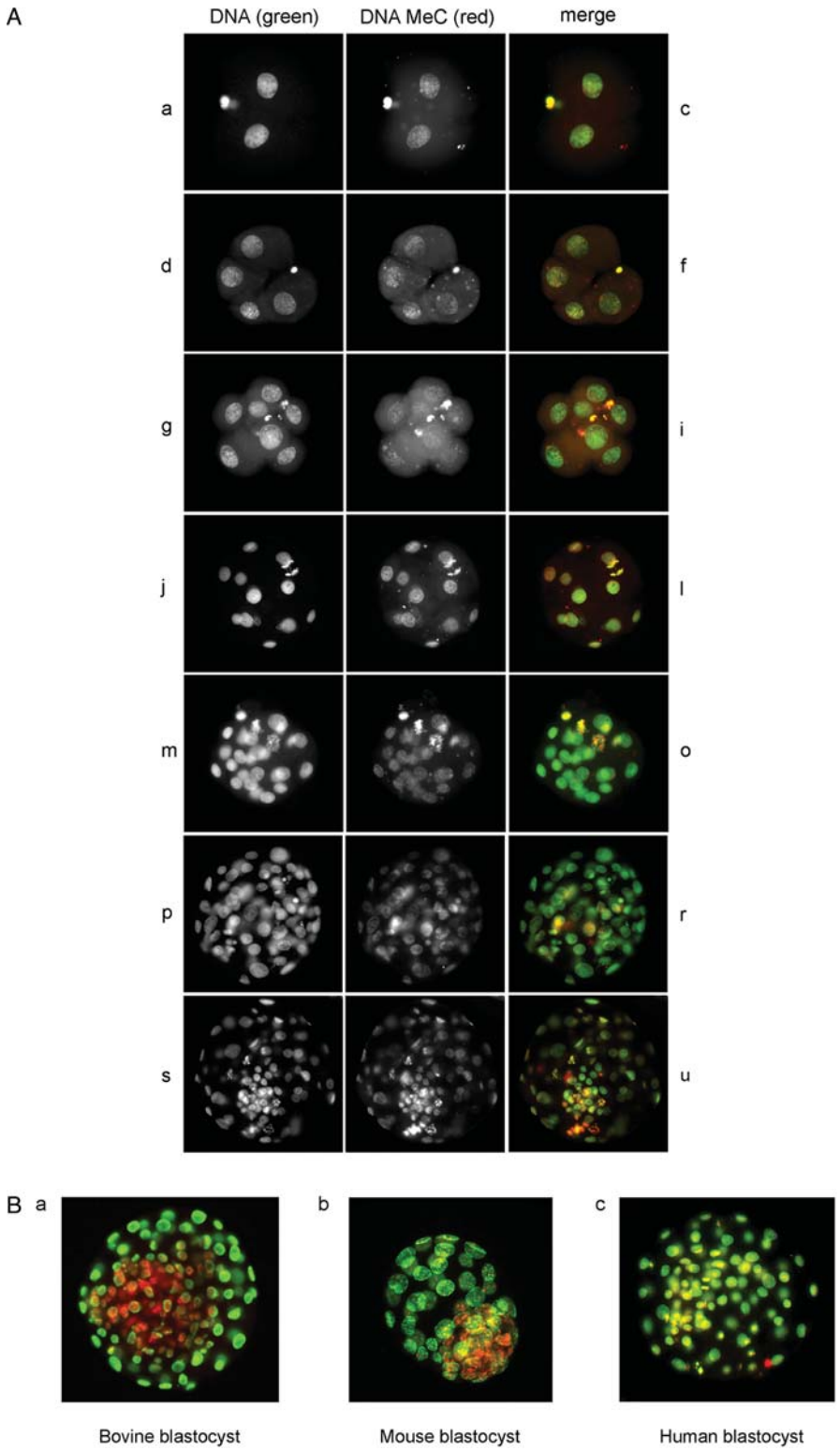

Figure 1 Pre-implantation embryos staining guide. (**A**) Developmental profile for human pre-implantation staged embryos; (a-v) guideline for normalcy. Embryos were collected following either IVF or ICSI, fixed and stained with an antibody to 5-methyl-cytidine (5MeC) as a measure of DNA methylation and a quantitative DNA dye, YOYO1™, to evaluate chromatin epigenotype. Embryos profiled in this figure represent the best estimate of normal pattern and intensity of DNA methylation of staged embryos from the 2-cell up to the expanded blastocyst. (**B**) Comparative illustration of qualitative features of DNA methylation asymmetry in the blastocyst. Examples of typical patterns for DNA methylation (red) and chromatin (green) in (a) bovine, (b) mouse and (c) human blastocysts. Collection of mouse and bovine embryos has been published previously (Dean *et al.*, 2001; Santos *et al.*, 2003).

differences were observed between these two groups) it remained formally possible that differences might become apparent at the expanded blastocyst stage, a time when distinctive epigenetic marks, including asymmetric DNA methylation, should be in place. To address this issue, we undertook a higher resolution analysis and focused a sample collection solely at the blastocyst stage. Blastocysts were scored in accordance with published and accepted guidelines (Gardner and Schoolcraft, 1999). The only departure from the ordinary use of this criterion was that embryos were scored after PFA fixation; however, it was still possible to score the degree of expansion and hatching and the size of the ICM (Supplementary Material, Fig. S2). Interestingly, blastocysts showed an overwhelmingly positive correlation with double normal epigenotype scores (Table III, $P < 0.001$) irrespective of the