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ORIGINAL ARTICLE *Embryology*

ART culture conditions change the probability of mouse embryo gestation through defined cellular and molecular responses

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STUDY QUESTION: Do different human ART culture protocols prepare embryos differently for post-implantation development?

SUMMARY ANSWER: The type of ART culture protocol results in distinct cellular and molecular phenotypes *in vitro* at the blastocyst stage as well as subsequently during *in vivo* development.

WHAT IS KNOWN ALREADY: It has been reported that ART culture medium affects human development as measured by gestation rates and birthweights. However, due to individual variation across ART patients, it is not possible as yet to pinpoint a cause–effect relationship between choice of culture medium and developmental outcome.

STUDY DESIGN, SIZE, DURATION: In a prospective study, 13 human ART culture protocols were compared two at a time against *in vivo* and *in vitro* controls. Superovulated mouse oocytes were fertilized *in vivo* using outbred and inbred mating schemes. Zygotes were cultured in medium or in the oviduct and scored for developmental parameters 96 h later. Blastocysts were either analyzed or transferred into fosters to measure implantation rates and fetal development. In total, 5735 fertilized mouse oocytes, 1732 blastocysts, 605 fetuses and 178 newborns were examined during the course of the study (December 2010–December 2011).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Mice of the B6C3F1, C57Bl/6 and CD1 strains were used as oocyte donors, sperm donors and recipients for embryo transfer, respectively. *In vivo* fertilized B6C3F1 oocytes were allowed to cleave in 13 human ART culture protocols compared with mouse oviduct and optimized mouse medium (KSOM(aa)). Cell lineage composition of resultant blastocysts was analyzed by immunostaining and confocal microscopy (trophectoderm, Cdx2; primitive ectoderm, Nanog; primitive endoderm, Sox17), global gene expression by microarray analysis, and rates of development to midgestation and to term.

MAIN RESULTS AND THE ROLE OF CHANCE: Mouse zygotes show profound variation in blastocyst (49.9–91.9%) and fetal (15.7–62.0%) development rates across the 13 ART culture protocols tested ($R^2 = 0.337$). Two opposite protocols, human tubal fluid/multiblast (high fetal rate) and ISM1/ISM2 (low fetal rate), were analyzed in depth using outbred and inbred fertilization schemes. Resultant blastocysts show imbalances of cell lineage composition; culture medium-specific deviation of gene expression (38 genes, $\geq$4-fold) compared with the *in vivo* pattern; and produce different litter sizes ($P \leq 0.0076$) after transfer into fosters. Confounding effects of subfertility, life style and genetic heterogeneity are reduced to a minimum in the mouse model compared with ART patients.

LIMITATIONS, REASONS FOR CAUTION: This is an animal model study. Mouse embryo responses to human ART media are not transferable 1-to-1 to human development due to structural and physiologic differences between oocytes of the two species.

WIDER IMPLICATIONS OF THE FINDINGS: Our data promote awareness that human ART culture media affect embryo development. Effects reported here in the mouse may apply also in human, because no ART medium presently available on the market has been

optimized for human embryo development. The mouse embryo assay (MEA), which requires ART media to support at least 80% blastocyst formation, is in need of reform and should be extended to include post-implantation development.

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Introduction

Assisted reproductive techniques (ART) were established more than 30 years ago in response to human infertility and the cultural shift towards childbearing at later age. ART account for 3.9% of all births in developed countries and for as much as 6% in certain countries, such as Denmark (Nyboe Andersen and Erb, 2006; Nyboe Andersen *et al*., 2007; de Mouzon *et al*., 2010). One of the most common ART is embryo culture. Numerous media are commercially available for the *in vitro* culture of human pre-implantation stage embryos prior to transfer into the uterus. The type of culture medium used has been correlated with gestation rates in some studies (Dumoulin *et al*., 2010; Xella *et al*., 2010) but not in others (Aoki *et al*., 2005; Hambiliki *et al*., 2011). It has been suggested that the choice of culture media can also affect the birthweight of ART children (Dumoulin *et al*., 2010). Confirming or confuting these data is important for society in general, and for individuals undergoing ART in particular; at stake is the awareness that the choice of an embryo culture medium may, in part, determine which human embryos develop into healthy babies. Gathering solid evidence to address this issue is impeded by the broad variation (genetic, environmental, physiologic, nutritional, etc.) of human populations, and is confounded by the subfertility of the ART subpopulation. In simple words, it is difficult to resolve the effects of embryo culture *per se*, from those of other factors that relate to the genotype and life history of ART patients. The quest for post-implantation data can be modeled in an animal species under carefully controlled experimental conditions, provided that excessive generalization between mammalian species is avoided and that differences of oocyte structure and physiology are considered (Menezo and Herubel, 2002; Benkhalifa *et al*., 2010). While polytocous (litter-bearing) model species like the mouse are in many aspects distinct from singlet pregnancy humans, they allow an extra level of information in that the size of the litter can be examined. This information was decisive to show that culture medium supplemented with granulocyte-macrophage colony-stimulating factor supported more mouse embryos to term compared with unsupplemented medium and *in vivo* control (Sjoblom *et al*., 2005).

