Different pre-implantation phenotypes of bovine blastocysts produced *in vitro*

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Abstract

Embryo transfer in cattle is performed with blastocysts produced *in vivo* or *in vitro* using defined media. However, outdated systems such as those that use serum and co-culture remain of interest for research purposes. Here, we investigated the effect of additional culture time on *in vitro*-produced embryos. Specifically, we compared embryos that formed a blastocoel at different times after fertilisation to those that stayed in culture for up to two additional days with respect to their development *in vivo* after temporary transfer to oestrus-synchronised recipients. A pre-transfer set (D6, D6+1, D6+2, D7, D7+1, D8) was examined using microarray analyses and correlated with a post-transfer set that included two different days of transfer (D6-T6, D6+2-T8, D7+1-T8, D8-T8). All surviving conceptuses reached primitive-streak stages and filamentous sizes similarly to *in vivo* (D18) or *in vitro* controls (D7/T7). The recovery rate differed between D6 and D8 embryos that were immediately transferred (58 vs 25%). With an intermediate survival rate (33%), the D6 embryos with two additional days in culture produced nine times more IFN-tau (IFNT) at D18 than the D6 embryos that were immediately transferred. At the end of culture, D6 and D6+2 embryos displayed the highest number of gene expression differences. Despite a mortality of 40–60%, no signature was detectable in any of the transferred groups that would account for the embryos' fates. Initially reputed to be beneficial in producing more blastocysts, our culture system of B2 medium plus serum and co-culture generated blastocysts that were distinct from those developed *in vivo* (D7).

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Introduction

Embryo transfer (ET) is used worldwide in cattle as a basis for genomic selection to improve animal productivity (Hasler 2014, Stroebech *et al.* 2015). The embryos used in this process can be produced either *in vivo* or *in vitro*. Although *in vitro* culture conditions have been optimised over the years, they do not perfectly mimic the environment of the oviduct. Embryos must therefore compensate for this as much as they are able to, activating survival processes such as the oxidative stress response and changes in cellular metabolism (for example, Thompson *et al.* 2007, Bertoldo *et al.* 2015). Furthermore, once transferred to the uterus of synchronised recipients, not all embryos reach term and live birth. Indeed, 40% are lost between days 8 and 17 (Mamo *et al.* 2011) so that about 60% survive until D17 (Farin *et al.* 2001), a time at which pregnancy is recognised in the mother through the secretion of IFN-tau (IFNT; Lonergan *et al.* 2016, Bazer *et al.* 2018), a

cytokine whose expression is temporally and spatially regulated (Ealy & Yang 2009, Sakurai *et al.* 2013).

Increasing the survival rate has thus been a major goal of the scientific community. This has led to the optimisation of many aspects of ET protocols, including recipient management, time of transfer or the number of embryos per transfer (Jones & Lamb 2008, Berg *et al.* 2010, Randi *et al.* 2016). While one embryo per recipient is the gold standard, research efforts sometimes involve the transfer of multiple embryos in order to evaluate the quality of *in vitro*-produced embryos. The features of these embryos are then compared with developmental landmarks for blastocyst elongation and gastrulation known from *in vivo* blastocyst differentiation (Maddox-Hyttel *et al.* 2003, Hue 2016). Generally speaking, multiple ETs led to either (i) lower retrieval rates and reduced average conceptus length in some but not all cases (Berg *et al.* 2010), number of embryos transferred per recipient: $n=3-30$; but see (Clemente *et al.* 2009,

Machado *et al.* 2013) or (ii) the development of smaller or no embryonic discs or deficient gastrulation or neurulation patterns (Fischer-Brown *et al.* 2004, Alexopoulos *et al.* 2008, Machado *et al.* 2013, van Leeuwen *et al.* 2015).

Another feature of *in vitro* development that has been the target of optimisation studies is the culture system used. Many different media and culture systems have been proposed, with one of the first promising ones utilising co-culture with feeder cells (African green monkey kidney (VERO) cells, for instance: Desai & Goldfarb 1998) on B2 medium supplemented with serum. Initially, it was found that co-culture with VERO cells increased the number of expanding and hatching human blastocysts with respect to single-culture conditions (Menezo *et al.* 1990). The same procedure was likewise found to produce more high-viability bovine blastocysts, leading to a pregnancy rate at D90 of 58% (Menck *et al.* 1997). The feeder principle was concomitantly confirmed using other cells, such as buffalo rat liver or bovine oviduct epithelial cells (BRL, BOEC, Farin *et al.* 1997, Hasler 2000) on other serum-containing culture media (such as M-199, Catt 1994). Co-cultures *ab initio* appeared to support earlier formation of blastocysts and in higher numbers (Farin *et al.* 1997, Hasler 2000). A decade later it became clear, though, that *in vitro* culture induced gene expression changes in embryos and that high concentrations of human serum (20%) in culture media generated developmental consequences that remained long after ETs were carried out (Sinclair *et al.* 1999, Rief *et al.* 2002). For this reason, *in vitro*-produced (IVP) embryos began to be commercially produced worldwide using semi-defined or fully defined media with no added serum or bovine serum albumin (BSA; Farin *et al.* 2001, Murillo-Rios *et al.* 2017).

Numerous studies have investigated the molecular changes at work in different IVP embryos (bovine, mouse, rabbit) in order to evaluate the influence of different parameters of culture systems on subsequent *in vitro* development (Gutierrez-Adan *et al.* 2015, Salvaing *et al.* 2016, Thompson *et al.* 2016, Duranthon & Chavatte-Palmer 2018). This work has led to the recognition of signatures for certain *in vitro* culture conditions (Cagnone & Sirard 2016, Feuer *et al.* 2017), but these have only rarely been compared to those of *in vivo*-developing embryos that are able to reach term (Ghanem *et al.* 2011, Gad *et al.* 2012a). Furthermore, a few studies have recently re-evaluated the use and impact of serum (Heras *et al.* 2016, Murillo *et al.* 2017) or feeder cells (BOEC; Schmaltz-Panneau *et al.* 2015) on embryos, while others have evaluated new options such as the use of oviductal and/or uterine fluids (Hamdi *et al.* 2018). For example, Cordova *et al.* (2014) obtained more blastocysts at D6 or D7 when BOECs were added to synthetic oviduct fluid (SOF) for 4 of the 8 days of culture. To the best of our knowledge, only one study to date has compared BOEC or VERO cells added to SOF with

respect to their impact on the embryonic transcriptome (Vitorino Carvalho *et al.* 2017). However, no study has yet examined the molecular effects of co-culture in serum-containing B2 medium on the whole embryonic transcriptome. To investigate this, in this study, we used VERO cells in serum-containing B2 medium during the whole culture period to sort blastocysts that formed at D6, D7 or D8.

Since ET is classically performed at D7 or D8 using unsorted blastocysts, we also tested the effect of extending the culture time of the D6- or D7-forming embryos by 2 or 1 days, respectively, and transferring them at D8. Overall, we evaluated (i) molecular differences between these groups at the end of culture and (ii) the developmental features of a subset of embryos 10–12 days after temporary transfer *in vivo*. Using this system, we aimed to (i) tease apart the differences among embryos, which may be caused by their developmental potential or ability to compensate for a suboptimal culture environment and (ii) evaluate whether some of these compensatory/adaptive pathways would resemble those reported for the embryos of wild species that pause their development, in a reversible manner, in response to unfavourable conditions (diapause; Fenelon *et al.* 2014).