procedure used to derive them (Fig. 4A: IVF = 78%; ICSI = 69%). A small number of blastocysts were observed to have inappropriate DNA methylation (see Table III) but otherwise normal nuclear organisation (Fig. 4A). At the blastocyst stage, the assessment of DNA methylation would, by necessity, require that the ICM was hypermethylated (Fig. 4B: ICM includes NANOG positive cells), while the trophectoderm was hypomethylated (Fig. 4B). Thus, temporal elements together with lineage asymmetry would need to be correct (Fig. 4B: Image J co-localisation). Together these results suggest that development to the blastocyst stage is associated with a normal epigenotype for both ICSI and IVF treatments.

Table I Distribution of epigenetic abnormalities, chromatin organisation (Ch) and DNA methylation (5MeC), in assisted reproduction technologies (ART) generated embryos.

	Total 2004–2007[a]		Blastocysts 2005–2007	
	IVF	ICSI	IVF	ICSI
Ch+5MeC 'normal'	20*	14*	11[b]	9[b]
Ch 'abnormal'	5*	6*	0[b]	0[b]
5MeC 'abnormal'	10*	5*	2[b]	2[b]
Ch+5MeC 'abnormal'	40*	38*	1[b]	2[b]
Total	75	63	14	13

P-values were determined by Pearson's exact test.
[a]Developmentally arrested embryos at Day 6 of culture.
[b]No degrees of freedom to perform the test.
*$P > 0.5$.

Discussion

Epigenetic programming during early development is a conserved process in mammals, modulating critical quantitative features of DNA methylation and remodelling chromatin (Dean *et al.*, 2001; Beaujean *et al.*, 2004). These heritable instructions, imposed during the early stages of development, can be influenced by environmental perturbations such as those encountered during oocyte retrieval and embryo culture and manipulation, all necessary steps in ART as part of the treatment of human infertility (Dean *et al.*, 2001; Khosla *et al.*, 2001; Rivera *et al.*, 2008).