We modified the 'mouse embryo assay, MEA' (FDA, 1998; Byers *et al*., 2006; Taft, 2008; Punt-van der Zalm *et al*., 2009), which requires ART culture media to support at least 80% blastocyst formation, to include post-implantation development. Furthermore, we adopted outbred and inbred fertilization schemes to mimic, if only in part, the large and the smaller allelic pools of the general human population and its ART subpopulation, respectively. While it is known that mouse embryo culture can induce deviation from the normal range of genomic, physiological and behavioral parameters (Ecker *et al*., 2004; Fernandez-Gonzalez *et al*., 2004; Watkins *et al*., 2007; Rivera *et al*., 2008; Yu *et al*., 2009; Market-Velker *et al*., 2010; Scott *et al*., 2010), these effects are typically associated with low, e.g. $<$30% full-term rates (Ecker *et al*., 2004) or small, e.g. three to four pups litter sizes (Rivera *et al*., 2008). It is debatable whether the genomic, physiological and behavioral effects would still occur for gestations with a regular density of embryos in the female genital tract, which is important for the timing of parturition in a polytocous species like the mouse (McLaren, 1970). In humans, the first and foremost aim of fertility clinics is to ensure the delivery of healthy babies, and only in suborder, to increase the pregnancy rates—a sensible ranking of priorities since the natural fecundity of our species is low anyway (Racowsky, 2002). However, when losses are high during early development, it seems unrealistic that the surviving embryos have remained completely unaffected. Therefore, in the mouse model, we became interested in determining whether ART culture conditions support the same fetal rates as the oviduct they are meant to substitute for; and if not, which genes and processes correlate with the potential differences. To this end, we allocated *in vivo* fertilized mouse oocytes to 13 culture protocols transposed from the fertility clinic to our mouse laboratory; we collected the embryos from culture at the blastocyst stage, and we transferred these blastocysts to the reproductive tract in order to assess fetal development (E12.5).

Blastocyst implantation and post-implantation development relies on the establishment of two founder cell lineages: the trophectoderm (TE; Strumpf *et al*., 2005) and the inner cell mass (ICM; Nichols *et al*., 1998); the latter divides further into primitive ectoderm (pEct; Mitsui *et al*., 2003) and primitive endoderm (pEnd; Artus *et al*., 2011). These three lineages represent the founder stem cells of the embryo, and their origin and function are thought to be largely conserved in mammals (Rossant, 2007; Cockburn and Rossant, 2010). In the context of ART, blastomeres and their cell progenies undergo commitment to the founder lineages of the embryo while being under *in vitro* culture conditions. Culture media, while elaborate, remain a suboptimal environment (Bavister, 1988) in which cell fate and lineage establishment may be perturbed; and the specific metabolic needs of ICM and TE (Hewitson and Leese, 1993; Houghton, 2006) may only partly be supported. It has been observed that ICMs of mouse blastocysts cultured *in vitro* yield fewer embryonic stem (ES) cell lines compared with their counterparts grown *in vivo* (17 versus 38%; Tielens *et al*., 2006). This observation lends support to the proposition that culture conditions affect the stem cells of the embryo (Watkins *et al*., 2007).

Here, we report that fertilized mouse oocytes cultured under various ART conditions form blastocysts that differ in their competence for post-implantation development. While imbalances of the founder cell lineages have been previously reported after embryo bisection, or for high-glucose culture conditions, our results demonstrate that small but significant (±10%) imbalances in allocation to TE, pEct and pEnd occur in ART blastocysts compared with their *in vivo* counterparts. Gene ontology (GO) enrichment analysis of transcriptomes reveals effects of ART culture on gene expression that could not have been predicted, that is, altered expression of metabolic instead of cell-fate genes. Of 38 transcripts with levels differing at least 4-fold between ART and *in vivo* blastocysts, 36 have ontologies related to cytoplasm and metabolism, two encode transcription factors and none is imprinted. We conclude that culture media exert a priming effect on the ability of mouse blastocysts for postimplantation development through very selective alterations of metabolic gene expression. Our study exemplifies hypothesis-driven, animal-based basic research that should always precede the introduction of new reagents and treatments in ART (Harper *et al*., 2012).

Materials and Methods

Ethics statement

This animal study was performed in strict accordance with the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). Mice were used for experiments according to the ethical permit issued by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of the state of North Rhine-Westphalia, Germany (Permit Number: G 9.93.2.10.36.07.254).

Collection of fertilized mouse oocytes

Six- to eight-week-old B6C3F1 females were primed with 10 IU each pregnant mare serum gonadotrophin (PMSG) and hCG 48 h apart, and paired with proved stud males of the CD1 or C57Bl/6 strain. Fertilized (pronucleated) oocytes were isolated from oviducts at 12/noon on the day of the copulation plug, using Hepes-buffered CZB medium (Chatot *et al*., 1989). Mice were housed under 14L:10D photoperiod and fed *ad libitum* on Harlan Teklad 2020SX chow.

Pre-implantation development

Within 1 h of collection, oocytes were randomly allocated to the experimental groups and allowed to cleave to the blastocyst stage (defined by the unequivocal presence of a cavity). ART culture protocols were tested two at a time against the control group *in vivo* (CD1 oviduct) or *in vitro* (KSOM(aa) medium; Ho *et al*., 1995; Biggers *et al*., 2000). ART media were obtained from Universitätsklinikum Münster's pharmacy and used as prescribed (e.g. monoculture or sequential culture) resulting in 13 culture protocols (Table I). For each culture group (≥3 replicates), 30–40 fertilized oocytes were placed directly after retrieval in 400 µl of culture medium in a 4-well Nunc plate, without oil overlay, at 37°C under 5.5% CO_2 in air. For the *in vivo* control group, fertilized oocytes were recovered on the late morning of the copulation plug and transferred within 1 h to the left oviduct of pseudo-pregnant CD1 recipients (10 oocytes/recipient) that had been paired with vasectomized CD1 males 3 days prior and had a copulation plug on the day of embryo transfer (ET). All embryos were recovered 3 days later from the culture plate or from the uterus and scored for blastocyst formation. These blastocysts were then transferred to pseudo-pregnant recipients to allow for further development (see 'Post-implantation development'). Remaining embryos forming blastocysts until the next day were taken into account to calculate blastocyst rates at the end-point (E4.5).