Materials and methods

All experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

Definition of embryo groups to be compared

We wanted to evaluate whether embryos that formed a blastocyst cavity at different times after fertilisation would develop equally well after *in vivo* transfer if cultured for up to 2 more days. To do this, we first randomly assigned presumptive zygotes to microdrops of B2 medium that contained foetal calf serum (FCS, 5%) and were co-cultured with VERO cells, under oil. Blastocysts forming at D6, D7 or D8 were sorted, scored on the basis of previous reports (Mori *et al.* 2002, Hoelker *et al.* 2006) and assigned to new drops at each evaluation. For the post-transfer analysis, we included only those groups transferred at the earliest (D6) or latest (D8) days of transfer; D7 and D6+1 blastocysts were not transferred into oestrus-synchronised recipients at D7. As shown in Fig. 1, we therefore created the following four experimental groups: (i) blastocysts that formed early (at D6) and were transferred at D6 (experimental group D6-T6); (ii) blastocysts that formed early (D6) and were maintained in culture for two additional days before being transferred at D8 (experimental group D6+2-T8); (iii) blastocysts that formed at D7 and were maintained one extra day in culture before being transferred at D8 (experimental group D7+1-T8) and (iv) blastocysts that formed at D8 and were transferred at D8 (experimental group D8-T8). Blastocysts were recorded as expanded (i.e. those formed at D6 or D8), as intermediate or close to hatching

Figure 1 Experimental design. After *in vitro* fertilisation, bovine zygotes were cultured in batches of 25 embryos in 50 μ L drops, at 39°C with 5% O₂, 5% CO₂ and 90% N₂, until blastocoels formed at D6, D7 or D8 (D6 to D8, in black). For the *in vitro*-extended group, blastocysts that formed at D6 or D7 were maintained in culture for one or two additional days, up to a total culture time of eight days (D7+1, D6+1, D6+2, in blue). A subset of newly formed (D6, D8) or *in vitro*-extended (D6+2; D7+1) blastocysts (grade 1–2) were transferred to oestrus-synchronised recipient heifers at D6 (T6) or D8 (T8) to evolve *in utero* up to D18 (D6-T6, D6+2-T8, D7+1-T8, D8-T8). Thin black line: *in vitro* development; thick dashed blue line: additional day in culture after blastocyst formation (+1 or +2); thick grey line: *in utero* development; grey arrows: time of *in utero* transfer; small (black and blue) points: time of blastocyst sampling for molecular profiling; large grey point: time of conceptus flushing for developmental evaluation and quantification of IFN- τ production.

(D6+1, D7), or as hatched (D7+1, D6+2). These experimental groups were compared with a control dataset obtained from *in vivo* D7 blastocysts, which had been previously analysed in another study conducted by the EmbryoGENE network (Gad *et al.* 2012a). Additional control data were obtained from embryos that experienced full *in vivo* development up to D18 as well as classical D7-T7 embryos, both of which also originated from another study which used the identical IVP system (culture in B2 medium plus FCS with VERO cells: (Degrelle *et al.* 2012)). In sum, six pre-transfer and four post-transfer experimental groups were evaluated: the pre-transfer set (D6, D6+1, D6+2, D7, D7+1, D8) was examined using microarray profiles (see below), while the post-transfer set (D6-T6, D6+2-T8, D7+1-T8, D8-T8; Fig. 1) was evaluated with respect to differences in conceptus recovery rate, morphology, Brachyury (*TBX7*) mRNA expression and IFNT Western blots.

In vitro-produced blastocysts

Briefly, cow ovaries were collected at a local slaughterhouse and transported within 3 h to the laboratory in phosphate-buffered saline (PBS, BR0014G, Oxoid Limited, Basingstoke, UK) at 30°C. Immature oocytes were aspirated from antral follicles (2–7 mm in diameter), and we selected cumulus-oocyte complexes (COCs) with more than three compact layers of cumulus cells. These were pooled and rinsed in fresh medium before maturation. The COCs were cultured for 24 h

at 39°C under a humidified atmosphere of 5% CO₂ in air. At the end of the maturation period, the oocytes were fertilised *in vitro* in TALP medium supplemented with 1 μ g heparin/mL (H3149, Sigma-Aldrich) and PHE (Penicillamine–Hypotaurine–Epinephrine). Frozen semen from a single bull was used in all the *in vitro* experiments; this was the same semen that Degrelle *et al.* (2012) used to produce their *in vivo* D18 and D7-T7 controls (but different from that used for the *in vivo* D7 by Gad *et al.* (2012a)). Sperm was added at a concentration of 10⁶ spermatozoa/mL and the day of *in vitro* insemination was denoted D0. Eighteen hours after insemination, COCs were removed from the fertilisation medium; presumptive zygotes were denuded of cumulus cells by vortexing and randomly assigned to microdrops. *In vitro* development up to the blastocyst stage was carried out using the co-culture technique with VERO cells as reported in Menck *et al.* (1997). Blastocyst formation was evaluated successively at days 6, 7 and 8 post-insemination but total, ICM or TE cell numbers were not determined. At each evaluation, newly formed blastocysts were isolated and scored. Some blastocysts that formed on D6 or D7 were selected to remain in culture for an additional 1 or 2 days. In general, the phenotypes of D6 and D8 blastocysts were recorded as expanded (diameter of 165–180 μ m, ZP-enclosed), the intermediate groups (D6+1, D7) were close to hatching (diameter of 200–220 μ m, breaking ZP) and the two groups that spent the longest time in culture (D7+1, D6+2) were hatched (diameter of 230–250 μ m, re-expanded after hatching). Degeneration of the zona pellucida was observed in about 1% of most groups, in 5% of the most-extended *in vitro* group (D6+2) and in 10% of the late-forming blastocysts (D8). Only high-grade embryos (Q1, or 80% Q1–20% Q2) were transferred, and transfer was performed within the 2 h following the end of IVP. Embryos destined for molecular profiling were washed twice in sterile PBS, and then frozen and stored at –80°C for subsequent RNA extraction. All groups of blastocysts were collected from multiple IVP replicates and pooled ($n=15$ –20 per sample for array hybridisation; three replicates per group; $n=25$ –45 per sample for RT-qPCR; three replicates per group).

Temporary transfer of in vitro-produced blastocysts

Beef-breed heifers were treated to induce oestrus synchronisation (Crestar; Intervet, France). Each heifer received six blastocysts that were non-surgically transferred at D6 or D8. Two recipient heifers were used per blastocyst group (D6, D6+2, D7+1 and D8) but not exchanged between groups, so that a different heifer was used for each transfer. Ten to twelve days after transfer, conceptuses were non-surgically collected (details in Richard *et al.* 2015). After flushing, each recovered conceptus was carefully rinsed in fresh PBS, the embryonic disc was dissected and fixed in 4% paraformaldehyde, and the extra-embryonic tissues were snap-frozen and stored at –80°C prior to Western blotting. As a control, we used data from previously examined embryos that had been prepared using classical ET parameters (usual unsorted D7 embryos transferred at D7) that came from an earlier study of the same IVP system (culture in groups on VERO cells, in B2 medium plus FCS; Degrelle *et al.* 2012).

