Human Reproduction, Vol.32, No.11 pp. 2348–2357, 2017
Advanced Access publication on September 23, 2017 doi:10.1093/humrep/dex286

human reproduction

ORIGINAL ARTICLE ***Reproductive genetics***

Genome stability of bovine *in vivo*-conceived cleavage-stage embryos is higher compared to *in vitro*-produced embryos

Olga Tšuiko[1,2,3,4,†], Maaike Catteeuw[5,†], Masoud Zamani Esteki[6,†], Aspasia Destouni[1], Osvaldo Bogado Pascottini[5], Urban Besenfelder[7], Vitezslav Havlicek[7], Katrien Smits[5], Ants Kurg[4], Andres Salumets[2,3,8,9], Thomas D'Hooghe[10], Thierry Voet[6,11], Ann Van Soom[5], and Joris Robert Vermeesch[1,*]

[1]Laboratory of Cytogenetics and Genome Research, Center of Human Genetics, KU Leuven, Leuven 3000, Belgium [2]Institute of Bio- and Translational Medicine, University of Tartu, Tartu 50411, Estonia [3]Competence Centre on Health Technologies, Tartu 50410, Estonia [4]Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Tartu 51010, Estonia [5]Department of Obstetrics, Reproduction and Herd Health, Ghent University, Merelbeke 9820, Belgium [6]Laboratory of Reproductive Genomics, Center of Human Genetics, KU Leuven, Leuven 3000, Belgium [7]Institute of Animal Breeding and Genetics, University of Veterinary Medicine Vienna, Vienna 1210, Austria [8]Department of Obstetrics and Gynaecology, Institute of Clinical Medicine, University of Tartu, Tartu 51014, Estonia [9]Department of Obstetrics and Gynaecology, University of Helsinki and Helsinki University Hospital, Helsinki 00029, Finland [10]Department of Obstetrics and Gynaecology, Leuven University Fertility Centre, University Hospital Leuven, Leuven 3000, Belgium [11]Sanger-EBI Single Cell Genomics Centre, Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, UK

*Correspondence address. Laboratory of Cytogenetics and Genome Research, Center of Human Genetics, KU Leuven, Leuven, Belgium. E-mail: Joris.Vermeesch@med.kuleuven.be

Submitted on May 19, 2017; resubmitted on August 2, 2017

STUDY QUESTION: Is the rate and nature of chromosome instability (CIN) similar between bovine *in vivo*-derived and *in vitro*-cultured cleavage-stage embryos?

SUMMARY ANSWER: There is a major difference regarding chromosome stability of *in vivo*-derived and in *vitro*-cultured embryos, as CIN is significantly lower in *in vivo*-derived cleavage-stage embryos compared to *in vitro*-cultured embryos.

WHAT IS KNOWN ALREADY: CIN is common during *in vitro* embryogenesis and is associated with early embryonic loss in humans, but the stability of *in vivo*-conceived cleavage-stage embryos remains largely unknown.

STUDY DESIGN, SIZE, DURATION: Because human *in vivo* preimplantation embryos are not accessible, bovine (*Bos taurus*) embryos were used to study CIN *in vivo*. Five young, healthy, cycling Holstein Friesian heifers were used to analyze single blastomeres of *in vivo* embryos, *in vitro* embryos produced by ovum pick up with ovarian stimulation (OPU-IVF), and *in vitro* embryos produced from *in vitro* matured oocytes retrieved without ovarian stimulation (IVM-IVF).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Single blastomeres were isolated from embryos, whole-genome amplified and hybridized on Illumina BovineHD BeadChip arrays together with the bulk DNA from the donor cows (mothers) and the bull (father). DNA was also obtained from the parents of the bull and from the parents of the cows (paternal and maternal grandparents, respectively). Subsequently, genome-wide haplotyping and copy-number profiling was applied to investigate the genomic architecture of 171 single bovine blastomeres of 16 *in vivo*, 13 OPU-IVF and 13 IVM-IVF embryos.

MAIN RESULTS AND THE ROLE OF CHANCE: The genomic stability of single blastomeres in both of the *in vitro*-cultured embryo cohorts was severely compromised ($P < 0.0001$), and the frequency of whole chromosome or segmental aberrations was higher in embryos

[†]These authors are contributed equally to this work.

produced *in vitro* than in embryos derived *in vivo*. Only 18.8% of *in vivo*-derived embryos contained at least one blastomere with chromosomal anomalies, compared to 69.2% of OPU-IVF embryos ($P < 0.01$) and 84.6% of IVM-IVF embryos ($P < 0.001$).

LARGE SCALE DATA: Genotyping data obtained in this study has been submitted to NCBI Gene Expression Omnibus (GEO; accession number GSE95358)

LIMITATIONS REASONS FOR CAUTION: There were two main limitations of the study. First, animal models may not always reflect the nature of human embryogenesis, although the use of an animal model to investigate CIN was unavoidable in our study. Second, a limited number of embryos were obtained, therefore more studies are warranted to corroborate the findings.

WIDER IMPLICATIONS OF THE FINDINGS: Although CIN is also present in *in vivo*-developed embryos, *in vitro* procedures exacerbate chromosomal abnormalities during early embryo development. Hence, the present study highlights that IVF treatment compromises embryo viability and should be applied with care. Additionally, our results encourage to refine and improve *in vitro* culture conditions and assisted reproduction technologies.

STUDY FUNDING/COMPETING INTEREST(S): The study was funded by the Agency for Innovation by Science and Technology (IWT) (TBM-090878 to J.R.V. and T.V.), the Research Foundation Flanders (FWO; G.A093.11 N to T.V. and J.R.V. and G.0392.14 N to A.V. S. and J.R.V.), the European Union's FP7 Marie Curie Industry-Academia Partnerships and Pathways (IAPP, SARM, EU324509 to J.R.V., T.V., O.T, A.D., A.S. and A.K.) and Horizon 2020 innovation programme (WIDENLIFE, 692065 to J.R.V., O.T., T.V., A.K. and A.S.). M.Z.E., J.R.V. and T.V. are co-inventors on a patent application ZL913096-PCT/EP2014/068315-WO/2015/028576 ('Haplotyping and copy-number typing using polymorphic variant allelic frequencies'), licensed to Cartagenia (Agilent Technologies).

Key words: *in vivo* embryo / preimplantation embryo / chromosome instability / CIN / haplarithmisis

Introduction

Infertility is a rising health problem that leads to reduced fecundity rates in developed countries. As a consequence, more couples turn to ART to overcome childlessness and age-related infertility (Dyer *et al.*, 2016). On the other hand, the increased access to ART provides the opportunity for more couples to better plan and manage pregnancies. Since the birth of the first 'test tube baby' (Steptoe and Edwards, 1978), over 6 million children have been conceived via ART and fertility treatments have become standard care in many countries (Maheshwari *et al.*, 2016). Despite the progress made in clinical and laboratory ART protocols, including embryo selection for transfer, the pregnancy rate per embryo transfer after ART still lingers at around 30% worldwide (Dyer *et al.*, 2016).

Embryonic chromosomal aneuploidy is a major factor contributing to embryo implantation failure as well as spontaneous miscarriage and may explain the relatively low success rate of IVF procedures (Vanneste *et al.*, 2009b). Studies on human IVF preimplantation embryos have revealed a remarkably high incidence of chromosomal instability (CIN) with 70–90% of cleavage-stage embryos carrying at least one aneuploid cell (Vanneste *et al.*, 2009a, b; Voet *et al.*, 2011; Chow *et al.*, 2014; Huang *et al.*, 2014; Zamani Esteki *et al.*, 2015). Moreover, terminal deletions and duplications are also found in naturally conceived newborns (Riegel *et al.*, 2001). In addition, several cases of mosaic individuals, exhibiting mixoploidy or chimaerism have also been well documented (Jarvela *et al.*, 1993; Edwards *et al.*, 1994; Yamazawa *et al.*, 2010). Therefore, we hypothesized that CIN would also occur at similar rate *in vivo*. Due to the ethical and legal constraints associated with human embryo research, naturally conceived human cleavage-stage embryos are not accessible, and CIN in human IVF and *in vivo* embryos cannot be compared directly. As an alternative, animal models have been widely used for investigating chromosomal abnormalities in *in vivo* and *in vitro* preimplantation embryos (Viuff *et al.*, 2001; Rambags *et al.*, 2005; Coppola *et al.*, 2007). However, the major limitation of previous studies was the application of low-resolution karyotyping methods that can neither detect CIN at the single-cell level nor reveal subtle sub-chromosomal aberrations. Thus, the knowledge about the genomic stability of *in vivo*-conceived embryos remained limited, largely due to the lack of robust genome analysis technologies.