Post-implantation development

Ten blastocysts were transferred into the infundibulum of the left-side oviduct of pseudo-pregnant CD1 recipients that had been paired with vasectomized CD1 males 3 days prior and had a copulation plug on the day of ET (Caperton *et al*., 2007). Fetal rates were scored 12 days later (E12.5) after Caesarean section following cervical dislocation. In the case of B6C3F1xC57Bl/6 embryos, pregnancies were also let to term. The right-side oviduct served as a control that the vasectomized males were indeed consistently sterile. Mice that had been subjected to ET but found not to be pregnant were excluded from analysis, as this outcome may be independent of the embryos (e.g. ET failed by the operator). We included empty decidua in the count for implantation rates, but of course not in the count for fetal rates. Three experimental replicates were conducted for each medium.

Analysis of cell lineage allocation and apoptosis

Blastocysts were processed by immunostaining coupled with confocal microscopy to identify the cell lineages, as described (Balbach *et al*., 2010). The following primary antibodies were applied simultaneously to the specimens: anti-Cdx2 mouse IgG1κ (Emergo Europe, The Hague, Netherlands, cat. no. CDX2-88); anti-Nanog rabbit IgG (Cosmo Bio, Tokyo, Japan, cat. no. REC-RCAB0002P-F); anti-Sox17 goat IgG (R&D Systems, cat no. AF1924). Appropriate Alexa Fluor-tagged secondary antibodies were matched to the primaries. Apoptosis was detected using the cell membrane-impermeable dyes propidium iodide (PI) and YOPRO-1 according to Gawlitta *et al*. (2004).

Transcriptome analysis

Blastocysts of three biological replicates were lysed and processed for transcriptome analysis via microarray using the Illumina BeadStation 500 (Illumina, San Diego, CA, USA) platform. Total RNA was extracted using the ZR RNA Microprep Kit (Zymo Research Corporation, Irvine, USA) without DNase digestion step. A two-round linear amplification protocol employing a linear 2-step TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre) was used to generate biotin-labeled cRNA, 150 ng/µl of which was used for each hybridization reaction. Washing, Cy3-streptavidin staining and scanning were performed on the Illumina BeadStation 500 (Illumina, San Diego, CA, USA) platform. Purified and labeled cRNA was then hybridized for 17 h onto MouseWG6 v2 expression BeadChips (Illumina). After washing, chips were stained with streptavidin-Cy3 (GE Healthcare) and scanned using the iScan reader (Illumina) and accompanying software. The bead intensities were mapped to gene information using BeadStudio 3.2 (Illumina). Background correction was performed using the Affymetrix Robust Multi-array Analysis (RMA) background correction model (Irizarry *et al*., 2003). Variance stabilization was performed using the $\log_2$ scaling, and gene expression normalization was calculated with the quantile method. The raw microarray data were deposited in NCBI Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE31414 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31414).

Statistical analysis of developmental rates, cell lineage allocation and microarray data

Blastocyst, implantation and fetal rates were analyzed as proportions according to Bailey (1959) using normal approximations to binomial

Table I ART culture protocols used in this study (by alphabetical order of the medium).

Protocol	Monoculture medium	Sequential culture media	Manufacturer
A		Early Cleavage Medium (ECM) → MultiBlast	Irvine Scientific
B		Ferticult IVF → G3	Fertipro, Fertipro
C		G-1 Plus → G-2 Plus	Vitrolife
D	GM501 gentamicin		Gynemed
E	GM501 pen/strep		Gynemed
F		Human tubal fluid (HTF) medium → MultiBlast	Irvine Scientific
G		Innovative Sequential Medium (ISM)-1 → ISM2	Origio
H		ISM1 → BlastAssist	Origio
I		P1 → MultiBlast	Irvine Scientific
J		Quinn's Advantage Cleavage → Blastocyst	SAGE
K	Single Step Medium (SSM)		Irvine Scientific
L		Sydney cleavage → Sydney blastocyst	Cook medical
M	Universal		Origio
Detrimental control	ISM1 not switched to ISM2		Origio
In vitro control	KSOM(aa)		made in house by M.B.
In vivo control	Oviduct		CD1 mouse

distributions. All culture groups were compared with the same reference (*in vivo* control group) at the significance level of 0.05 (two tails). Pearson correlation and cluster analysis of blastocyst, implantation and fetal rates were performed with JMP 7 (SAS). Dunnett test of litter sizes and YOPRO-1 stainings were performed with JMP 7 (SAS). The allocation of cells to TE, pEct and pEnd was compared across media and *in vivo* control using the χ^2 test. Microarray data post-processing and graphics were performed using in-house developed functions in Matlab. Venn diagrams (Chow and Rodgers, 2005) were used to visually represent the differently expressed genes of each culture protocol with respect to the *in vivo* situation for each genomic background, based on a fold change threshold of 2 in $\log_2$. To perform the GO enrichment analysis, the GO terms were taken from the AMIGO GO database (Ashburner *et al.*, 2000). The significance (*P*-value) of the GO terms of the differently expressed genes was calculated using an enrichment approach based on the hypergeometric distribution. The multitest effect influence was corrected by controlling the false discovery rate using the Benjamini–Hochberg correction at a significance level $P = 0.005$.