RNA isolation, amplification and microarray hybridisation

The microarray procedure was performed as described in Hosseini *et al.* (2015). Total RNA (pool of 15–20 embryos per group, in triplicate) was extracted and purified using the Arcturus PicoPure RNA Isolation Kit (Thermo Fisher Scientific), including a DNase digestion (Qiagen) on the column. The quality and concentration of the extracted RNA were analysed with a bioanalyser (Agilent Technologies). All extracted samples were of high quality, with an RNA integrity number above 8.5.

For microarray purposes, purified RNA was amplified using T7-based *in vitro* transcription with the RiboAmp HSPlus RNA Amplification Kit (Thermo Fisher Scientific), and labelled with Cy3 using the ULS Fluorescent Labeling Kit (Kreatech, Amsterdam, Netherlands). The labelled anti-sense RNA (aRNA) was quantified using an ND-1000 Nanodrop apparatus (Thermo Fisher Scientific). Labelled aRNAs (825 ng per replicate) were hybridised on Agilent-manufactured EmbryoGENE slides (GPL13226, Robert *et al.* 2011) in a one-colour design using the Gene Expression Hybridisation Kit. After 17 h of hybridisation at 65°C, microarray slides were washed for 3 min in Gene Expression Wash Buffer 1 (room temperature), 3 min in Gene Expression Wash Buffer 2 (42°C), 10 s in 100% acetonitrile (room temperature) and 30 s in Stabilisation and Drying Solution (Agilent) at room temperature. Slides were scanned with a PowerScanner (Tecan, Männedorf, Switzerland) and feature extraction was performed with Array-pro 6.3 (Media Cybernetics, Rockville, MD, USA). The expression dataset (or GSE) is available on the GEO database (GSE96925).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from batches of embryos (pools of 25–45 embryos per group, in triplicate) using the Arcturus PicoPure RNA extraction kit (Thermo Fisher Scientific). Prior to extraction, carrier RNA (2.5 µg of 16S–28S carrier per sample; Roche Diagnostics) was added to restrict RNA loss during column purification and to estimate RNA recovery after purification. A purification procedure was performed, using DNase I (Qiagen) treatment at 25°C for 15 min, prior to elution. cDNAs were synthesised from total isolated RNA using the Superscript III enzyme (Invitrogen) and hexamer random primers (Roche Diagnostics) in 20 µL final volume, following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out with SYBR Green Master Mix (Applied Biosystems) and the Step One Plus system (Applied Biosystems). Three technical replicates were performed for each sample and a standard curve protocol was used to evaluate gene expression. Reactions were performed using the equivalent of 0.01 and 0.5 embryos of cDNA per well in 96-well plates, depending upon the gene of interest. Primer sequences are provided in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article). To assess the amplification of the correct cDNA fragments, each amplicon was sequenced and BLASTed against the NCBI RNA bovine database (RefSeq Release 70). For each gene of interest, relative expression was normalised to the expression of three housekeeping genes (*GAPDH*, *YWHAZ* and *SDHA*)

using qBasePLUS 2 software (Biogazelle, Gent, Belgium; Peynot *et al.* 2015).

Western blotting

Total proteins of D18 extra-embryonic tissues were prepared using RIPA buffer as previously described (Hue *et al.* 2015). Protein samples (30 µg) were resolved by SDS-PAGE and immunoblotted with antibodies to IFN-tau (kindly provided by M. Guillomot, INRA, UMR BDR, France; diluted 500-fold), β-ACTIN (0.4 µg/mL, Santa-Cruz), CDX2 (0.5 µg/mL, H43, Santa-Cruz), ETS2 (0.5 µg/mL, C20, Santa-Cruz) and AP-1/c-JUN (0.5 µg/mL, sc-45X, Santa-Cruz). After incubation with the appropriate Alexa Fluor-conjugated secondary antibody (680 or 790 conjugate, Molecular Probes), blots were revealed using the Odyssey infrared fluorescent system (Li-Cor), and the amount of each protein was determined. Signal intensity was quantified using ImageJ software (NIH). The arbitrary pixel densities of each protein were standardised to β-ACTIN levels and normalised to the *in vivo* control. Measurements were made in triplicate ($n=3$). The data are expressed as the mean ± s.d. Statistical analysis (*t*-test) was performed using GraphPad Prism 6 software. Results were considered significant if the *P* value was <0.05 (*), <0.01 (**), or <0.001 (***).

Microarray analysis

To provide context for the data generated in this study from the IVP groups ($n=6$), our analysis included a molecular dataset from an *in vivo* control. The microarray data from Gad *et al.* (2012a) were obtained by the same person as in the current study, using the same slides (Robert *et al.* 2011), within the EmbryoGENE framework (bovine microarray platform; GPL13226). Only the probes ($n=43,795$) that were common to the seven groups (D6, D6+1, D7, D6+2, D7+1, D8, *in vivo* D7) and 21 samples (three biological replicates per group) were analysed. Statistical analyses of microarray data were performed with BioConductor version 3.2 (Gentleman *et al.* 2004) and R version 3.2.4 (R Core Team 2016). A comprehensive examination of expression patterns was performed by principal components analysis (PCA) and between-class analysis (BCA), using the R package ade4 (Dray & Dufour 2007). A between-class principal components analysis focuses on between-group variability by performing a PCA on group means. The importance of the difference between groups is assessed using the ratio of the between-group inertia to the total inertia. The statistical significance of differences between groups is then checked with a Monte-Carlo permutation test. To identify probes that were differentially expressed, the data were analysed using the package Limma (Smyth 2005), based on a linear mixed-model approach. For each Limma comparison, *t*-tests were performed. A Benjamini–Hochberg correction for multiple testing (Benjamini 1995) was applied and an adjusted *P* value cut-off at 0.05 was applied.

Gene Set Enrichment Analysis

The whole expression dataset was analysed using the Gene Set Enrichment Analysis (GSEA) database (hallmark v6.1;

Broad Institute; Subramanian *et al.* 2005) as well as with 17 additional gene sets. Two were downloaded from the Amigo web site (bovine metabolic sensors and stress sensors) while the remaining 15 were collected from published supplementary data; these included relevant gene sets such as those devoted to bovine cell lineage descriptors (from whole tissues (inner cell mass and trophectoderm) *in vivo* and *in vitro* (Ozawa *et al.* 2012, Nagatomo *et al.* 2013, Hosseini *et al.* 2015, Zhao *et al.* 2016) or single cells (epiblast, trophectoderm and hypoblast; Negrón-Pérez *et al.* 2017, Wei *et al.* 2017), bovine markers of embryonic quality (Cagnone & Sirard 2016) or of *in vitro* culture with FCS (at 5%; Heras *et al.* 2016)), expression modules of mouse embryonic lineages in diapause or WT (E2.5 to E5.5; Boroviak *et al.* 2015) and conserved FOXO target genes (Webb *et al.* 2016). The parameters we used were FDR <5%, 1000 permutations (on gene sets instead of phenotypes), use of the median value of probes (instead of the maximum) and gene set with a minimum size of 30, instead of the default size of 15. In doing so, 20,393 (out of 27,607) expressed IDs were considered and processed.

Ingenuity pathway analysis

To identify biological functions as well as the putative upstream regulators affected in the different groups of blastocysts, differentially expressed (DE) probes with an adjusted *P* value <0.05 were selected. The significant DE probes with an annotated gene symbol were then analysed with ingenuity pathway analysis (IPA; build version 470319M, content version 43605602; Ingenuity Systems, Mountain View, CA; USA).