To compare *in vitro* versus *in vivo* CIN directly, we used bovine cleavage-stage embryos, which represent a suitable model mimicking human early embryogenesis (Menezo and Herubel, 2002; Destouni *et al.*, 2016). In addition, similarly to human only 40–55% of dairy cows produce an offspring after single insemination (Diskin *et al.*, 2012), and aneuploidy was reported in one-fifth of bovine aborted fetuses and non-viable neonates (Coates *et al.*, 1988). We applied a genome-wide single-cell analysis method that enabled haplotyping and copy-number profiling, called haplarithmisis (Zamani Esteki *et al.*, 2015), on all individual bovine blastomeres obtained from *in vivo* embryos derived from oocytes that were matured and fertilized *in vivo* after ovarian stimulation of donor animals (referred to as *in vivo* embryos). In parallel, we tested *in vitro*-produced embryos derived from *in vitro* matured and fertilized oocytes that were retrieved from the same donor animals using ovarian stimulation and ovum pick up (referred to as OPU-IVF embryos) and *in vitro*-produced embryos derived from *in vitro* matured and fertilized oocytes that were retrieved from donor animals without ovarian stimulation (referred to as IVM-IVF embryos). All single blastomeres were analyzed for the presence of chromosomal aberrations.

Materials and Methods

Ethical approval

This study was approved by the Ethical Committee of the Faculty of Veterinary Sciences of Ghent University, Belgium (EC2013/197, EC2015/71).

Study design

The aim of the study was to evaluate CIN in naturally conceived preimplantation embryos. In parallel, we investigated the influence of different IVF procedures on embryo development using bovine as a model for human early embryogenesis (Menezo and Herubel, 2002; Destouni *et al.*, 2016). Five young, healthy, cycling Holstein Friesian heifers (*Bos taurus*) between 18 and 36 months of age were used as oocyte and embryo donors. All donor cows were subjected to hormonal stimulation with subsequent ovum pick up or *in vivo* embryo collection (Besenfelder *et al.*, 2008). Blood samples from the donor cows (mothers) and semen from the bull (father) were used to extract bulk DNA (DNeasy Blood and Tissue kit, Qiagen, Germany). Bulk DNA was also obtained from the parents of the bull (paternal grandparents) and the available parents of the cows (maternal grandparents; only for crosses 4757, 8301 and 9617). After hormonal treatments, the cows were left untreated for one month before they were slaughtered. After collection of ovaries, oocytes were retrieved and embryos were produced *in vitro* by routine procedures (Catteeuw *et al.*, 2017). Subsequently, single blastomeres were isolated, whole-genome amplified (WGA) and hybridized on BovineHD BeadChip arrays (Illumina Inc., USA). The acquired array data was used for single-cell genome-wide haplotyping and copy-number profiling (Zamani Esteki *et al.*, 2015).

Media and reagents

Basic Eagle's Medium amino acids, Minimal Essential Medium (MEM) non-essential amino acids (100×), TCM-199-medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium) and all other components were obtained from Sigma (Schnelldorf, Germany), unless otherwise stated. All the media were filter-sterilized using a 0.22 µm filter (Pall Corporation, Ann Arbor, MI, USA) before use.

Stimulation protocol and ovum pick up

Stimulation protocol for ovum pick up was used to generate OPU-IVF embryos and was performed 3 to 6 times in all animals with at least one week interval between OPU sessions. On Day 0, heifers were given an epidural anesthesia using 3 ml of Procaine Hydrochloride 2% (VMD, Belgium) to decrease peristalsis and discomfort. An ultrasound probe was inserted in the vagina, and follicles larger than 5 mm were removed by puncturing the ovaries. Animals received dinoprost (prostaglandin F2α ($PGF_{2\alpha}$)) intramuscularly (i.m.) (Dinolytic®, Zoetis, Belgium), and a CIDR (controlled internal drug release, Progesterone, Zoetis, Belgium) was administered in the vagina. In following days, pFSH injections (Stimufol®, Reprobiol, Belgium) were given i.m. twice a day. The CIDR was removed 40–44 h after the last pFSH injection and OPU was performed. On the day of OPU animals were given an epidural anesthesia using 3 ml of Procaine Hydrochloride 2%. All follicles were aspirated using an ultrasound probe, a 7.5 MHz transducer and a stainless steel guide. Puncturing was performed using disposable 19 G needles connected to a 50 ml tube via silicon tubing. Needles were changed between ovaries of the same animal and between animals, further tubing was also renewed between animals. Follicular fluid containing the oocytes was collected in 5 ml HEPES-buffered TCM-199 supplemented with 18 IU/ml heparin, 50 µg/ml gentamicin and 0.1% fetal calf serum (FCS). Immediately following recovery, the collected follicular fluid was filtered through a 75 µm mesh filter with HEPES-buffered TCM-199. Oocytes were grouped per donor and embryos were produced according to the standard *in vitro* embryo production protocol.

In vitro bovine embryo production protocol

Bovine OPU-IVF and IVM-IVF embryos were produced per donor by previously described methods (Catteeuw *et al.*, 2017). Briefly, oocytes retrieved via ovum pick up and oocytes retrieved from ovaries of slaughtered animals were placed per donor in 500 µl maturation medium, consisting of modified bicarbonate-buffered TCM-199 supplemented with 50 µg/ml gentamycin and 20 ng/ml epidermal growth factor (EGF) for 22 h at 38.5°C in 5% CO_2 in humidified air. After maturation, frozen–thawed semen of a previously tested Holstein Friesian bull was used for fertilization. Spermatozoa were separated over a discontinuous Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden) and sperm concentration was adjusted to 1×10^6 spermatozoa/ml using IVF-TALP (Tyrode's Albumin Lactate Pyruvate), which is supplemented with 6 mg/ml BSA (Sigma A8806) and 25 µg/ml heparin. Matured oocytes were incubated per donor in 500 µl IVF-TALP with spermatozoa for 21 h at 38.5°C in 5% CO_2 in humidified air. Presumptive zygotes were transferred to synthetic oviductal fluid (SOF) supplemented with essential and non-essential amino acids (SOFaa), 0.4% BSA (Sigma A9647) and ITS (5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium) and were placed per donor in a droplet of culture medium. The droplet size differed between donors depending on the number of zygotes, an embryo:medium ratio of 1:2 was maintained with a minimal droplet size of 20 µl. Each droplet was covered by mineral oil and incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2.