Results

Mouse ART blastocysts of high and low fetal potential are determined by the choice of culture medium

In vivo fertilized oocytes ($n = 3864$) of B6C3F1 females mated with CD1 males were collected from the oviductal ampullae on the morning of the detection of a copulation plug. Fertilized oocytes were allocated to 13 ART culture protocols and controls (Table I), followed by development to blastocyst stage and transfer of these blastocysts to pseudo-pregnant CD1 recipients. All mice and embryos were subjected to equal manipulation, including ovarian stimulation before mating, and ET after *in vitro* culture, such that the pre-implantation environment of the embryos was the sole difference between experimental groups (Table I). It is important to note that the same number ($n = 10$) of normal-looking blastocysts were transferred per recipient in all groups. Pooled results of at least three biological replicates were used for comparative analysis of developmental rates (Table II).

We compared the fetal rates of blastocysts that formed under ART culture protocols versus oviduct. When B6C3F1xCD1 fertilized oocytes were collected from the oviductal ampullae, like all embryos of this study, and promptly returned to oviduct (*in vivo* control), 71.1% of the oocytes were able to form blastocysts, as determined by flushing of the uterine horns 3 days later; after ET, 47.5% of the blastocysts had become fetuses, as determined by hysterectomy on gestation day 12.5 (Table II). Only females with implantations were considered, and all of the E12.5 concepti were outwardly normal as judged by surface anatomy (data not shown; Gruneberg, 1943; Wahlsten and Wainwright, 1977). Compared with *in vivo* control, developmental rates subsequent to culture under ART protocols were generally lower but occasionally higher (Fig. 1A). Linear correlation analysis revealed low but significant association between blastocyst and fetal (E12.5) rates across the environments ($R^2 = 0.337$, $P > F$ 0.0183; Fig. 1A; raw data presented in Table II). Hierarchical cluster analysis (Ward) based on blastocyst, implantation and fetal rates reveals the existence of three groups, namely, Group 1: ART protocols that outperform or perform similar to *in vivo* (B, E, F); Group 2: ART protocols outperformed by *in vivo* (H, G, M); and Group 3: intermediate ART protocols (A, C, D, I, J, K, L; Fig. 1B).

To rule out major contribution of manipulation-induced embryonic stress to the outcomes described above, we examined the developmental rates of fertilized oocytes that were retrieved from oviduct and promptly returned to it, when compared with fertilized oocytes that were left *in situ*. Fertilized oocytes of stimulated B6C3F1 females mated with CD1 stud males formed 91.7% blastocysts when left

Table II Development of B6C3F1xCD1 and B6C3F1xC57Bl/6 fertilized oocytes sorted by decreasing global rate of development*.

Embryo genotype	Culture protocol	Global rate*	St.dev.	*n*	Blastocyst rate	St.dev.	*P*-value**	*n***	Implantation rate	E12.5¶ fetal rate	St.dev.	*P*-value**
B6C3F1xCD1	*In vitro* control	0.546	0.041	650	0.866	0.013	≤0.002	160	0.850	0.631	0.038	0.009
	E	0.436	0.077	98	0.704	0.046	0.897	50	0.680	0.620	0.069	0.085
	F	0.427	0.077	347	0.853	0.019	≤0.002	40	0.600	0.500	0.079	0.787
	K	0.360	0.073	139	0.899	0.026	≤0.002	50	0.520	0.400	0.069	0.373
	B	0.354	0.077	141	0.787	0.034	0.084	40	0.625	0.450	0.079	0.787
	In vivo control	0.338	0.044	356	0.711	0.024	1	120	0.617	0.475	0.046	1
	I	0.333	0.070	162	0.877	0.026	≤0.002	50	0.500	0.380	0.069	0.258
	J	0.289	0.053	219	0.826	0.026	≤0.002	80	0.438	0.350	0.053	0.080
	L	0.294	0.068	148	0.919	0.022	≤0.002	50	0.380	0.320	0.066	0.063
	A	0.289	0.062	150	0.867	0.028	≤0.002	60	0.433	0.333	0.061	0.070
	C	0.269	0.071	164	0.829	0.029	0.003	40	0.425	0.325	0.074	0.099
	D	0.177	0.055	94	0.766	0.044	0.289	52	0.404	0.231	0.058	0.003
	H	0.136	0.029	403	0.504	0.025	≤0.002	100	0.520	0.270	0.044	≤0.002
	G	0.111	0.028	375	0.499	0.026	≤0.002	90	0.544	0.222	0.044	≤0.002
	M	0.089	0.029	281	0.569	0.030	≤0.002	70	0.643	0.157	0.043	≤0.002
	Detrimental control	0.034	0.027	137	0.343	0.041	≤0.002	20	0.100	0.100	0.067	≤0.002
B6C3F1x C57Bl/6	*In vitro* control	0.255	0.037	308	0.786	0.023	≤0.002	160	0.551	0.325	0.037	0.010
	F	0.206	0.029	581	0.685	0.019	≤0.002	180	0.367	0.300	0.034	0.002
	In vivo control	0.100	0.027	147	0.211	0.034	1	100	0.474	0.470	0.050	1
	G	0.067	0.013	835	0.398	0.017	≤0.002	220	0.806	0.168	0.025	≤0.002

With the exception of the *in vivo* control, fertilized oocytes developed to blastocyst stage *in vitro* and were then transferred to pseudo-pregnant CD1 females.
*Global developmental rate is calculated by multiplying blastocyst and fetal rates with each other.
***P*-value of the comparison with *in vivo* control (two-tail test).
***Ten blastocysts were transferred per recipient.
¶Proportion of blastocysts that developed to fetuses scored 12 days after transfer.