A series of retrospective studies of children with imprinting disorders identified that a number of these cases were associated with children born following treatment for infertility (Cox *et al.*, 2002; Bowdin *et al.*, 2007; Amor and Halliday, 2008). In response to these reports, we initiated a study to ascertain whether differences existed between human embryos derived from IVF and ICSI procedures. Ideal experimental situations are frequently not achievable while operating within the limitations of ethical and practical guidelines for use of human embryos. This study was constrained by restrictions allowing use of materials only when embryos generated in the course

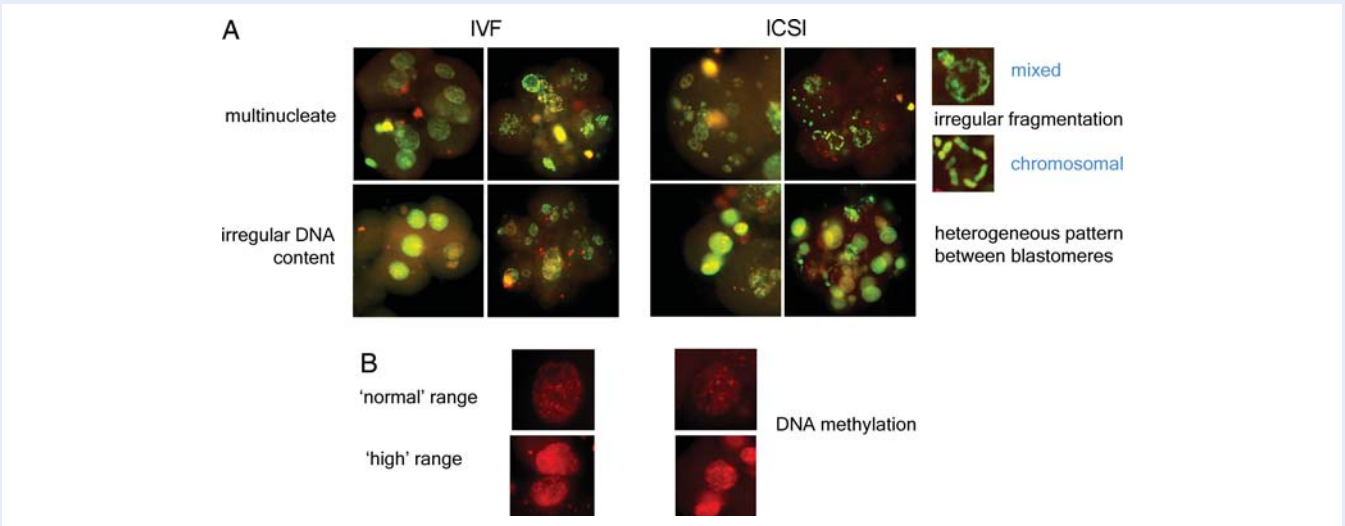

Figure 2 Epigenetic errors are inherent to arrested IVF and ICSI derived embryos. Embryos were collected, fixed and assessed for DNA methylation and chromatin organisation. (**A**) Merged images (DNA methylation- red and chromatin-green) of representative error classes are depicted. (**B**) Examples of blastomeres deemed to fall within the 'normal' and 'high' range of DNA methylation are included from both groups reinforcing an underlying shared aetiology of the abnormalities.

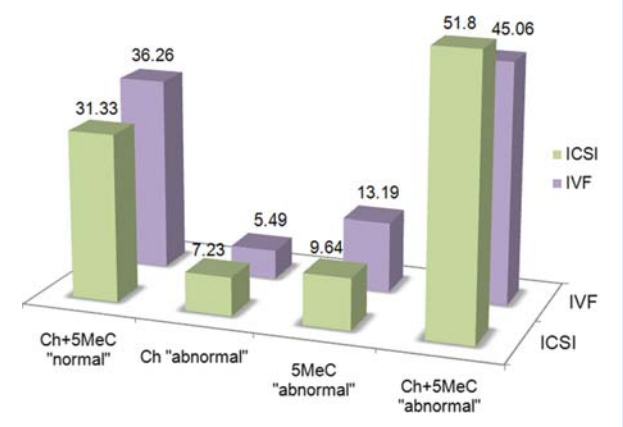

Figure 3 Comparison of epigenetic error frequency (%) between arrested embryos derived by ICSI ($n = 63$) and IVF ($n = 75$). Embryos obtained over a three year period and at all stages between 1-cell and morula were scored for two measures of epigenetic quantities, DNA methylation (5MeC) and chromatin integrity (Ch). These were plotted in a discontinuous gradient from normal in both regards to abnormal in both regards. Embryos were grouped according to treatment.