Whole-mount in situ hybridisation

Isolated embryonic discs that were dissected out from D18 conceptuses (*n*=17) were fixed in 4% paraformaldehyde, stored and hybridised with a DIG-labelled *Brachyury* riboprobe as described by Hue *et al.* (2013). The hybridised

embryos were mounted in Mowiol and photographed with a Coolsnap camera (Photometrics).

Results

Patterns of commonly enriched genes and developmental functions confirmed the three main embryo phenotypes: fully formed blastocysts, additional day in vitro and equal culture period

Once profiled on the EmbryoGENE platform, we searched for enriched features that could identify common signatures across IVP groups using GSEA. Using the hallmark database from the Broad Institute (v6.1) and a homemade one that included 17 relevant datasets (detailed in the 'Materials and methods' section), we identified 17 molecular features that characterised the six *in vitro*-produced embryonic pools (Supplementary Tables 2, 3, 4 and 5).

At first glance, the enrichment of a core of eight shared functions (in bold, Supplementary Tables 2, 3, 4 and 5) supported the existence of a universal '*in vitro*' phenotype (apoptosis, cholesterol homeostasis, embryonic quality, mTORC1 and P53 signalling, Myc targets, Oxphos, whole TE). However, at the gene level, patterns of enrichment (Fig. 2 and Supplementary Table 6) provided evidence for three additional phenotypes: (i) fully formed blastocysts (D6, D7, D8; 34 genes and 3 functions in common: mTORC1, Oxphos, TE), (ii) same total culture period of 8 days (D6+2, D7+1, D8; 82 genes and 4 functions in common: Glycolysis, Oxphos, Ros, TE) and (iii) one extra day in culture for blastocyst formation at D6 or D7 (D6+1, D7+1; 87 genes and four functions in common: Apoptosis, mTORC1, P53, TE).

With respect to the expression of bovine metabolic sensors, all *in vitro* groups segregated to the upper expression limit set by the *in vivo* control; one-third

D6 vs D7

D6+2 vs D8

D6+2 vs D7+1

D6 vs D8

D7+1 vs D7

Figure 2 Common gene enrichment sets across IVP transcriptomes. GSEA identified enriched genes and functions for every paired comparison within the experimental design (9 pairs; Supplementary Tables 2, 3, 4 and 5). A search for shared genes and functions across these comparisons identified three clusters of 2–3 pairs: (D6 vs D7; D7 vs D8; D6 vs D8), (D6+2 vs D8; D6+2 vs D7+1) and (D6+1 vs D6; D7+1 vs D7). Each cluster was characterised by 2–3 shared functions, but these did not necessarily involve the identical genes in each group. The enriched functions in each subset also tended to be predominantly found in the trophectoderm cells (TE) of the embryos, though, again, not with exactly the same genes (Supplementary Table 7 and the corresponding gene lists will be provided on request). In brackets are the numbers of unique gene IDs.

of these probes represented both metabolic and stress sensors (Supplementary Table 7). This feature – absent from intra-IVP analyses, as all IVP groups expressed these genes faintly compared to the *in vivo* group – underlined a unique difference in micro-environment sensing between *in vitro*- and *in vivo*-produced blastocysts.

Molecular differences among groups with respect to early vs late blastocyst formation and equal time in culture vs without extra time in culture

Using a few shared functions (8 out of the 17 initially identified) and small sets of commonly enriched genes (100, at most), we examined the differences between the groups of embryos (IVP: $n=6$; *in vivo*, $n=1$). Using a PCA of all samples (seven groups, three replicates per group; Supplementary Fig. 1), we were able to distinguish the *in vivo* embryos (D7, Gad et al. 2012a) from all the IVPs (D6, D6+1, D7, D6+2, D7+1, D8); the D8 embryos from the D6+2 (Supplementary Fig. 1A) or D7+1 groups (Supplementary Fig. 1C) and the D6 embryos from the D7+1 (Supplementary Fig. 1B) or D6+2 groups (Supplementary Fig. 1C). However, the degree of variability among replicates within the D7, D6+1 or D7+1 groups (in the PCA) prompted us to also perform a BCA which averaged expression values for each embryo group (seven groups, Fig. 3). There too, three main axes distinguished *in vivo*- from *in vitro*-produced embryos, D6 from D6+2 (Fig. 3A and C) and D8 from D6 (Fig. 3B) or D7+1 embryos (Fig. 3C). The genes whose expression significantly contributed to these axes are represented in the violin plots in Fig. 3D by high gene scores (red tails).

These analyses thus revealed that (i) D8 and D6 blastocysts differed at a molecular level even though blastocysts in both groups were recorded as newly formed and expanded and (ii) despite being cultured for the same length of time, embryos in groups D8, D7+1 and D6+2 also demonstrated recognisable differences. This latter finding was less surprising, though, in view of the differences in developmental status between these groups, i.e. expanded (D8) vs hatched (D7+1, D6+2). In this context, the gene enrichment patterns (Fig. 2) that were shared among the extended-culture groups (D6+1–D7+1: Fig. 3A, B and C; D6+2–D6+1: Fig. 3B and C) supported the hypothesis that an extra day in culture generated similar adaptations in both D6 and D7 blastocysts.

Differential gene expression highlighted a major distinction between *in vivo*- and *in vitro*-produced embryos

As was observed with GSEA and BCA, patterns of gene expression in *in vitro*-produced embryos were different from those that developed *in vivo*. Specifically, more than 15,000 probes (corresponding to 6746 genes) were DE, equally distributed between

Figure 3 Molecular differences between *in vitro* and *in vivo* transcriptomes. Between-class analysis (BCA) on all groups of blastocysts, experimental and control (*in vitro*, $n=6$; *in vivo*, $n=1$). In A to C, projections based on expression values of a subset of probes that were shared among all groups, averaged on a per-group basis, along three main axes (1–3). In red, green and pink: the D6, D7 and D8 blastocysts. In yellow, blue and light green: the D6+1, D7+1 and D6+2 embryo groups. In D, the violin plots illustrate the gene scores along a normal distribution (in blue) or along each of the axes of the BCA (in red). Long distribution tails are indicative of high gene scores (or high contributions to the axis).

over- and under-expression (Table 1). In addition, 60–70% of the expression differences among IVPs were part of the ‘*vivo/vitro*’ contrast, so that in two of the intra-IVP contrasts (D6 vs D8 and D8 vs D6+2) the contrast-specific differences were quite small (reduced to 53 and 23 genes, respectively). However, the comparison between D6 and D6+2 revealed a much higher number of differentially expressed genes (DEGs; $n=237$; 4 and 10 times more than D8 vs D6 and D8 vs D6+2, respectively; Supplementary Table 8). D6+1, D7 and D7+1 were included in the analysis and in the ‘all IVP’ group, but are not presented in Table 1 due to the fact that the paired contrasts involving these groups yielded limited numbers of contrast-specific DEGs (<15), with most being shared with the *vivo/vitro* contrast and the other contrasts presented here.