Oviductal flush and collection of *in vivo* embryos

The *in vivo* collection of embryos was performed by oviductal flush as described earlier (Besenfelder *et al.*, 2008). First, estrous cycles of the donor animals were pre-synchronized by i.m. administering 2 ml $PGF_{2\alpha}$ (500 µg Cloprostenol, Estrumate, Belgium) twice within 11 days. Forty-eight hours after both $PGF_{2\alpha}$ treatments, the animals received i.m. About 21 µg Gonadotropin Releasing Hormone (GnRH) (Receptal®, MSD AH, Belgium). Dominant follicles were ablated 9 days after heat detection. Thirty-six hours later, pFSH was administered in decreasing dosages twice a day for 4 days (1.5, 1.4, 1.2, 1.1, 0.8, 0.6, 0.5 and 0.5 ml), and in total 380 µg follitropine was given. The donor animals received two $PGF_{2\alpha}$ treatments 60 and 72 h after the initial pFSH treatment. Finally, 24 h after the last pFSH treatment, 21 µg GnRH was administered to induce ovulation, simultaneously animals were inseminated with frozen–thawed semen. Artificial insemination (AI) was repeated 12 and 24 h later. Embryos were flushed bilaterally 36 h after the last AI. Briefly, donor animals were given epidural anesthesia using 5 ml of Procaine Hydrochloride 2%. An embryo flushing catheter was directed through the cervix and fixed in the uterine horn. An integrated device consisting of a universal tube, an endoscope and flushing system was inserted through the vaginal wall into the peritoneal cavity, which was passively filled with air. Oviducts were flushed with 40–60 mL flushing medium (PBS supplemented with 1% FCS) to pass the embryos through the uterotubal junction. Once in the uterine horn, flushing medium containing the embryos was collected via the uterus flushing catheter into an embryo filter. Finally, the uterine horn was flushed with another 300–500 mL medium through the uterine flushing catheter. This procedure was repeated for flushing the other oviduct and uterine horn. The collected medium was transferred to petri dishes and examined for embryos using a stereomicroscope.

Single blastomere isolation and SNP genotyping

IVM-IVF, OPU-IVF and *in vivo*-derived embryos were treated with pronase (0.1% protease for IVM-IVF and OPU-IVF embryos and 1% protease for *in vivo* embryos from S. *griseus*, Sigma P88110) in Hepes-buffered TCM-199 (Life Technologies Europe, Belgium) to dissolve zona pellucida. The zona-free embryos were then washed in HEPES-buffered TCM-199 with 10% FCS followed by Ca^{+2}/Mg^{+2} free PBS with 0.05% BSA to stimulate

blastomere dissociation. Next, each blastomere was washed three times in wash medium (Ca^{+2}/ Mg^{+2} free PBS with 0.1% polyvinylpyrrolidone) and subsequently transferred into a 0.2-mL PCR tube containing 2 µl of PBS and WGA using a commercial multiple displacement amplification kit according to the manufacturer's fast 3 h protocol (REPLI-g Single Cell Kit, Qiagen, Germany). WGA products were purified with SPRI-beads (Beckman Coulter Inc., USA) at 0.8× total reaction volume and SNP genotyped on BovineHD SNP arrays using the Infinium HD whole-genome genotyping assay. Genotyping data obtained in this study has been submitted to NCBI Gene Expression Omnibus (GEO; accession number GSE95358; http://www.ncbi.nlm.nih.gov/geo/).

Single blastomere whole-genome analysis

SNP genotypes, logR Ratio (logR) and B Allele Frequency (BAF) values were obtained for each sample by applying the GenCall algorithm, embedded in the GenomeStudio software Genotyping Module v.3.1 (Illumina Inc.). SNP genotypes were called by setting the GenCall score at 0.75. Next, computational workflow 'siCHILD-bovine' was used to acquire genome-wide haplarithm plots for each sample as described previously (Destouni *et al.*, 2016). Briefly, the acquired single-cell SNP data underwent quality control (QC) using a combination of unsupervised hierarchical clustering on the discrete SNP genotype calls and cumulative chromosome-specific standard deviation on the logR-values. Substandard samples were excluded from further investigations. The entire process of haplarithmisis was then applied for data analysis as previously described (Zamani Esteki *et al.*, 2015). Briefly, haplarithmisis uses single-cell SNP BAF-values and phased parental genotypes to determine genome-wide haplotypes, copy-number state of the haplotypes, as well as the parental and segregational origin of putative haplotype anomalies in the cell. The parental genotypes are phased via SNP genotype calls derived from a close relative, e.g. sibling or the grandparents. In this study, we have used paternal and maternal grandparents. Next, specific combinations of phased parental genotypes are retrieved that consequently define single-cell SNP BAF-values. Consequently, these values are plotted on paternal and maternal haplarithms. All the haplarithm plots obtained from this study are provided in Supplementary Figure S3. In parallel with haplarithmisis, genome-wide haplotypes of single blastomeres were also reconstructed. Data were visualized with siCHILD, Circos (Krzywinski *et al.*, 2009) and R (https://www.r-project.org/).

Statistical analysis

Statistical calculations were carried out using GraphPad Prism 6 software (GraphPad Software Inc., USA). The prevalence of CIN and the nature of detected chromosomal abnormalities were compared between the three embryo groups and corresponding single blastomeres by two-tailed Fisher's exact test with Bonferroni correction for multiple testing. The differences in the frequencies of CIN between the three embryo cohorts were considered to be statistically significant when the multiple testing corrected *P*-value was < 0.01. When comparing monospermic embryos, a *P*-value <0.05 was considered to be statistically significant.

Results

Embryo collection and genome-wide analysis of single bovine blastomeres

Five healthy, cycling Holstein Friesian heifers (*Bos taurus*) were used to produce IVM-IVF, OPU-IVF and *in vivo* embryos (Fig. 1A), and the incidence of CIN was evaluated for all three groups. The use of the same cows and bull seed to study the effect of CIN *in vivo* and *in vitro* reduces potential genetic background confounding effects. First, donor animals were subjected to varying numbers of ovum pick up sessions depending on the ovarian response to hormonal stimulation and the number of oocytes retrieved per session (Table S1). Overall, 49 oocytes were collected, of which 13 (26.5%, $n = 49$) were good quality oocytes with homogeneous non-granulated cytoplasm and at least three compact layers of cumulus cells. On Day-1 post-insemination (pi), 28 (57.1%, $n = 49$) of the presumed zygotes cleaved, and subsequently 77 single blastomeres were collected from 10 OPU-IVF embryos on Day-2 pi (median 5.0 blastomeres per embryo) and five OPU-IVF embryos on Day-3 pi (median 6.0 blastomeres per embryo).

Next, a total of 42 *in vivo* oviductal-stage embryos were retrieved on Day-2 pi by oviduct flushing (Besenfelder *et al.*, 2008), from which 34 (81.0%) have cleaved. Due to ovarian stimulation and OPU, the cow from cross 4757 developed scar tissue and no flushing of *in vivo* embryos was possible, because of the obstruction of the oviduct. Of all the cleaved *in vivo* embryos, 12 either had indigestible zona pellucida or lysed during washing and single-cell collection. As a result, 22 zona-free embryos were collected (median 4.45 blastomeres per embryo) and 18 were hybridized on SNP arrays after successful amplification of at least half of the blastomeres per embryo ($n = 73$).

Following OPU-IVF and *in vivo* embryo collection, donor animals were slaughtered, ovaries were collected, and *in vitro* embryos were produced (Catteeuw *et al.*, 2017). Thirty-one oocytes were aspirated, of which 16 (51.6%, $n = 31$) were of good quality. On Day-1 pi, 20 (64.5%, $n = 31$) of the presumed zygotes cleaved. Subsequently, 72 blastomeres were isolated from 13 Day-2 pi IVM-IVF embryos (mean 5.54 blastomeres per embryo) and further analyzed.

In summary, a total of 222 individual bovine blastomeres were collected from 13 IVM-IVF, 15 OPU-IVF and 18 *in vivo*-derived bovine cleavage-stage embryos. Following QC and initial data analysis, 171 (77.0%, $n = 222$) blastomeres were considered for further data interpretation (66 blastomeres from 13 IVM-IVF embryos, 46 blastomeres from 13 OPU-IVF embryos and 59 blastomeres from 16 *in vivo*-derived embryos, respectively; Table S2). For crosses 4757, 8301 and 9617 we applied haplarithmisis using both maternal and paternal grandparents as seeds for parental genotype phasing to reconstruct haplotypes of single blastomeres (Fig. 1B–C). For crosses 4006 and 4770, lacking maternal grandparental DNA samples, only parents of the bull were used as a seed for creating the paternal haplarithm profile.