Table II Association between epigenetic abnormalities, Ch and 5MeC, in ART generated embryos.

	Chromatin organisation			
	'normal'		'abnormal'	
	5MeC 'normal'	5MeC 'abnormal'	5MeC 'normal'	5MeC 'abnormal'
IVF	31*	12*	5*	41*
ICSI	23[a]	7[a]	6[a]	40[a]

Total sample, embryos collected between 2004 and 2007. *P*-values were determined by Pearson's exact test.
[a]$P < 0.001$.
*$P < 0.001$.

of ART treatments were either unsuitable or not required for transfer or cryopreservation. Despite these restrictions, valuable information can be obtained from embryos supplied where each and every embryo is assessed individually.

Comparison of epigenotypes (DNA methylation staining and chromatin assessment) arising from arrested or delayed human embryos derived by IVF or ICSI suggested that approximately half of all embryos analysed met expectations defined for normal human embryos (Fig. 1A). Notably, among the remaining abnormal embryos, those possessing inappropriate DNA methylation also frequently had aberrant chromatin. DNA methylation is normally modulated during the immediate post-fertilisation period in humans, although the extent of this reprogramming is a matter of some debate (Fulka *et al.*, 2004). We reasoned that errors in DNA methylation may be correlated with errors in chromatin suggesting a mechanistic link leading to the epigenotype, an observation previously reported in bovine embryos (Santos *et al.*, 2003). Unexpectedly, no significant difference in the distribution of epigenotypes of embryos was observed between the two procedures (Figs. 3 and 4A). Remarkably, the spectrum of abnormalities was not unique to either group with all classes and levels of severity shared between IVF and ICSI-derived embryos (Fig. 2 and Supplementary Material, Fig. S1). *A priori*, it was not necessarily expected that these two groups would share similar repertoires of anomalies. In general, ICSI is typically elected for cases of male infertility owing to reduced sperm count, motility and/or poor morphology (Palermo *et al.*, 2009) and is more invasive than conventional IVF.

We extended our study to also evaluate DNA methylation and chromatin organisation at the blastocyst stage. An overwhelming majority of embryos assessed at this stage were assigned double normal scores pointing to a biological connection between the developmental stage and normal epigenotypes. While this observation will be welcomed into the debate concerning the effects of extended culture and the normalcy of embryos, our study does not preclude the possibility of changes in the epigenotype at the molecular level. The fidelity of the epigenotype at the blastocyst stage will have important implications for the derivation of human embryonic stem cells (hESC). Notably, the epigenotype of the blastocyst, prior to hESC derivation, is particularly important as it is this extraordinarily faithful reiteration of epigenetic information that reinforces lineage commitment throughout development and in cellular commitment in all tissues undergoing renewal in adult life (Hemberger *et al.*, 2009).

Table III Association of epigenetic abnormalities with developmental arrest in ART generated embryos.

	Chromatin organisation			
	'normal'		'abnormal'	
	5MeC 'normal'	5MeC 'abnormal'	5MeC 'normal'	5MeC 'abnormal'
Arrested	34*	15*	11*	78*
Blastocysts	20*	4*	0*	3*

Total sample, embryos collected between 2004 and 2007.
P-value was determined by Pearson's exact test.
*$P < 0.001$.