By performing an IPA, we found that these DEGs (Table 2) played a part in metabolic, developmental and cellular functions that were shared among either *in vitro*-produced (lipid metabolism, cell morphology) or *in vivo*-developed blastocysts (amino acid metabolism, cellular movement, gene expression). DEGs that were putative upstream regulators encoded 22 transcriptional regulators (*CDX2*, *CDKN2A*, *CITED2*, *GATA4*, *HDAC6*, *HSF1*, *ID2*, *ID3*, *MECP2*, *MYC*, *MYCN*, *NANOG*, *NCOA1*, *PDX1*, *PML*, *POU5F1*, *RB1*, *STAT3*, *TFAP2A*, *TP53*, *WT1*, *YY1*), four ligand-dependent nuclear

Table 1 Differential gene expression between *in vitro*-produced blastocysts that formed at D6 or D8 or were isolated at D6 and cultured two more days (D6+2).

	Contrasts			
	D6+2 (vs D6)	D8 (vs D6)	D8 (vs D6+2)	D7 <i>in vivo</i> vs all IVP*
Adjusted <i>P</i> value <0.05	6.2–6<0: 322 6.2–6>0: 864	8–6<0: 92 8–6>0: 234	8–6.2<0: 82 8–6.2>0: 56	<i>in vivo</i> -IVP<0: 7102 <i>in vivo</i> -IVP>0: 8691
DE probes	1186	326	138	15,793
Annotated DE probes	778	196	64	8001
Unique DE IDs (or DEGs)	702	188	64	6746
(<i>in vivo</i> -all IVP) shared genes	465 (66%)	135 (72%)	41 (64%)	524
Contrast-specific DE genes	237 (34%)	53 (28%)	23 (34%)	6222
Shared DEGs among IVPs	46	40	19	

*Transcriptomes were obtained from six experimental groups of bovine blastocysts, but three (D7, D6+1 and D7+1) do not appear here in any paired contrast due to the limited number of differentially expressed genes that characterised them. However, all six groups (D6, D6+1, D7, D7+1, D8, D6+2) were combined to form the 'all IVP' group which was compared to *in vivo* D7 development. The *in vivo* controls which originated from a previous study that used the same array platform within the EmbryoGENE network (Gad et al. 2012a). The numbers of differentially expressed probes/genes are indicated if they were significant at an adjusted *P* value of <0.05.

receptors (*ESR1*, *NR1H2*, *NR1H3*, *NR1H4*) and one enzyme (*DNMT3B*). Only a few DEGs were initially identified through gene set enrichment (166 in D6+2 vs D6, 45 in D8 vs D6, 6 in D8 vs D6+2), among which were four putative regulators identified within the first contrast (*ID2*, *MYC*, *STA3*, *TFAP2A*, D6+2 vs D6).

Across all group contrasts, eight genes were selected for further validation with RT-qPCR, using three independent biological replicates per condition (D6, D6+2, D8). The genes were expressed across different cell lineages (*AKR1A1*, *DNMT3A*, *FADS3*, *PTGS2* in TE; *APOA1*, *DNMT3A*, *STAT3* in PrE; *NANOG* in ICM), two were identified as regulators (*NANOG* in D6+2 and D8, *STAT3* in D6; Table 2) and one appeared in only one contrast (*PTGS2* in D8 vs D6). The results for the genes that had demonstrated differential expression (log ratios) below 1.3 (0.6, 0.9, and 1.2) were not corroborated with RT-qPCR. However, *APOA1*, *NANOG* and *PTGS2* (expression log ratios of 1.7–2.5) were indeed found to be more expressed in D6+2 than in D6 or D8 embryos (Fig. 4). Here, the RT-qPCR analysis did not validate the changes in expression found for *AKR1A1*, *FADS3*, *PTGS2* and *STAT3*, but a meta-analysis including 20 studies within the EmbryoGENE network also reported that the expression levels of these genes were affected by culture and/or culture conditions, as were those of the 18 DEGs identified here as putative regulators (Gad et al. 2012a, Plourde et al. 2012b, Khan et al. 2016; Fig. 5).

ET revealed two phenotypes: embryos that survived to D18 and a subset that produced IFN-tau at levels similar to controls

Before transfer, the IVP embryos from the D6, D6+2, D7+1 or D8 groups were similar in some ways but distinct in others (see results above of GSEA, BCA and DEG analysis). Moreover, an analysis of negative pregnancy predictors in these groups was inconclusive; these predictors had been previously established using

biopsied *in vitro*-produced embryos and are associated with the inability to reach term (no calf, no pregnancy, embryonic resorption: El-Sayed et al. 2006, Ghanem et al. 2011). Specifically, D6 and D6+2 blastocysts expressed similar numbers of negative pregnancy predictors (five vs six DEGs), while D8 blastocysts did not express any of those genes as DEGs, and the D7+1 group only expressed two (Table 2, D7+1 not shown). Four groups of IVP embryos were then transferred to recipient heifers at either D6 (D6 group) or D8 (D6+2, D7+1, D8) and recovered at D18; each recipient was used for only one group of embryos. Although this aspect of the study design had drawbacks (specifically, we were unable to evaluate interactions between different embryos and recipient heifers), we still believed that the intrinsic embryo viability would be at least partly mirrored by the post-transfer phenotypes (Fig. 6). The uterine fluids, which serve as *in vivo* modulators of embryonic viability, were not evaluated in the current work (Fig. 1). All conceptuses were elongated (17/17; Fig. 6A) and most displayed a gastrulation landmark (such as a primitive streak, 13/17). The expression of a mesoderm marker gene (*TBXT* mRNA) was detected at early-, mid-, or late-primitive streak stages (HH3–HH5, 13/17; Fig. 6B) and even in the youngest stage (HH2; 4/17), which exhibited a posterior pole containing a small *TBXT* domain but no visible streak (not shown). The most frequent stage observed in the IVP embryos at D18 was stage 3 (or mid-streak: 10/17) regardless of group designation (D6-T6, D6+2-T8, D7+1-T8, D8-T8, IVP control (D7-T7)); this was slightly delayed in comparison to embryos that developed entirely *in vivo*, which by D18 had mostly reached stage 4 (7/10). All conceptuses produced IFNT protein, either at an equal level to (D6 and D7+1) or at higher levels (4–9 times more in the D8 and D6+2 embryos, respectively; Fig. 6C) than control embryos that were developed entirely *in vivo* to D18. However, *CDX2*, *ETS2* and *JUN* proteins were undetectable at D18. In comparison, *IFNT* mRNA levels in the pre-transfer embryos were 2.4 and 3.6 times higher in D8

Table 2 Functions and regulators associated with the differential expression among *in vitro*-produced blastocysts that formed at D6 or D8, or that were isolated at D6 and cultured two more days (D6+2).