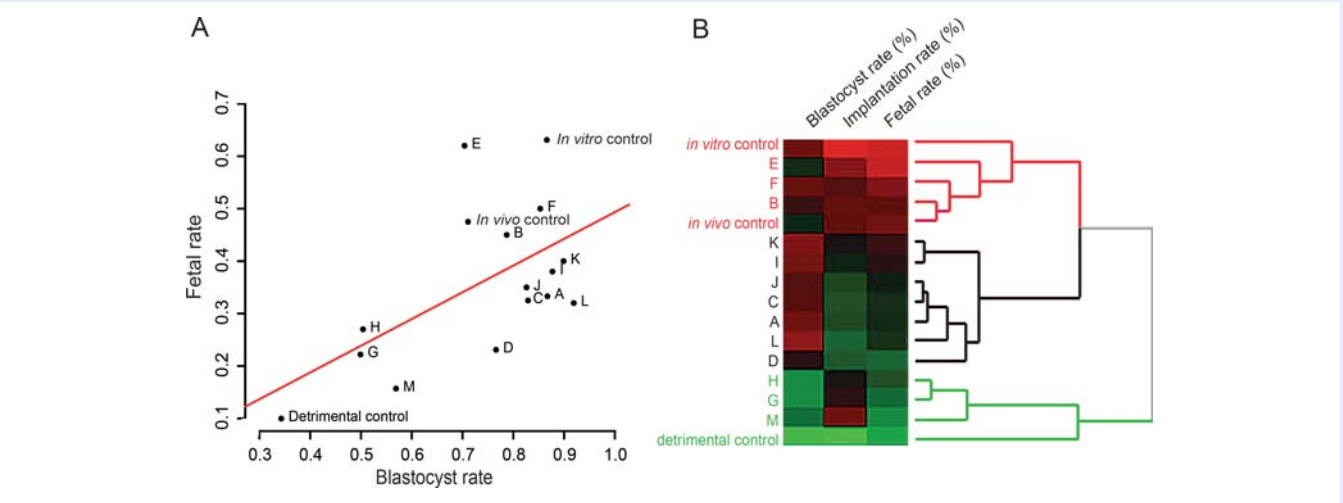

Figure 1 Scatter plot with linear correlation (**A**) and hierarchical cluster (**B**) analysis of the ART culture protocols tested in this study, based on blastocyst, implantation and fetal rates of B6C3F1xCD1 zygotes. Detrimental control is ISM1 employed alone without switch to ISM2. (A) Linear regression line is drawn in red. (B) The higher the rate, the more red the color (red > black > green).

Table III Developmental potential of fertilized mouse oocytes (B6C3F1xCD1) left *in situ* after mating, or subjected to recovery and transfer (ET) back to oviduct.

	Mice with embryos developing *in situ*	Mice with embryos transferred[a]	Embryos recovered on E3.5	Blastocysts (% of embryos)	Fetuses E12.5 (% of blastocysts)
In situ	18	n.a.	384	352 (91.7)	n.p.
In situ	18	n.a.	n.p.	n.a.	299 (84.9)
ET	n.a.	36	356	253 (71.1)	n.p.
ET	n.a.	9	n.p.	n.a.	55 (85.9)

[a]Ten embryos/recipient.
n.a, not applicable; n.p, not performed; E3.5/12.5, embryonic day 3.5 and 12.5, respectively.

in situ, as determined by flushing of the B6C3F1 uterine horns; these blastocysts expressed 84.9% fetal potential, as determined by hysterectomy of an equivalent set of mice. In comparison, fertilized oocytes that were collected and immediately transferred to CD1 oviduct formed 71.1% blastocysts, which expressed 85.9% fetal potential (Table III). Thus, cellular stress associated with oocyte collection and ET (Rivera *et al.*, 2008) affects pre-implantation (χ^2 test, $P = 4.18E-13$) but not post-implantation (χ^2 test, $P = 0.84$) development.

Blastocysts grown in the two extremes of the battery of ART protocols, namely protocol F (Quinn *et al.*, 1985) and protocol G (Xella *et al.*, 2010) were chosen for in-depth characterization also in a different genetic background. Characterization included developmental rates, cell lineage composition and global gene expression. When B6C3F1 females were mated to C57Bl/6 instead of CD1 males, and the resultant fertilized oocytes ($n = 1871$) were allocated to ART protocols F and G as well as to control groups, previous observations were confirmed: ART protocols can support higher global rates of development compared with *in vivo* control (Table II). Specifically, the rank positions of the embryos with C57Bl/6 paternal background were the same as those of the CD1-fathered embryos, i.e. *in vitro* control > protocol F > *in vivo* control > protocol G (Table II). In addition to the overall fetal rate at E12.5, we examined the size of litters that were dropped by the individual mothers, each of which received a transfer of 10 blastocysts. Litter sizes were significantly smaller for ART protocols than for *in vivo* control (Dunnett test, $P \leq 0.0076$), and for protocol G than for protocol F (Fig. 2). Taken together, these data show unequivocally that the observed post-implantation differences are due to the ART culture protocols.