Characteristics of CIN in embryos developed *in vitro* and *in vivo*

We first aimed to assess the prevalence of CIN in IVM-IVF, OPU-IVF and *in vivo*-derived bovine cleavage-stage embryos. To evaluate the genomic stability of embryos, we investigated chromosome segregation patterns in all analyzed blastomeres ($n = 171$, Supplementary Fig. S1). In this analysis, euploid blastomeres, irrespective of their ploidy, that lacked full chromosome or segmental aberrations were scored as balanced. The genomic integrity of single blastomeres was higher in *in vivo* embryos than in OPU-IVF and IVM-IVF embryos (in both cases $P < 0.0001$, Fisher's exact test; Fig. 2A). At the embryonic level, the number of abnormal embryos carrying at least one blastomere with a full or segmental chromosomal aberration increased from 18.8% in *in vivo* embryos (3/16) up to 84.6% in IVM-IVF embryos (11/13) ($P < 0.001$, Fisher's exact test; Fig. 2B). The CIN rate in OPU-IVF embryos

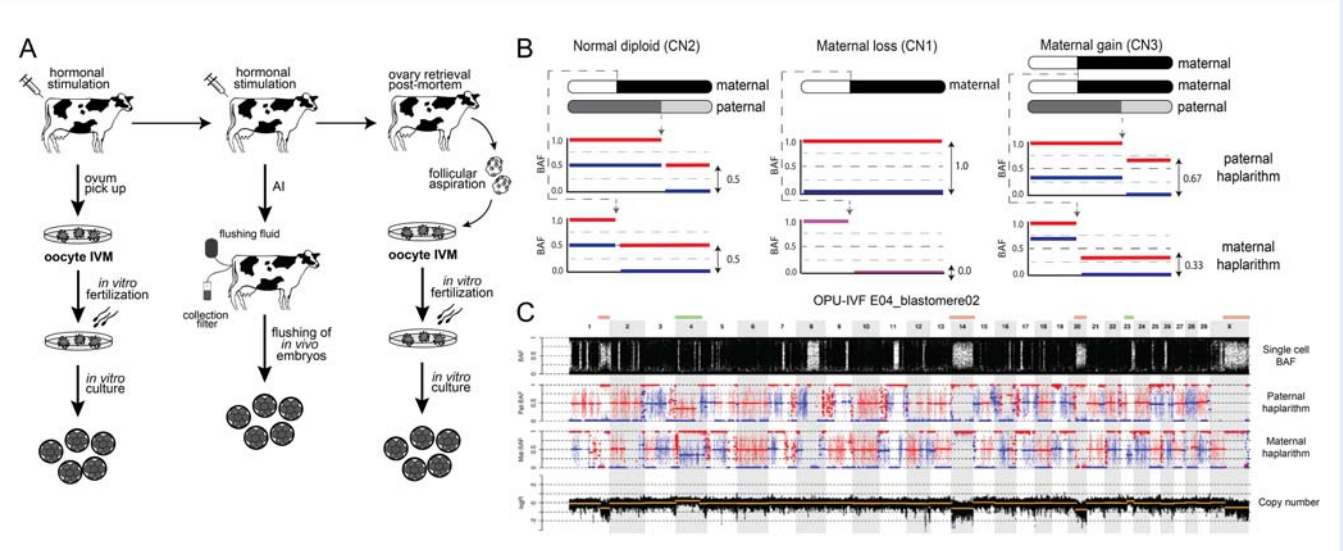

Figure 1 Schematic overview of study design and data analysis. (**A**) Different protocols used for embryo production. First, five donor cows were used to obtain OPU-IVF embryos (left). Next, *in vivo* embryos were derived from the same donor cows via oviduct flush (middle). Finally, donor cows were slaughtered and IVM-IVF embryos were produced (right). (**B**) Schematic representation of haplarithm profiles for different genomic rearrangements. During initial parental phasing using informative SNPs, single-cell B allele frequency (BAF) values are assigned to P1 or M1 (blue lines) and P2 or M2 (red lines) subcategories (for more detail see (Zamani Esteki *et al.*, 2015)). Defined single-cell BAF-values of the segmented P1, P2, M1 and M2, as well as the distance between the P1-P2 or M1-M2 denote the origin and nature of copy-number (CN) alterations. Dashed gray arrows showing the pairwise P1-P2 and M1-M2 breakpoints in the haplarithm profiles signify homologous recombination sites, accompanied by the switch in the haplotype blocks. (**C**) An example of genome-wide haplarithm profile of single blastomere of OPU-IVF embryo E04 with segmental and full chromosome losses (light red) and gains (light green), corroborated by the corresponding paternal and maternal haplarithm patterns and the normalized logR-values.

(69.2%, 9/13) was comparable to the CIN rate in IVM-IVF embryos ($P > 0.05$, Fisher's exact test), but was higher than in *in vivo*-derived embryos ($P < 0.01$, Fisher's exact test). Because OPU-IVF group also contained five Day-3 pi embryos that may have undergone at least one more cell division that can lead to mitotic error, we decided to analyze Day-2 pi OPU-IVF embryos ($n = 8$) separately to obtain more consistency between the groups. Comparison of Day-2 OPU-IVF and *in vivo* embryos provided similar results, demonstrating that CIN is higher in Day-2 pi OPU-IVF embryos (75%, 6/8) than in *in vivo*-derived Day-2 pi embryos ($P = 0.02$, Fisher's exact test). Likewise, OPU-IVF embryos contained significantly larger proportion of unbalanced blastomeres that can subsequently lead to altered embryonic development (16/26, 61.5%, $P < 0.0001$, Fisher's exact test).

Next, we determined the nature of chromosomal aberrations in *in vitro*-produced and *in vivo*-derived embryos. As was expected, aneuploidy was the most prevalent type of error and all abnormal *in vivo*-derived, OPU-IVF and IVM-IVF embryos contained whole-chromosome aberrations. Therefore, the number of embryos with aneuploidy was significantly higher in OPU-IVF embryos (69.2%, 9/13) and in IVM-IVF embryos (84.6%, 11/13) than in the *in vivo* group (18.8%, 3/16, $P < 0.01$ and $P < 0.001$, respectively, Fisher's exact test; Fig. 3A). Meiotic errors were observed only in Cross 9617, once within the OPU-IVF group and once within the IVM-IVF group, in which case the embryos showed the same chromosomal aneuploidy in all of the sister blastomeres (OPU-IVF E01_Cross9617 and IVM-IVF E09_Cross9617 were monosomic for chromosomes 26 and 24, respectively; Supplementary Fig. S1). The remaining aberrations were of mitotic origin and resulted in either whole chromosome or segmental imbalances. Segmental imbalances were most prevalent in IVM-IVF embryos (9/13, 69.2%) when compared to OPU-IVF embryos (2/13, 15.4%, $P = 0.01$, Fisher's exact test) and *in vivo* embryos (1/16, 6.3%, $P = 0.001$, Fisher's exact test; Fig. 3A).