	Comparisons			
	D6+2 (vs D6)	D8 (vs D6)	D8 (vs D6+2)	D7 <i>vivo</i> (vs all IVP : 6, 6+1, 7, 7+1, 8, 6+2)
Molecular and cellular functions (on the total numbers of DEG)	Cellular movement Metabolic disease Amino acid metabolism Carbohydrate metabolism Lipid metabolism Cell morphology	Cellular movement Cellular compromise Lipid metabolism	Cell morphology Gene expression Lipid metabolism Molecular transport	Cellular movement RNA post-transcriptional modification Protein trafficking Gene expression Protein synthesis Amino acid metabolism
Upstream regulators				
<i>In silico</i> identified	<u>CITED2</u> (−1.08/7/ 2.4)	<u>GATA4</u> (−2.3/6/ 1.7)	<u>DNMT3B</u> (−1/3)	<u>DNMT3B</u> (−1.8/6/− 2.2)
Differentially expressed in the dataset (ExprLogRatio)	<u>POU5F1</u> (−0.98/23/− 0.45)	<u>MECP2</u> (2/4)		<u>NR1H3</u> (−1.5/12/− 2.2)
Target molecules in the dataset	<u>STAT3</u> (−0.96/30/− 0.7)	NANOG (1.8/4)		<u>TP53</u> (−1.4/100/− 3.2)
IPA activation Z-score	<u>HDAC6</u> (−0.83/5/ 0.9) <u>CDX2</u> (0.75/8/ 1.5) <u>DNMT3B</u> (0.97/11/− 0.3) <u>ID2</u> (1.92/10/ 1) <u>MYC</u> (2.07/78/ 3.1) <u>NANOG</u> (2.55/9/− 0.5) <u>TFAP2A</u> (2.75/11/− 0.3)			<u>YY1</u> (−1.1/20/− 1.1) <u>MYCN</u> (−0.8/38/− 3.3) <u>NR1H2</u> (−0.6/8/− 1.1) <u>NR1H4</u> (−0.6/11/ 0.4) <u>ID3</u> (−0.6/6/ 2.2) <u>HSF1</u> (−0.3/11/− 2.2) <u>ESR1</u> (0.3/92/ 2.2) <u>WT1</u> (0.4/19/ 0.2) <u>PML</u> (0.4/13/ 2.2) <u>CDKN2A</u> (0.4/21/− 1.7) <u>RB1</u> (0.6/25/− 3.1) <u>PDX1</u> (0.6/11/− 0.08) <u>TFAP2A</u> (0.8/8/ 0.1) <u>NCOA1</u> (1.2/6/− 2) <u>DNMT3A</u> , <u>FADS3</u>
Genes selected for validation	<u>AKR1A1</u> , <u>APOA1</u> , <u>DNMT3A</u> , <u>FADS3</u> , <u>NANOG</u> , <u>PTGS2</u> , <u>STAT3</u>	<u>APOA1</u> , <u>NANOG</u> , <u>PTGES2</u>	<u>FADS3</u>	
Negative pregnancy predictors (no calf, no pregnancy, embryonic resorption)	<u>AKR1B1</u> , <u>CSRP2</u> , <u>ELOVL1</u> , <u>LGALS3</u> , <u>PRSS23</u> , <u>S100A10</u> <u>CDX2</u> , <u>PLAC8</u> , <u>PLAU</u> , <u>PTGS2</u> , <u>TXN</u>	<u>PLAU</u>	<u>PRSS23</u>	<u>ACTG1</u> , <u>CEP164</u> , <u>DDX5</u> , <u>DSTN</u> , <u>EEF1A1</u> , <u>MLH1</u> , <u>MRPL33</u> , <u>ODC1</u> , <u>PLAU</u> , <u>RIOK3</u> , <u>RPLP0</u> , <u>TPT1</u> , <u>TUBA1C</u> , <u>ACHE</u> , <u>AKR1B1</u> , <u>ALDOA</u> , <u>ATPIF1</u> , <u>B2M</u> , <u>CD9</u> , <u>CLIC1</u> , <u>COOZ</u> , <u>ELOVL1</u> , <u>ESRRG</u> , <u>FADS1</u> , <u>ETH1</u> , <u>GNB2L1</u> , <u>HNRNPAL</u> , <u>HSDP1</u> , <u>KRT8</u> , <u>NANOS1</u> , <u>PLAC8</u> , <u>PRSS23</u> , <u>RGS2</u> , <u>S100A14</u> , <u>SDF2</u> , <u>SHEM1</u>

Three to six functions were identified per contrast that were unique to the contrast, shared among IVP groups (in bold and in black), or shared with the 'vivo-vitro' contrast (in bold and in grey). Many upstream regulators were identified. Shown are those that were differentially expressed (ExprLogRatio) and whose target molecules were also in the dataset (Target number). Activation scores are provided as calculated by IPA (Z-scores, in bold): (+) indicates activation, (−) indicates inhibition. Each putative regulator is therefore described by three features: (ExprLogRatio/Target number/Z-score). The underlined genes are those whose expression increased in the underlined group of the contrast. As depicted for the functions, the regulators that are in bold and in grey are those that were shared with the 'vivo-vitro' contrast.

and D6+2 blastocysts, respectively, compared to D6 levels (array data confirmed by RT-qPCR; Fig. 7). The expression profile of *IFNT* mRNA was similar to that of *JUN*, whereas *ETS2* and *CDX2* levels appeared to be either similar in all groups (D6, D6+2, D8: *ETS2*) or to demonstrate patterns opposite to those of *IFNT* and *JUN* (similar in D6+2 and D6 embryos, lower in D8: *CDX2*; Fig. 7). Recovery rates were quite similar between D6 and T6 (58%) and controls (unsorted D7-T7 blastocysts or full *in vivo* development: 62% and 66%, respectively), but generally lower in the D6+2-T8, D7+1-T8 or D8-T8 groups (33–25%).

Discussion

Although there is abundant literature that has questioned the ability of *in vitro*-produced embryos to replicate the development of those produced *in vivo*, in the current study, all conceptuses in the experimental groups that survived up to D18 gastrulated and elongated almost as well as *in vivo* (D18) or *in vitro* controls (classical D7/T7). Two other notable results of this study were that the recovery rate differed markedly between D6 and D8 embryos that were immediately transferred (58 vs 25% in D6/T6 vs D8/T8), and that,

Figure 4 Gene expression differences ($n=8$) observed in microarrays (left panel) and RT-qPCRs (right panel). RT-qPCR data were normalised by qBasePLUS based on *GAPDH*, *YWHAZ* and *SDHA* expression. Significant differences are indicated by stars: (*) for a P value < 0.05 , (**) for a P value < 0.01 , (***) for a P value < 0.001 and (****) for a P value < 0.0001 . PCR primers are provided in Supplementary Table 1.

despite a mortality of 40–60% in the 2 weeks following ET, no signature of dying or surviving was detectable in any of the transferred groups (D6, D6+2, D7+1, D8). Finally, we were intrigued by our finding of a '+1' phenotype in the D6+1 and D7+1 embryos: these groups overlapped in the BCA and shared a larger gene set than day 6 and day 7 blastocysts did, which could be a possible sign of a similar adaptation to the *in vitro* environment.