ART blastocysts of high and low fetal potential present imbalances of the founder cell lineages

Differences of pre- and post-implantation development observed across ART protocols may be rooted in the blastocyst lineages that enable implantation and fetal development: the TE and the ICM, respectively, of which the latter splits into pEct and pEnd. Using immunoconfocal microscopy, we determined how many cells of each

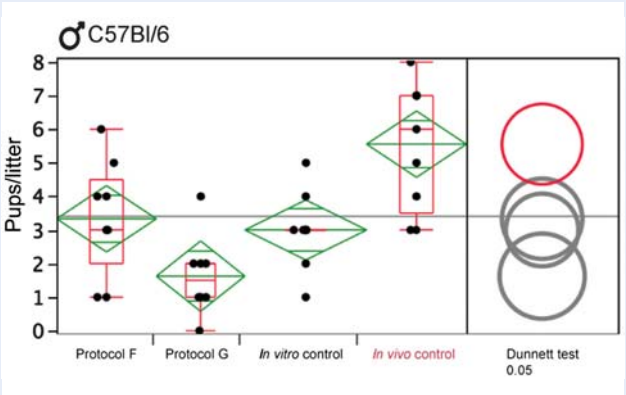

Figure 2 Sizes of litters obtained after transfer of 10 blastocysts (B6C3F1xC57Bl/6) to recipients (CD1). Blastocysts were obtained from protocol F, G as well as from *in vitro* and *in vivo* control. Black dots indicate the individual litters after natural delivery.

lineage are present in blastocysts originating from ART, *in vitro* control and *in vivo* control. TE nuclei (positive to Cdx2 protein; Strumpf *et al.*, 2005), pEct nuclei (positive to Nanog protein; Mitsui *et al.*, 2003) and pEnd nuclei (positive to Sox17 protein; Artus *et al.*, 2011) were counted on stacks of confocal *z*-series images (Fig. 3A). Counts were added to each other to give the total number of cells present in E4.5 blastocysts, of which 169 from CD1 and 148 from C57Bl/6 father (Supplementary data, Table S1). Linear correlation between total cell numbers and fetal rates across the four developmental conditions was $R^2 = 0.303$ in B6C3F1xCD1 blastocysts and $R^2 = 0.113$ in B6C3F1xC57Bl/6 blastocysts, neither of which is significant ($P >$ F 0.663 and 0.158, respectively).

Of the total blastocysts spanning a range of cell numbers, the subset with 40 ± 10 cells was analyzed for TE, pEct and pEnd composition (Supplementary data, Table S1). Due to the fact that the embryos were fertilized *in vivo*, they were naturally asynchronous; the choice for a cell number interval offsets the natural differences of cell cycle progression between embryos, thereby making the blastocysts comparable across the environments of ART culture protocols and oviduct. Eighty eight and 57 such blastocysts were scored in the B6C3F1xCD1 genotype and the B6C3F1xC57Bl/6 genotype, respectively (Fig. 3B). Overall, taking the counts of Cdx2-, Nanog- and Sox17-positive cells together, χ^2 frequencies are different in ART blastocysts compared with *in vivo* and *in vitro* controls ($P \leq$ 8.47E-48 in CD1 paternal background; $P \leq$ 4.94E-13 in C57Bl/6 paternal background; Supplementary data, Table S2). In particular, we examined the role of the first wave of embryonic lineage formation by taking the sum of Nanog- and Sox17-positive nuclei as a valid approximation of the cells present in the ICM. In the CD1 paternal background, χ^2 frequencies of the ICM (Sox17 + Nanog) and TE (Cdx2) cell populations are conserved across ART protocols ($P \geq 0.083$). However, when the pEnd (Sox17) and pEct (Nanog) cell populations are compared with each other, a significant difference is apparent across ART protocols relative to *in vivo* control ($P \leq$ 1.28E-04). Conversely, in the C57Bl/6 paternal background, χ^2 frequencies of pEnd (Sox17) and pEct (Nanog) cell populations are conserved when compared with *in vivo* control ($P > 0.654$). The χ^2 frequencies of ICM and TE cells are different in protocol G compared with *in vivo* control, and in protocol G compared with protocol F ($P \leq$ 2.11E-03; see Supplementary data, Table S2 for all possible pairwise comparisons).

Because cell lineage imbalances may be active or passive in origin—active when cells take on a different fate autonomously, passive when cells die disproportionately in another lineage—we quantified the prevalence of cell death *in situ*. Apoptotic cells exclude the red-fluorescent dye PI but let the green-fluorescent dye YOPRO-1 leak in, whereas necrotic cells let both dyes in (Gawlitta *et al.*, 2004). Staining of live ART blastocysts with PI and YOPRO-1 resulted in PI signal invariably present in a single cell (second polar body), and YOPRO-1 signal diffused in the whole embryo, with different intensities across the culture protocols and controls (Supplementary data, Fig. S1). Although cultured blastocysts have a higher YOPRO-1 signal compared with *in vivo* counterparts, and ART blastocysts from protocol F have higher YOPRO-1 signal than *in vivo* control blastocysts in both genetic backgrounds (Dunnett test, $P < 0.0004$), they present no dead cells except the second polar body. Therefore, we ascribe the observed imbalance between TE, pEct and pEnd to an active process, whose mode of action is consistent with an alteration of the first cell lineage decision in B6C3F1 × C57Bl/6 blastocysts, as opposed to an alteration of the second cell lineage decision in B6C3F1 × CD1 blastocysts (Fig. 3B).