We also observed a number of embryos that had at least one blastomere with an abnormal ploidy state (Fig. 3A). Single-cell haplarithm profiles uncovered the presence of only paternal (androgenetic) or only maternal (gynogenetic) genomes in a single blastomere and enabled triploid blastomeres to be classified as diandric or digynic in origin. Upon comparing IVM-IVF, OPU-IVF and *in vivo* embryos, we observed that IVM-IVF embryos were burdened with mixoploidy. In this study, mixoploidy is defined by the presence of cell lineages of different parental origin and/or different genome-wide ploidy states within the same embryo. As such, mixoploid embryos harbor simultaneously haploid, diploid and/or triploid cells (Fig. 3B, Supplementary Fig. S1). Mixoploidy in IVM-IVF embryos can be attributed to the dispermic fertilization (69.2%, 9/13) that was identified by the presence of two different paternal haplotypes within the same embryo. In seven IVM-IVF embryos one of the extra paternal genomes segregated into a separate androgenetic cell line carrying only paternal DNA, a phenomenon that was recently discovered in *in vitro*-produced bovine cleavage-stage embryos and termed heterogoneic cell division (Destouni *et al.*, 2016). For example, a 10-cell IVM-IVF embryo contained eight androgenetic blastomeres, one biparental and one triploid blastomere (E10_Cross4770, Fig. 3B). In addition, we observed amplified shattered paternal chromosomal DNA fragments and no maternal DNA in blastomere Bl005 of E07_Cross4770 (Supplementary Fig. S1), and in E11_Cross4770 two blastomeres (Bl002 and Bl003) contained

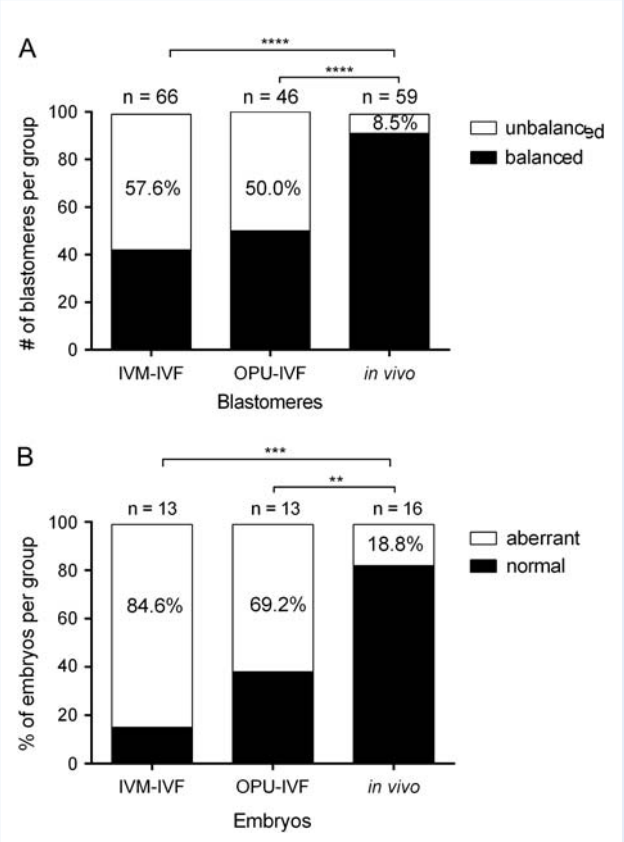

Figure 2 The rate of CIN in IVM-IVF, OPU-IVF and *in vivo* embryos. The numbers above the columns represent the total numbers of blastomeres (**A**) and embryos (**B**) included in the study after QC. (A) The comparison of balanced and unbalanced blastomeres represents the chromosome dynamics of single blastomeres in IVM-IVF ($n = 66$), OPU-IVF ($n = 46$) and *in vivo*-derived embryos ($n = 59$); ****$P < 0.0001$, two-tailed Fisher's exact test for multiple testing. (B) The proportion of normal diploid embryos and aberrant embryos in IVM-IVF ($n = 13$), OPU-IVF ($n = 13$) and *in vivo* group ($n = 16$); **$P < 0.01$, ***$P < 0.001$, two-tailed Fisher's exact test for multiple testing. QC, quality control.

residues of paternal DNA that were reciprocal in nature (Supplementary Fig. S1). Such replication and division of the remnants of sperm genome were also observed in a previous study on bovine *in vitro*-produced embryos (Destouni *et al.*, 2016). In contrast, only one OPU-IVF embryo underwent dispermic fertilization resulting in a diandric triploid embryo (E03_Cross4757, Fig. S1). Notably, no abnormal fertilization events occurred in the *in vivo*-derived embryos.

Because dispermy might influence CIN in embryos, we then analyzed only those embryos that developed from monospermic zygotes. For this purpose, we combined monospermic IVM-IVF ($n = 4$) and OPU-IVF ($n = 12$) embryos into a single group (referred to as *in vitro*) and compared them to *in vivo*-derived embryos ($n = 16$). The CIN rates confirmed a considerable difference between the *in vitro*-produced and cultured embryos, and *in vivo*-derived embryos (10/16, 62.5% vs 3/16, 18.8%, $P = 0.03$, Fisher's exact test; Fig. S2A). In addition, it was clear that *in vitro* procedures had a highly significant negative impact on CIN, when we compared the low frequency of chromosomal aberrations and aberrant ploidy states in blastomeres of *in vivo*-derived embryos (7/59, 11.9%) with the high frequency chromosomal aberrations and aberrant ploidy states of *in vitro*-produced and cultured embryos (27/57, 47.4%, $P < 0.0001$, Fisher's exact test; Fig. S2B). Similarly, when analyzing only Day-2 pi embryos, seven *in vitro* embryos were classified as abnormal (7/11, 63.6%, $P = 0.04$), while the total number of abnormal blastomeres in the *in vitro* group reached up to 45.9% (17/37, $P < 0.001$, Fisher's exact test). Together, these results strongly suggest that *in vitro* procedures, such as maturation, fertilization and culture, enhance embryonic CIN and consequently impede embryo developmental potential.

Discussion

Over the past decades, we have witnessed a profound impact of assisted reproduction on society, as the use of ART continues to increase globally. The number of women voluntarily or involuntarily delaying motherhood is also steadily increasing, and for those women, fertility preservation and IVF procedures are becoming a mainstream approach to achieve motherhood (Lallemant *et al.*, 2016). In this study, we applied an advanced genome analysis method to scrutinize the characteristics of chromosomal aberrations in *in vivo*-derived embryos and to investigate the potential influence of ART treatments on the rate and nature of CIN during early embryo development. We demonstrated that the genomic stability of *in vivo* embryos is significantly higher compared to OPU-IVF and IVM-IVF embryos.

Because only 30% of human conceptions result in live births (Macklon *et al.*, 2002) and because chromosomal abnormalities can be detected in at least half of all spontaneous miscarriages (Menasha *et al.*, 2005; Levy *et al.*, 2014), it was speculated that post-zygotic events leading to CIN in IVF embryos would also occur at similar frequency in naturally conceived embryos. However, in contrast to the initial hypothesis, CIN was found to be significantly more frequent in *in vitro*-produced rather that *in vivo*-derived bovine embryos. Since CIN was observed in less than 20% of *in vivo* embryos compared to at least 70% in *in vitro* embryos, *in vivo*-conceived embryos will most likely be overall more viable. This observation has two major implications: (i) directing individuals towards ART programs should be done cautiously as it may compromise embryo quality and (ii) improvements to the embryo *in vitro* environment are likely still possible to enhance ART success.

When oocytes are matured *in vivo*, they originate from ovulatory follicles that undergo strongly regulated processes of selection, growth and dominance, until LH surge induces the meiotic maturation of the fully grown oocytes into fertilizable oocytes (Li and Albertini, 2013). In addition, during preimplantation embryo development *in vivo*, the female reproductive tract provides the appropriate environment and the essential nutrition that guide physiological processes of mammalian early embryogenesis (Gardner *et al.*, 1996). In contrast, during preimplantation embryo development *in vitro*, even minor alterations during *in vitro* culture and the micromanipulation of oocytes and embryos may negatively impact embryo quality and subsequent fetal development (Wale and Gardner, 2016). Indeed, a study in cattle investigated the separate effect of *in vitro* procedures (maturation, fertilization and culture) on embryo developmental potential, and it was clear that *in vivo* oocyte maturation and *in vivo* embryo development show consistently more favorable outcome in terms of embryo quality