Due to the limited size of the dataset (48 embryos transferred; 14 recovered) and the non-physiological use of the recipient uteri (six embryos each instead of one), our data do have limits. However, the broad patterns apparent in our analyses are worthy of further discussion and investigation. Generally speaking, few D7+1 and D8 embryos survived *in utero* (25%), while those in the D6+2 and D6 groups fared better (33 and 58%, respectively). It is worth noting that the 58% survival rate observed for this last group was not far from the recovery rate that we had previously reported for D7 embryos produced and transferred under the very same conditions (Degrelle *et al.* 2012). The D6-T6 experimental group (expanded blastocysts formed at D6 and immediately transferred) was also similar to the IVP control group (D7-T7) in terms of the number of embryonic tissues that reached stage 3 (4/7) and the amount of IFNT that was produced by elongated tissues. In the other three experimental groups, elongation and gastrulation patterns were also identical to controls (10/10 filamentous; 5/7 at stage HH3), which was inconsistent with previous reports that described shorter conceptuses and smaller embryonic discs at D16 (after transfer of D7 IVP blastocysts cultured in SOF plus BOEC; Bertolini *et al.* 2002) or no disc at D14 after culture in KSOM or SOF (Fischer-Brown *et al.* 2004, Berg *et al.* 2010). There was a substantial increase in the amount of IFNT produced by the elongated tissues of the D8-T8 and D6+2-T8 groups compared to D6-T6 embryos ($\times 4$ and $\times 9$, respectively), which we could not relate to the expression of *CDX2*, *ETS2* or *JUN* proteins (Sakurai *et al.* 2013) since none of these transcription factors was detectable in these conditions. In this, our results differed from those reported by Xavier *et al.* (1997) for the very same antibodies, which perhaps could be attributable to differences in the protein extraction buffers or the revealing methods used. Our Western blot results for IFNT were mimicked in the amount of *IFNT* transcript detected prior to transfer in the D8 and D6+2 blastocysts ($\times 2.4$ and $\times 3.6$ compared to D6). Interestingly, the profile of *IFNT* mRNA was similar to that of *JUN*, whereas those of *CDX2* and *ETS2* differed. Earlier studies within the EmbryoGENE network likewise reported that *IFNT* and *JUN* expressions were similarly affected by culture conditions that included

Whole EmbryoGENE dataset meta-analysis		D6+2-D8-D6
Impact of culture	Culture Conditions	Current DEG
Adjusted P-Value	Adjusted P-Value	Gene
1.60 e-10	0.0735	AKR1A1
5.24 e-06	3.67 e-05	APOA1
0.0653	0.1665	DNMT3A
0.0032	0.0003	FADS3
2.06 e-14	0.0972	NANOG
0.5911	0.0007	PTGES2
0.0138	0.0012	PTGS2
7.98 e-05	0.1243	STAT3

Whole EmbryoGENE dataset meta-analysis		D6+2-D8-D6
Impact of culture	Culture Conditions	Current upstream regulators
Adjusted P-Value	Adjusted P-Value	Gene
0.1503	0.0717	CDX2
0.0029	2.42 e-05	CDKN2A
5.58 e-10	4.37 e-06	CITED2
0.9204	0.0827	DNMT3B
0.2705	7.55 e-05	ESR1
0.0095	0.0774	GATA4
0.3249	0.7057	HDAC6
0.5609	2.10 e-05	HSF1
0.0008	0.1461	ID2
4.73 e-08	2.27 e-06	ID3
0.0003	0.0004	MECP2
4.10 e-06	0.1461	MYC
0.6067	0.2033	MYCN
9.04 e-07	0.0002	NCOA1
0.0001	0.4639	NR1H2
0.0408	0.1985	NR1H3
0.5722	0.0028	NR1H4
0.1204	0.1204	PDX1
0.3156	7.74 e-05	PML
3.47 e-12	0.0001	POU5F1
0.0041	0.0678	RB1
0.2940	0.0002	TFAP2A
0.2040	0.3827	TP53
0.7309	0.0092	WT1
0.0131	0.0013	YY1

Figure 5 Effect of *in vitro* culture or culture conditions on the expression of certain DEGs as recorded by studies within the EmbryoGENE network. A meta-analysis of the entire dataset from the EmbryoGENE network was used to investigate the differential expression of certain DEGs identified herein. Presented are the adjusted *P* values that report the statistical relevance in studies that investigated the effects of culture conditions (SOF, co-culture in B2-BRL), origin of the oocytes (culture post ovum pick-up) and full or partial *in vitro* vs full or partial *vivo* development (including the D7 control that was used herein: full *vivo*; Gad *et al.* 2012a). Among these DEGs are the eight that were selected for biological validation with qRT-PCR (in A), and the 27 that were identified as putative upstream regulators in the current dataset (in B). Note that: (i) the putative regulators encoding *NANOG* and *STAT3* appear only once (A panel), (ii) *DNMT3A* was not confirmed as a DEG either internally (current RT-qPCR) or externally (meta-analysis) and (iii) the differential patterns of *CDX2*, *DNMT3B*, *HDAC6*, *MYCN*, *PDX1* and *TP53* were not validated beyond the current study. Adjusted *P* value: not validated, in grey

co-culture with VERO cells in B2 medium without serum (Plourde *et al.* 2012a).

D6+2 and D6 embryos differed substantially in gene expression, possibly due to dysregulations resembling the epigenetic ones associated with IFNT (Sakurai *et al.* 2009) as well as those at other loci following *in vitro* production (Salilew-Wondim *et al.* 2015, Barrera *et al.* 2017). Of the embryos that could be classified as high producers of IFNT (D6+2, D8), 67–75% died prior to D18, which was consistent with the hypothesis of Wrenzycki *et al.* (2001) that high-IFNT producers are blastocysts of poor quality. Even though 5.8% of the genes analysed were DE between the D6+2 and D6 transcriptomes (1186 out of 20,393 expressed probes), we were not able to detect any signatures that could point to the mechanisms responsible for death (or survival). Future work focused on individuals instead of pooled embryos, using RNA sequencing instead of microarrays, may enable such a discovery, but here, the between-group analysis of D6+2, D6 and D8 embryos found these groups to be relatively homogeneous in gene expression (less than 6% to less than 1% differential expression).

Of all the extended culture groups, D6+2 embryos had the highest number of DEGs with respect to the group they derived from, D6. These differences may have accumulated as a consequence of the extended stay *in vitro* (+2 days). Two additional observations were consistent with this hypothesis: first, many of the enriched genes shared between the D6+2 and D6 groups were also shared between the D6+1 and D6 blastocysts ($n=242$), and second, the number of genes that were unique to each comparison was seven times greater in the former pair than in the latter (286 vs 40). Alternatively, since D6+2 embryos were hatched while D6 were only expanded, the molecular differences between the two groups may have merely resulted from ordinary developmental functions involved in the differentiation of trophoctoderm, primitive endoderm or inner cell mass. Evidence to support this hypothesis was found with the observation that a great deal of the gene expression that was enriched in D6+2 embryos was from the trophoctoderm tissue, while enriched gene expression in the D6 embryos tended to be in the inner cell mass. Although the enriched gene expression was not entirely specific to any of these cell lineages, none of these gene patterns was expressed in both ICM and TE (Hosseini *et al.* 2015). Surprisingly, the gene enrichment analysis also revealed that D6+2 embryos were much less similar to D6 embryos than they were to the other group of expanded embryos, D8-forming blastocysts (D6+2 vs D8: 10 times fewer differences than in D6+2 vs D6). Could this be due to the fact that D6+2 and D8 blastocysts each spent a total of 8 days in culture and thus shared similar adaptations to the *in vitro* environment? Some evidence supporting this idea came from another finding of the gene enrichment analysis that 82 genes (linked with OXPHOS, ROS and glycolysis), which