ART blastocysts of high and low fetal potential present differences of gene expression in metabolic but not cell-fate genes

Imbalances of blastocyst lineage composition may be rooted in the function of relevant gene pathways, as documented for the ICM by both the loss and gain of function of the transcription factors Pard3 and Carm1, respectively (Plusa *et al.*, 2005; Parfitt and Zernicka-Goetz, 2010). We reasoned that genes accounting for the imbalance should exhibit a broader variation of mRNA level across ART protocols compared with housekeeping genes. We screened the transcriptomes of ART blastocysts for candidate genes using the Illumina MouseWG6 v2 expression BeadChip microarray, which covers the mouse genome with 25 697 probes for 19 100 non-redundant genes. Mean gene expression values of three biological replicates were used for comparative analysis.

Overall, the blastocyst transcriptomes across ART protocols display broad similarity (Fig. 4) that extends to imprinted genes (Supplementary data, Fig. S2). Among the imprinted genes, we examined the oppositely imprinted genes *H19* and *Igf2* for their highly characterized role in the regulation of feto-placental growth and in the pathogenesis of mouse and human imprinting disorders. Our data show no significant differences in the expression levels of these two genes, whatever the genetic background and culture environment (Fig. 5). For the non-imprinted genes, expression fold-changes were assessed based on 2 $\log_2$, i.e. 4-fold differences. In the C57Bl/6 paternal background, 104, 53 and 169 genes were called differently expressed in protocols F, G and *in vitro* control, respectively, relative to *in vivo* control; of these genes, 62, 36 and 129 were called differently expressed only in the culture protocol concerned (Fig. 6A). In the CD1 paternal background, calls were made for 241, 209 and 674 genes in protocols F, G and *in vitro* control, respectively; of these genes, 91, 109 and 518 were

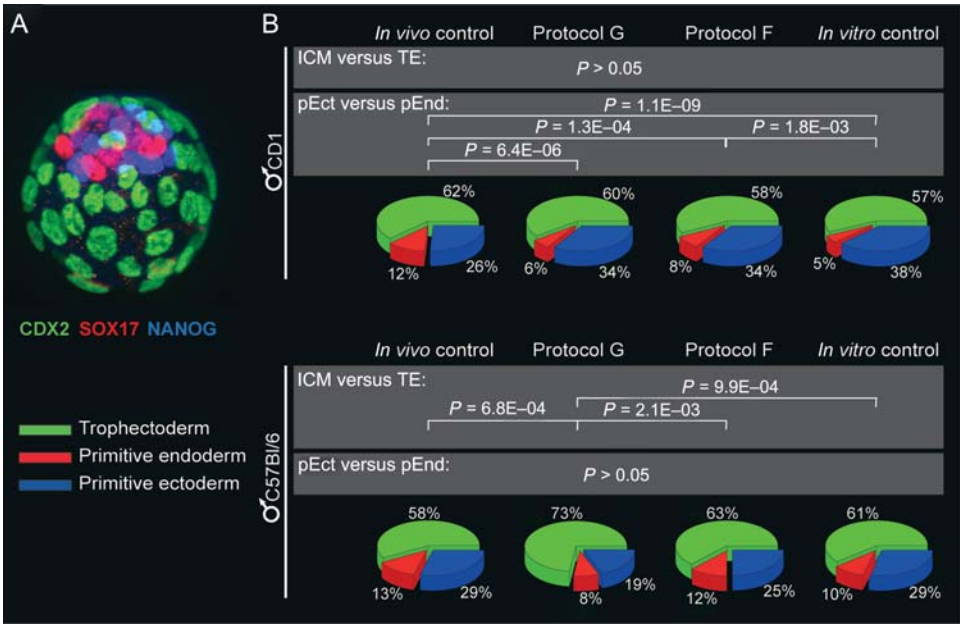

Figure 3 (**A**) Stack of *z*-confocal series of a representative blastocyst after immunostaining. (**B**) Pie charts showing the proportion of cells of the three lineages within blastocysts, sorted by growth environment and paternal genetic background (CD1 or C57Bl/6). *P*-values are based on χ^2.