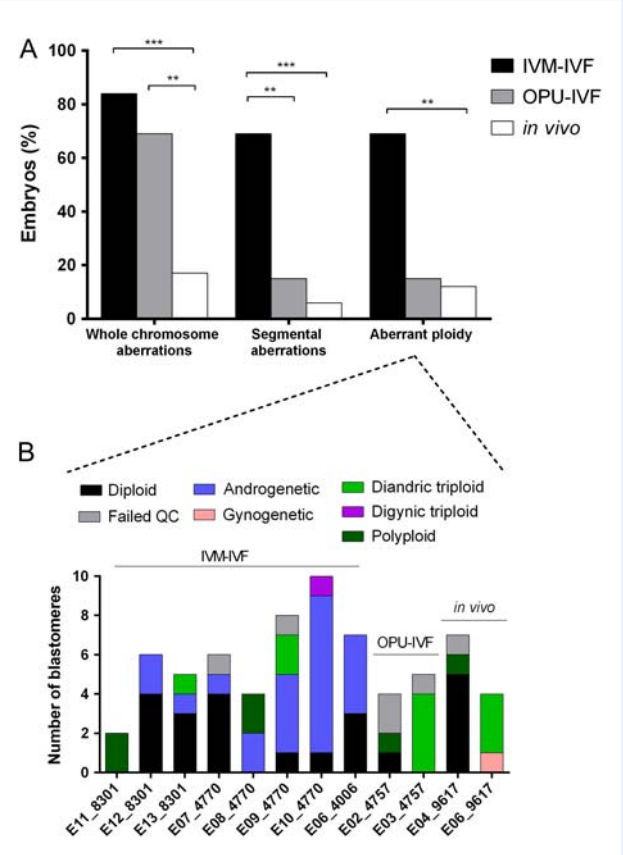

Figure 3 Incidence and nature of CIN in IVM-IVF, OPU-IVF and *in vivo* embryos. (**A**) The proportion of embryos with different genomic rearrangements. Abnormal ploidy state of blastomeres was not considered in evaluating whole-chromosome aberrations to avoid bias; for whole-chromosome aberrations *P*-values stand as $^{**}P < 0.01$, $^{***}P < 0.001$; for segmental imbalances and aberrant ploidy *P*-values stand as $^{**}P = 0.01$, $^{***}P = 0.001$, two-tailed Fisher's exact test for multiple testing. (**B**) Examples of embryos containing at least one blastomere with an abnormal ploidy status. The stacked bar plots depict the number of blastomeres with different genomic anomalies per embryo. Blastomeres with normal karyotype and with single aneuploidies and/or segmental losses or gains that have both maternal and paternal alleles present in their genome (diploid embryos) are depicted in black.

compared to *in vitro* conditions (Rizos *et al.*, 2002). Moreover, the oocyte plays a central role in maintaining genomic integrity before major embryonic genome activation (EGA), as first post-zygotic divisions are highly dependent on the large pool of maternal mRNAs and proteins provided by the oocyte (Braude *et al.*, 1988). This view is supported by the time-lapse study, demonstrating that the generation of embryonic aneuploidy precedes the major wave of EGA (Chavez *et al.*, 2012), while the inheritance of an aberrant oocyte transcriptome has been associated with abnormal first post-zygotic cleavage (Vera-Rodriguez *et al.*, 2015). Therefore, in the current study, the higher rate of chromosomal abnormalities in *in vitro* embryos may also arise from the defective maternal resources of the oocytes; however more research should target the precise impact of the intrinsic quality of the oocyte on the incidence of chromosomal aberrations in cleavage-stage embryos. Finally, although it was recently demonstrated that mosaic embryos may be viable, as abnormal cells get depleted during embryo development, there needs to be a sufficient proportion of normal cells within the embryo to ensure its survival (Bolton *et al.*, 2016). Moreover, aneuploid cells in trophectoderm lineage were proposed to contribute to the formation of confined placental mosaicism (Robberecht *et al.*, 2010), a condition that has been associated with intrauterine growth restriction (Wilkins-Haug *et al.*, 2006; Baffero *et al.*, 2012).

Controlled ovarian stimulation (COS) is widely used in women to retrieve numerous oocytes for fertilization per cycle and to enhance the cumulative pregnancy rate. In humans, COS is considered to be a safe procedure, since embryo quality, implantation rates and pregnancy outcomes are comparable between stimulated and natural IVF cycles (Ziebe *et al.*, 2004; Sunkara *et al.*, 2016). In our study, although both *in vivo* and OPU-IVF embryos were obtained after hormonal stimulation of donor animals, we observed more chromosomally normal diploid embryos and blastomeres in *in vivo*-derived embryos than in OPU-IVF embryos. This indicates that *in vitro* maturation, fertilization and culture are the major causes of embryonic CIN, rather than ovarian stimulation itself. This further suggests that improvements in culture conditions are necessary to increase IVF success rates. However, there is still an ongoing debate on the potential deleterious effect of ovarian stimulation on oocyte and embryo quality, so the technology of human IVM of retrieved oocytes with no or very little follicle stimulating hormone (FSH) stimulation has been proposed as an alternative approach to conventional ovarian stimulation followed by IVF (Dal Canto *et al.*, 2006; Ellenbogen *et al.*, 2014). Although in humans, achieving pregnancy and live birth via IVM is less successful than by using conventional IVF (Buckett *et al.*, 2008; Gremeau *et al.*, 2012), it is a much safer and beneficial option for women with polycystic ovaries or cancer patients for whom hormonal stimulation is contraindicated. The main advantage of using IVM in humans is the ability to eliminate the risk of developing ovarian hyperstimulation syndrome, a possible life-threatening complication, and to reduce the overall cost associated with hormonal therapy. In human IVM procedure, subsequent oocyte fertilization is typically performed by intracytoplasmic sperm injection (ICSI), but traditional IVF (human IVM-IVF) has also been used to fertilize *in vitro* matured oocytes (Soderstrom-Anttila *et al.*, 2005; Walls *et al.*, 2012). However, the IVM-IVF combination may be suboptimal for humans because *in vitro* matured oocytes have no contact with the oviductal environment, which enables the oocyte to reach full cytoplasmic maturation that modifies zona pellucida resistance, and thus zona pellucida of IVM oocytes may become less resistant to dispermic fertilization under *in vitro* conditions (Xia, 2013). Our results seem to corroborate this view as dispermic fertilization was almost exclusively found among IVM-IVF embryos. In addition, we observed that dispermic embryos segregate their extra paternal genome into a separate androgenetic cell lineage (Destouni *et al.*, 2016) leading to mixoploidy. These embryos would have a low developmental potential, but due to a highly proliferative cell lineage carrying paternal genome only, they would have a higher implantation capacity and could potentially give rise to molar pregnancies of androgenetic origin. Complete hydatidiform moles of androgenetic origin have been reported in both human (Ibrahim *et al.*, 1989; Kwon *et al.*, 2002; Sun *et al.*, 2012; Obeidi *et al.*, 2015) and in cattle (Meinecke *et al.*, 2002). Taken together, our results can possibly explain why IVM of human

eggs and subsequent IVF and embryo culture are associated with lower reproductive success rate (Pfeifer *et al.*, 2013).

Unfortunately, studies of *in vivo* embryogenesis rely on appropriate animal models and direct research of *in vivo* human embryogenesis awaits novel technical approaches. Another limitation of our study is the small number of embryos analyzed, and because some of the cells did not pass the QC, it was also not possible to determine the chromosomal status of those QC-failed blastomeres. In addition, due to small cohort sizes, we were not able to compare the pedigrees between each other to determine any cow-specific confounding factors, influencing the frequency of aneuploidy in embryos. Thus more studies are warranted to corroborate our findings. Also, *in vivo*-derived embryos do not entirely represent the natural conception, as donor cows underwent hormonal stimulation to increase the number of *in vivo*-derived embryos via oviductal flush. Moreover, the ovarian response to hormonal treatment is unpredictable and can vary from cycle to cycle, and may result in either 'low' or 'high' response to hormone treatment both in cattle (De Roover *et al.*, 2005; Durocher *et al.*, 2006) and human (Broekmans *et al.*, 2014; Rombauts *et al.*, 2015). Although the difference in oocyte and embryo quality after ovarian stimulation between donor animals was also noticed in this study, future research is needed to evaluate the impact of hormonal stimulation on CIN in embryos. However, the overall reduced CIN in *in vivo* embryos compared to OPU-IVF embryos suggests that the effect of hormonal stimulation will be minor.