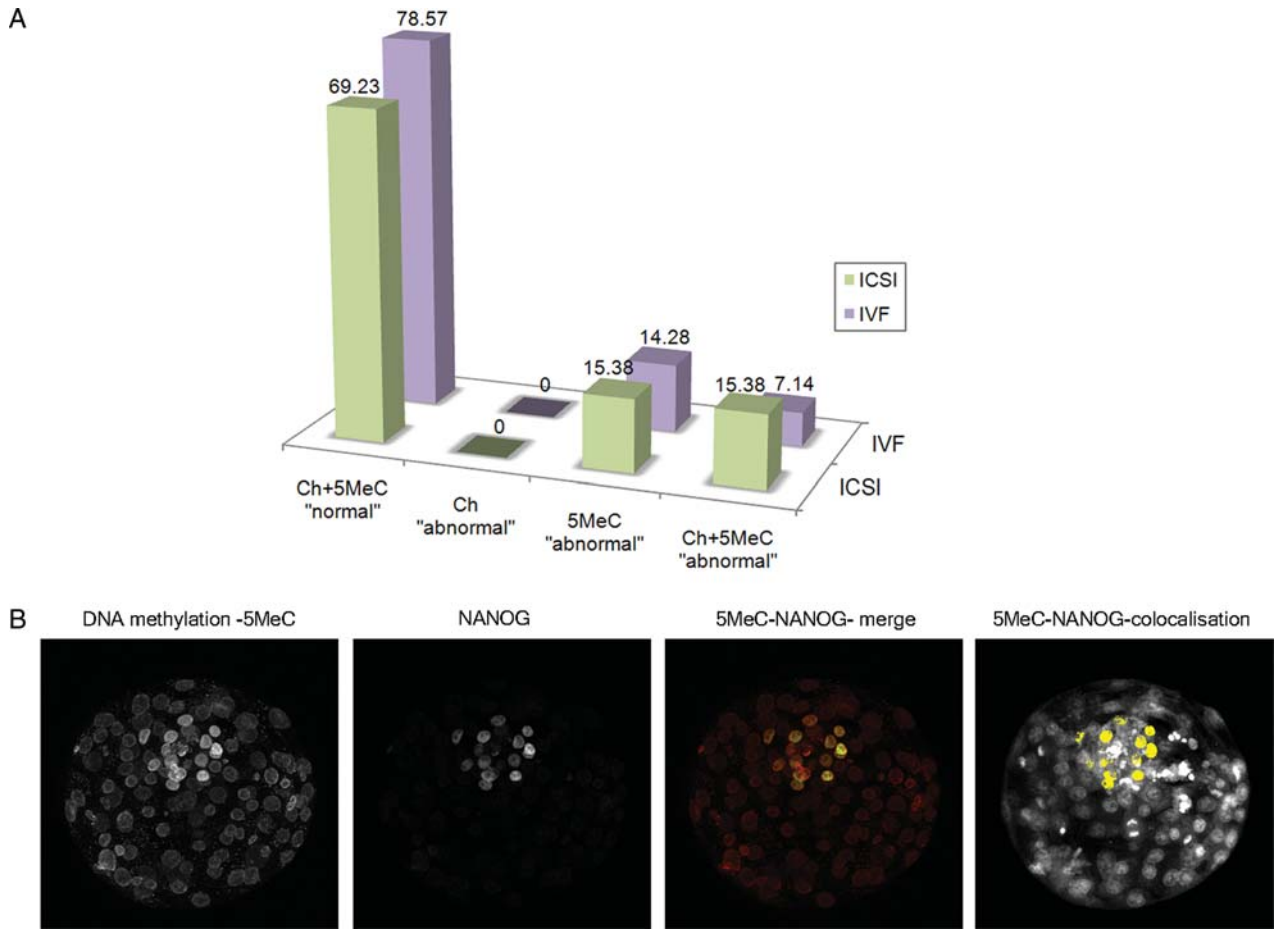

Figure 4 Epigenetic lineage asymmetry is conserved in human embryos. (**A**) Assessment of epigenotypes in blastocysts arising from ART procedures. Blastocysts generated by IVF ($n = 14$) or ICSI ($n = 13$) were stained and are portrayed in the discontinuous plot from double normal to double abnormal scores. In contrast to other embryo stages obtained throughout the study, blastocysts showed an overwhelmingly significant positive correlation with normal epigenotype scores (Table III, $P < 0.001$). (**B**) High morphology score expanded blastocysts were labelled for a lineage identification marker, NANOG, followed by staining for 5MeC. ICM cells were consistently hypermethylated and positive for NANOG. Co-localisation software, Image J, was used to analyse potential association between the epigenetic mark and the pluripotency factor. Bright yellow cells identified most, but not all, of the cells of the ICM, highlighting the intrinsic heterogeneity associated with this compartment. In the expanded blastocyst NANOG is highly specific for the ICM and hence is justifiably used as a lineage marker.