Figure 6 Post-transfer phenotypes at D18. Bovine conceptuses were collected 10–12 days after transfer of *in vitro*-formed blastocysts; blastocysts were fully formed at D6–D8 or maintained in culture 1 or 2 additional day(s). In (A), recovery rates are provided. With the exception of the D7+1-T8 and D8-T8 groups, recovery rates all fell within previously reported ranges for the transfer of multiple embryos (11 per transfer, 40% (Fischer-Brown *et al.* 2004); 3–30 per transfer, 53% (Berg *et al.* 2010); 5–6 per transfer, 62% (Degrelle *et al.* 2012)). Conceptus sizes were recorded and conceptuses were all identified as filamentous (length range: 108–182 mm). Embryonic discs were observed and identified as gastrulating (typical HH (Hamburger and Hamilton)) stages 2–5 as defined in (Hue *et al.* 2001). In grey, the two control datasets: from embryos which experienced full *in vivo* development and those that underwent classical ET at D7 (unsorted D7 IVP embryos transferred at D7). These originated from a previous study that used the identical IVP system (culture in groups, on VERO cells, and in B2 medium plus FCS; Degrelle *et al.* 2012). In (B), gastrulation landmarks are indicated: streak (for primitive streak), node (for Hensen's node), and folds (for amnion folds). *In situ* hybridisation with a low concentration of bovine *TBXT* riboprobe revealed a characteristic 'violin'-shaped pattern in the embryonic tissues. In (C), IFN-tau (17 kDa) production was evaluated within the elongated tissues by Western blotting (3 biological samples per condition) and quantified relative to ACTB (42 kDa). The control in this case was provided by the *in vivo*-developed embryos.

represented 58% of the shared enriched genes in the D6+2 vs D8 comparison, were also found to be enriched in D7+1 embryos (the third group that spent 8 days in culture). On the other hand, some of the differences between the D6+2 group and the D8 or D6 embryos may have originated in differences between these latter two groups: although both comprised embryos that were expanded, members of these two groups may have

differed a great deal at the molecular level due to the intrinsic features that made them form blastocoels at 8 or 6 days, respectively, post fertilisation. In agreement with this were the findings that the newly formed blastocysts (D6, D7 and D8) shared the smallest enriched gene set (34 genes, representing less than 20% of the enriched genes in D7 vs D8, D6 vs D7, or D6 vs D8 comparisons) and that the D6 and D8 groups were opposed in the

Figure 7 IFNT regulation. The expression of *IFNT* and its potential regulators, *CDX2*, *ETS2* and *AP-1/JUN* (within the dashed lines), was analysed in the D6, D6+2 and D8 groups. RT-qPCR data were normalised by qBasePLUS based on *GAPDH*, *YWHAZ* and *SDHA* expression. *JUN* was detected and quantified at very low levels in D6 embryos, as was *CDX2* in D8 embryos (lower limits of the standard curve). Significant differences are indicated by stars: (*) for a *P* value <0.05. PCR primers are provided in Supplementary Table 1.

BCA along axes 1–3 and 2–3, but not 1–2. Interestingly, though, the small amount of differential gene expression between D6 and D8 embryos would seem to be evidence for similarity between the two groups. Clearly, the biological complexity of this system goes far beyond the analyses that we performed here.

Although they spent different amounts of time in culture and originated from different blastocysts, D6+1 (vs D6) and D7+1 (vs D7) embryos shared an enriched gene set of 87 genes associated with three functions (mTORC1, P53, apoptosis); together, these genes represented 26–30% of those enrichment sets and possibly define a ‘+1’ phenotype. Some of these enriched genes (33 of 87) were even shared with the D6+2 embryos (which could be considered [D6+1] +1). This ‘+1’ phenotype made logical sense but it also reinforced the uniqueness of the D6+2 embryos, demonstrating the magnitude of the adaptive response of D6 blastocysts to an extended time in culture.

Many gene expression differences have been found between *in vitro*- and *in vivo*-produced embryos, as has often been reported in the literature (Gad *et al.* 2012b). The majority of these – over a thousand genes – were ascribed to culture conditions and mainly to serum addition ($n=1039/6746$, Heras *et al.* 2016). Despite the detrimental *in vitro* conditions, no developmental pause was observed in any of our *in vitro*-produced embryos. Indeed, in comparing the gene expression patterns from our embryos with those from a study of diapause in mice (Fu *et al.* 2014), the largest degree of overlap was not in genes associated with dormancy ($n=18$), but those linked to reactivation at the end of dormancy ($n=72$). We were thus surprised at the high number of DEGs (41% of genes overexpressed in IVP vs *vivo*) that were shared with mouse epiblast in diapause (as compared to WT epiblast and PrE at E4.5; Boroviak *et al.* 2015). The simplest explanation of this is that most of these genes are linked to the cellular machinery (cellular junctions, cell signalling, translation regulators, transporters), perhaps IVP embryos (as compared to *vivo*) engage pathways similar to those involved in ongoing diapause or a reactivation process at the end of dormancy (as compared to WTs). Whether embryonic adaptabilities could depend on such genes is an open question.

In conclusion, the current work found evidence for three main phenotypes. The patterns of gene enrichment we initially found seemed to confirm a phenotype for ‘fully formed’ blastocysts, but further investigation revealed that early- and late-forming blastocysts in fact appear to be quite different: they share a limited set of enriched genes and yield dramatically different survival rates when immediately transferred (58 vs 25% in D6-T6 vs D8-T8). Instead the ‘+1 phenotype’ was characterised by a shared gene enrichment set that was larger than that of the newly formed blastocysts, which argued for similar mechanisms of adaptation in both the D6 and D7 embryos to the *in vitro* environment. However, these adaptations did not

appear to benefit the D7+1 embryos, as their survival rate at D18 was as low as that of the D8-T8 group: 25%. The '+2 phenotype' was defined by the highest number of differences with the D6 embryos, both *in vitro* (differential gene expression) and *in vivo* (increased IFN-tau production). Despite these differences, D6+2 embryos survived slightly better than the D8 or D7+1 embryos, even though all three groups were transferred at D8. Finally, it was clear that the D6 embryos were the most successful once transferred (recovery rate and IFN-tau production equal to those of the *in vivo* control).

Regardless of the differences among the groups, the fact remains that no IVP group closely resembled D7 *in vivo*-developed blastocysts. One potential explanation could be that the IVP embryos engaged pathways partly similar to those of an ongoing diapause or a reactivation process at the end of dormancy. Much work remains to be done to decipher the abilities of *in vitro*-produced zygotes to adapt to culture conditions (del Collado *et al.* 2016, Desmet *et al.* 2016, Sutton-McDowall *et al.* 2016), the impact of such conditions upon developmental competence (Rizos *et al.* 2002) and their adaptive abilities *in vivo*. Although the intrinsic embryo viability was partly mirrored by the post-transfer phenotypes in this study, there is growing appreciation of the role that the uterine fluids play as *in vivo* modulators of embryonic viability (sensing/driving properties). Indeed, as a result of these sensor/driver properties (Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009), the uterine milieu may alternatively (i) 'rescue' embryos by advancing development or minimising transcriptional differences (Clemente *et al.* 2009, Machado *et al.* 2013), (ii) induce novel transcriptional differences in embryos (Forde *et al.* 2017) or (iii) filter out suboptimal embryos to prevent unsuccessful implantation and later miscarriages (Macklon & Brosens 2014). The next major goal for this field of research might therefore be a pairwise analysis of the intrinsic viability of embryos in the context of the uterine milieu in which they are transferred.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0439>.

Declaration of interest

Hue I is a member of the Editorial Board of Reproduction. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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