In summary, this is the first study to date that compared simultaneously the impact of three different embryo production protocols on subsequent embryo development using single-cell technologies. We showed that *in vitro* environment influence CIN and compromises cleavage-stage embryo development and survival. This highlights the importance of understanding *in vivo* regulation of mammalian oocyte maturation and subsequent embryonic development to refine assisted reproductive technologies in human. Some caution to extrapolate the results to human IVF is required, since oocytes in both OPU-IVF and IVM-IVF study groups were *in vitro* matured prior to fertilization. This procedure is rarely used in human ART. Still, in the absence of human data, it is of paramount importance to propose ART only to those couples who have a medical indication for IVF treatment, while the use of IVF for social reasons should be critically discussed, taking into account possible complications associated with assisted reproduction.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

The authors thank the CRV Belgium (www.crv4all.be) team for providing grandparental bovine genotyping data used in this study. We are grateful to Nathalie Sohier and Eftychia Dimitriadou for technical assistance.

Authors' roles

O.T., M.C., M.Z.E., A.V.S. and J.R.V. conceived and designed the study. M.Z.E. and T.V. developed haplarithmisis. M.C., K.S., O.B.P., U.B. and V.H. performed embryo production experiments. O.T. and M.C. collected single blastomeres and processed samples for SNP-array hybridization. O.T. and M.Z.E. performed data and statistical analysis. O.T., M.C., and M.Z.E. wrote the original version of the manuscript. O.T., M.C., M.Z.E., A.D., U.B., V.H., K.S., A.K., A.S., T.D'H., T.V., A.V.S. and J.R.V. wrote and edited the final version of the manuscript. J.R.V., A.V.S., T.V., A.K. and A.S. provided funding. A.V.S. and J.R.V. supervised the study.

Funding

The study was funded by the Agency for Innovation by Science and Technology (IWT) (TBM-090878 to J.R.V. and T.V.), the Research Foundation Flanders (FWO; G.A093.11N to T.V. and J.R.V. and G.0392.14N to A.V.S. and J.R.V.), the European Union's FP7 Marie Curie Industry-Academia Partnerships and Pathways (IAPP, SARM, EU324509 to J.R.V., T.V., O.T, A.D., A.S. and A.K.), Horizon 2020 innovation programme (WIDENLIFE, 692065 to J.R.V., O.T., T.V., A.K. and A.S.) and Estonian Ministry of Education and Research (IUT34-16 to A.S.).

Conflict of interest

M.Z.E., J.R.V. and T.V. are co-inventors on a patent application ZL913096-PCT/EP2014/068315-WO/2015/028576 ('Haplotyping and copy-number typing using polymorphic variant allelic frequencies'), licensed to Cartagenia (Agilent Technologies).

References

Baffero GM, Somigliana E, Crovetto F, Paffoni A, Persico N, Guerneri S, Lalatta F, Fogliani R, Fedele L. Confined placental mosaicism at chorionic villous sampling: risk factors and pregnancy outcome. *Prenat Diagn* 2012; **32**:1102–1108.

Besenfelder U, Havlicek V, Moesslacher G, Gilles M, Tesfaye D, Griese J, Hoelker M, Hyttel PM, Laurincik J, Brem G *et al.* Endoscopic recovery of early preimplantation bovine embryos: effect of hormonal stimulation, embryo kinetics and repeated collection. *Reprod Domest Anim* 2008;**43**: 566–572.

Bolton H, Graham SJ, Van der Aa N, Kumar P, Theunis K, Fernandez Gallardo E, Voet T, Zernicka-Goetz M. Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. *Nat Commun* 2016;**7**:11165.

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988;**332**:459–461.

Broekmans FJ, Verweij PJ, Eijkemans MJ, Mannaerts BM, Witjes H. Prognostic models for high and low ovarian responses in controlled ovarian stimulation using a GnRH antagonist protocol. *Hum Reprod* 2014;**29**:1688–1697.

Buckett WM, Chian RC, Dean NL, Sylvestre C, Holzer HE, Tan SL. Pregnancy loss in pregnancies conceived after in vitro oocyte maturation, conventional in vitro fertilization, and intracytoplasmic sperm injection. *Fertil Steril* 2008;**90**:546–550.

Catteeuw M, Wydooghe E, Mullaart E, Knijn HM, Van Soom A. In vitro production of bovine embryos derived from individual donors in the Corral(R) dish. *Acta Vet Scand* 2017;**59**:41.

Chavez SL, Loewke KE, Han J, Moussavi F, Colls P, Munne S, Behr B, Reijo Pera RA. Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. *Nat Commun* 2012;**3**:1251.

Chow JF, Yeung WS, Lau EY, Lee VC, Ng EH, Ho PC. Array comparative genomic hybridization analyses of all blastomeres of a cohort of embryos from young IVF patients revealed significant contribution of mitotic errors to embryo mosaicism at the cleavage stage. *Reprod Biol Endocrinol* 2014;**12**:105.

Coates JW, Schmutz SM, Rousseaux CG. A survey of malformed aborted bovine fetuses, stillbirths and nonviable neonates for abnormal karyotypes. *Can J Vet Res* 1988;**52**:258–263.

Coppola G, Alexander B, Di Berardino D, St John E, Basrur PK, King WA. Use of cross-species in-situ hybridization (ZOO-FISH) to assess chromosome abnormalities in day-6 in-vivo- or in-vitro-produced sheep embryos. *Chromosome Res* 2007;**15**:399–408.

Dal Canto MB, Mignini Renzini M, Brambillasca F, Cepparo H, Comi R, Villa A, Rangoni G, Mastrolilli M, Crippa M, de Ponti E *et al*. IVM–the first choice for IVF in Italy. *Reprod Biomed Online* 2006;**13**:159–165.

De Roover R, Bols PE, Genicot G, Hanzen C. Characterisation of low, medium and high responders following FSH stimulation prior to ultrasound-guided transvaginal oocyte retrieval in cows. *Theriogenology* 2005;**63**:1902–1913.

Destouni A, Zamani Esteki M, Catteeuw M, Tsuiko O, Dimitriadou E, Smits K, Kurg A, Salumets A, Van Soom A, Voet T *et al*. Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage-stage chimerism and mixoploidy. *Genome Res* 2016;**26**:567–578.

Diskin MG, Parr MH, Morris DG. Embryo death in cattle: an update. *Reprod Fertil Dev* 2012;**24**:244–251.

Durocher J, Morin N, Blondin P. Effect of hormonal stimulation on bovine follicular response and oocyte developmental competence in a commercial operation. *Theriogenology* 2006;**65**:102–115.

Dyer S, Chambers GM, de Mouzon J, Nygren KG, Zegers-Hochschild F, Mansour R, Ishihara O, Banker M, Adamson GD. International Committee for Monitoring Assisted Reproductive Technologies world report: Assisted Reproductive Technology 2008, 2009 and 2010. *Hum Reprod* 2016;**31**:1588–1609.

Edwards MJ, Park JP, Wurster-Hill DH, Graham JM Jr. Mixoploidy in humans: two surviving cases of diploid-tetraploid mixoploidy and comparison with diploid-triploid mixoploidy. *Am J Med Genet* 1994;**52**:324–330.

Ellenbogen A, Shavit T, Shalom-Paz E. IVM results are comparable and may have advantages over standard IVF. *Facts Views Vis Obgyn* 2014;**6**:77–80.

Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996;**65**:349–353.

Gremeau AS, Andreadis N, Fatum M, Craig J, Turner K, McVeigh E, Child T. In vitro maturation or in vitro fertilization for women with polycystic ovaries? A case-control study of 194 treatment cycles. *Fertil Steril* 2012;**98**:355–360.