DNA methylation is programmed during at least two critical periods during development in mammals (Reik and Walter, 2001). Erasure of DNA methylation leads to the expression of genes essential for both germ cell development and for the re-establishment of parent-specific methylation imprints (Hajkova *et al*., 2002; Li *et al*., 2004; Shovlin *et al*., 2008) during fetal development in males and post-natally in females (Davis *et al*., 2000; Lucifero *et al*., 2004a, b; Maatouk *et al*., 2006; Sasaki and Matsui, 2008). Thus, matured and ovulated oocytes and sperm will have germline imprints fully imposed prior to fertilisation. The second phase of reprogramming is initiated following fertilisation and continues throughout the period of pre-implantation. While the significance of this second wave is still not known, the result of this modulation is that somatic levels of DNA methylation are restored at the blastocyst stage (Dean *et al*., 2001; Santos *et al*., 2002). These details derive largely from studies in the mouse, however, they likely extend to other early mammalian embryos including humans. Collectively, these periods of reprogramming establish critical levels and the landscape of epigenetic marks which comprise the epigenotype.

The essential resetting in the mature gamete, prior to ART, may be particularly important where *in vitro* maturation (IVM) of immature oocytes is required. The IVM of oocytes is used clinically to avoid situations where ovarian hyperstimulation syndrome may occur (Chian *et al*., 2004; Manipalviratn *et al*., 2009). It would be expected that epigenetic marks, especially DNA methylation, that are acquired during these late stages of oogenesis may be particularly susceptible to epimutations. Indeed, studies in human and bovine where IVM was investigated reported increased incidences of imprinting errors, especially those associated with maternal DNA methylation (Borghol *et al*., 2006; Hiendleder *et al*., 2006; Sato *et al*., 2007).

Recent opinion has challenged the idea of a causal link of ART to epimutations, suggesting instead that it may be a result of the

underlying infertility (Doornbos *et al*., 2007). Our results would also hint in that direction, with the high degree of similarity of frequencies, and types of errors represented in both IVF and ICSI patient groups. Studies have frequently suggested that superovulation, together with underlying infertility, accounted for these perturbations of maternal alleles (Fortier *et al*., 2008). Importantly, a newly published study in the mouse has uncovered that both maternal and paternal alleles may be negatively affected by superovulation in a dosage-dependant manner (Market-Velker *et al*., 2009).

Many details, including the specific timing of events and identification of activities, remain to be worked out specifically for human pre-implantation embryos, as a prerequisite for understanding human reproductive health. Notably, the influence of the environment, specifically of *in vitro* culture and the impact it may have on the transcriptional regulation of activities critical for the establishment and maintenance of the normal epigenotype. While the present studies are largely descriptive, an inescapable limitation of working with these elite samples, we believe that the conclusions are essential to pave the way for future lines of investigation.

Authors' roles

The initial study was designed by M.S. and W.D. In later phases of the work, further study design was supplied by M.H. and F.S. P.S, M.S, C.L., L.H., M.H. and F.S. were involved in the execution of the study. M.S., W.D., F.S., M.H. and L.H. were involved at different phases of the work in the analysis of data. A.M. was responsible for regulatory and statutory issues relating to the project and for ensuring that patients were seen by an independent research nurse. F.S. stained and imaged all embryos and prepared figures and completed statistical analysis during the drafting of the manuscript. A.M., F.S., M.H., M.S. and W.R. offered critical commentary to W.D. who wrote the manuscript.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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