Huang J, Yan L, Fan W, Zhao N, Zhang Y, Tang F, Xie XS, Qiao J. Validation of multiple annealing and looping-based amplification cycle sequencing for 24-chromosome aneuploidy screening of cleavage-stage embryos. *Fertil Steril* 2014;**102**:1685–1691.

Ibrahim ZH, Matson PL, Buck P, Atkinson AD, Lieberman BA. Hydatidiform mole following in-vitro fertilization. Case report. *Br J Obstet Gynaecol* 1989;**96**:1238–1240.

Jarvela IE, Salo MK, Santavuori P, Salonen RK. 46,XX/69,XXX diploid-triploid mixoploidy with hypothyroidism and precocious puberty. *J Med Genet* 1993;**30**:966–967.

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA. Circos: an information aesthetic for comparative genomics. *Genome Res* 2009;**19**:1639–1645.

Kwon HE, Park EJ, Kim SH, Chae HD, Won HS, Kim CH, Kang BM. A case of twin pregnancy with complete hydatidiform mole and coexisting fetus following IVF-ET. *J Assist Reprod Genet* 2002;**19**:144–148.

Lallemant C, Vassard D, Nyboe Andersen A, Schmidt L, Macklon N. Medical and social egg freezing: internet based survey of knowledge and attitudes among women in Denmark and the UK. *Acta Obstet Gynecol Scand* 2016;**95**:1402–1410.

Levy B, Sigurjonsson S, Pettersen B, Maisenbacher MK, Hall MP, Demko Z, Lathi RB, Tao R, Aggarwal V, Rabinowitz M. Genomic imbalance in products of conception: single-nucleotide polymorphism chromosomal microarray analysis. *Obstet Gynecol* 2014;**124**:202–209.

Li R, Albertini DF. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nat Rev Mol Cell Biol* 2013;**14**:141–152.

Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. *Hum Reprod Update* 2002;**8**:333–343.

Maheshwari A, Raja EA, Bhattacharya S. Obstetric and perinatal outcomes after either fresh or thawed frozen embryo transfer: an analysis of 112,432 singleton pregnancies recorded in the Human Fertilisation and Embryology Authority anonymized dataset. *Fertil Steril* 2016;**106**:1703–1708.

Meinecke B, Kuiper H, Drogemuller C, Leeb T, Meinecke-Tillmann S. A mola hydatidosa coexistent with a foetus in a bovine freemartin pregnancy. *Placenta* 2002;**24**:107–112.

Menasha J, Levy B, Hirschhorn K, Kardon NB. Incidence and spectrum of chromosome abnormalities in spontaneous abortions: new insights from a 12-year study. *Genet Med* 2005;**7**:251–263.

Menezo YJ, Herubel F. Mouse and bovine models for human IVF. *Reprod Biomed Online* 2002;**4**:170–175.

Obeidi N, Tchrakian N, Abu Saadeh F, Mocanu E. Suspected ovarian molar pregnancy after assisted reproductive technology conception: a diagnostic challenge. *BMJ Case Rep* 2015;**2015**:pii: bcr2015209353. doi: 10.1136/bcr-2015-209353.

Pfeifer S, Fritz M, Goldberg J, Adamson G, McClure R, Lobo R, Thomas M, Widra E, Licht M, Collins J *et al*. In vitro maturation: a committee opinion. *Fertil Steril* 2013;**99**:663–666.

Rambags BP, Krijtenburg PJ, Drie HF, Lazzari G, Galli C, Pearson PL, Colenbrander B, Stout TA. Numerical chromosomal abnormalities in equine embryos produced in vivo and in vitro. *Mol Reprod Dev* 2005;**72**:77–87.

Riegel M, Baumer A, Jamar M, Delbecque K, Herens C, Verloes A, Schinzel A. Submicroscopic terminal deletions and duplications in retarded patients with unclassified malformation syndromes. *Hum Genet* 2001;**109**:286–294.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev* 2002;**61**:234–248.

Robberecht C, Vanneste E, Pexsters A, D'Hooghe T, Voet T, Vermeesch JR. Somatic genomic variations in early human prenatal development. *Curr Genomics* 2010;**11**:397–401.

Rombauts L, Lambalk CB, Schultze-Mosgau A, van Kuijk J, Verweij P, Gates D, Gordon K, Griesinger G. Intercycle variability of the ovarian response in patients undergoing repeated stimulation with corifollitropin alfa in a gonadotropin-releasing hormone antagonist protocol. *Fertil Steril* 2015;**104**:884–890 e882.

Soderstrom-Anttila V, Makinen S, Tuuri T, Suikkari AM. Favourable pregnancy results with insemination of in vitro matured oocytes from unstimulated patients. *Hum Reprod* 2005;**20**:1534–1540.

Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet* 1978;**2**:366.

Sun CJ, Zhao YP, Yu S, Fan L, Wu QQ, Li GH, Zhang WY. Twin pregnancy and partial hydatidiform mole following in vitro fertilization and embryos transfer: a novel case of placental mosaicism. *Chin Med J (Engl)* 2012;**125**:4517–4519.

Sunkara SK, LaMarca A, Polyzos NP, Seed PT, Khalaf Y. Live birth and perinatal outcomes following stimulated and unstimulated IVF: analysis

of over two decades of a nationwide data. *Hum Reprod* 2016;**31**: 2261–2267.

Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, Debrock S, Amyere M, Vikkula M, Schuit F *et al*. Chromosome instability is common in human cleavage-stage embryos. *Nat Med* 2009a;**15**:577–583.

Vanneste E, Voet T, Melotte C, Debrock S, Sermon K, Staessen C, Liebaers I, Fryns JP, D'Hooghe T, Vermeesch JR. What next for preimplantation genetic screening? High mitotic chromosome instability rate provides the biological basis for the low success rate. *Hum Reprod* 2009b;**24**:2679–2682.

Vera-Rodriguez M, Chavez SL, Rubio C, Reijo Pera RA, Simon C. Prediction model for aneuploidy in early human embryo development revealed by single-cell analysis. *Nat Commun* 2015;**6**:7601.

Viuff D, Hendriksen PJ, Vos PL, Dieleman SJ, Bibby BM, Greve T, Hyttel P, Thomsen PD. Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. *Biol Reprod* 2001;**65**:204–208.

Voet T, Vanneste E, Vermeesch JR. The human cleavage stage embryo is a cradle of chromosomal rearrangements. *Cytogenet Genome Res* 2011; **133**:160–168.

Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2016;**22**:2–22.

Walls M, Junk S, Ryan JP, Hart R. IVF versus ICSI for the fertilization of in-vitro matured human oocytes. *Reprod Biomed Online* 2012;**25**:603–607.

Wilkins-Haug L, Quade B, Morton CC. Confined placental mosaicism as a risk factor among newborns with fetal growth restriction. *Prenat Diagn* 2006;**26**:428–432.

Xia P. Biology of polyspermy in IVF and its clinical indication. *Curr Obstet Gynecol Rep* 2013;**2**:226–231.

Yamazawa K, Nakabayashi K, Kagami M, Sato T, Saitoh S, Horikawa R, Hizuka N, Ogata T. Parthenogenetic chimaerism/mosaicism with a Silver-Russell syndrome-like phenotype. *J Med Genet* 2010;**47**:782–785.

Zamani Esteki M, Dimitriadou E, Mateiu L, Melotte C, Van der Aa N, Kumar P, Das R, Theunis K, Cheng J, Legius E *et al*. Concurrent whole-genome haplotyping and copy-number profiling of single cells. *Am J Hum Genet* 2015;**96**:894–912.

Ziebe S, Bangsboll S, Schmidt KL, Loft A, Lindhard A, Nyboe Andersen A. Embryo quality in natural versus stimulated IVF cycles. *Hum Reprod* 2004;**19**:1457–1460.