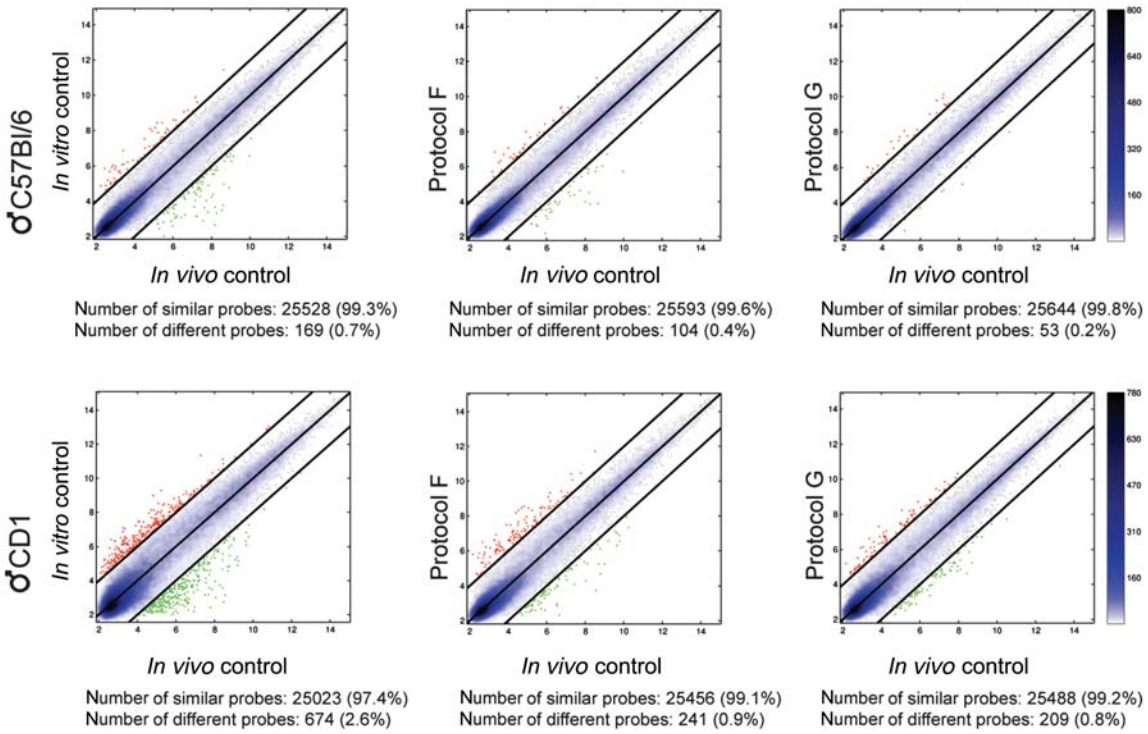

Figure 4 Pairwise comparisons (scatter plots) of gene expression values attained by blastocysts grown in medium versus *in vivo* counterparts. The black lines indicate 4-fold changes in gene expression levels between the paired embryo types. The color bar to the right indicates the scattering density (the higher the scattering density, the darker the blue). The gene expression levels are $\log_2$ scaled. The up- and down-regulated gene transcripts are marked as red and green dots, respectively.

called differently expressed only in the culture protocol concerned (Fig. 6B). The intersection of the gene sets of each culture protocol across the two genetic backgrounds (Fig. 6A and B) revealed a set of 38 genes that are differently expressed in a protocol-specific manner in both backgrounds: 8 genes in protocol F, 3 genes in protocol G and 27 genes in the *in vitro* control (Fig. 6C). The identified set of genes, while very small, is robust in that it is common to outbred and inbred mouse embryos.

These 38 genes are mostly lower expressed in cultured blastocysts compared with *in vivo* counterparts (22 of 38 genes), as visualized with the color code in Figure 7. Expression analysis using the coefficient of variation (CV; Hendriks and Robey, 1936) shows that the levels of the 38 mRNAs span a range 3.9 times broader than that of housekeeping genes. In contrast, mRNA levels of genes with established role in cell lineage regulation as well as genes proposed to be prognostic of embryo survival in uterus (*B3gnt5*, *Wnt3a*, *Eomes*; Parks *et al*., 2011) span the same range as housekeeping genes (Figure 7). Except for two transcription factors (*Elf5*, *Irx2*), the 38 genes have ontologies related to cytoplasm and enzymatic/metabolic activity. A GO enrichment analysis of the genes differently expressed by 1 $\log_2$, i.e. 2-fold in cultured blastocysts relative to *in vivo* control reveals that terms related to metabolism prevail among the top five significantly enriched terms of GO categories molecular function (MF), biological process (BP) and cellular component (CC) (Supplementary data, Table S3). Taken together, these results establish that the observed differences of gestation rate are preceded by selective effects of ART protocols on metabolic gene expression. The roles of these genes and how they might affect the blastocyst's cell lineage makeup are discussed.

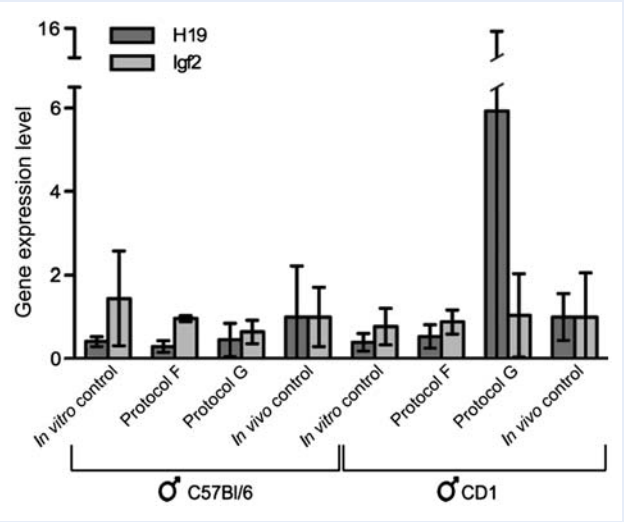

Figure 5 Expression levels of imprinted genes *H19* and *Igf2* in blastocysts are presented relative to expression levels of *in vivo* controls. Error bars indicate standard deviations (three biological replicates per protocol).

Discussion

Using a mouse model of human reproduction, we have established that the use of various pre-implantation stage culture protocols specified for ART results in different rates of fetal development and different litter size. As confounding effects of subfertility and genetic heterogeneity are reduced to a minimum in the mouse model compared with ART patients, and as mouse ART embryos were all transferred to recipients at the blastocyst stage, the observed differences in fetal development reveal that the ART blastocysts had acquired distinct developmental potentials depending on their culture environment. We reason that the effects reported here in the mouse may apply also in human, because none of the ART media presently available can practically be optimized for human embryo development, as exemplified by the requirement for 2160 mouse zygotes to optimize one culture medium for a single genotype (Lawitts and Biggers, 1991). Nonetheless, outcomes from a study comparing two media in consecutive rounds of human IVF are consistent with our findings and suggest that the choice of medium can influence gestation rates (Dumoulin *et al*., 2010). Likewise, a retrospective comparison of the developmental competence of human intracytoplasmic sperm injection embryos produced over several years has associated two different media types (other than the above) with different gestation rates

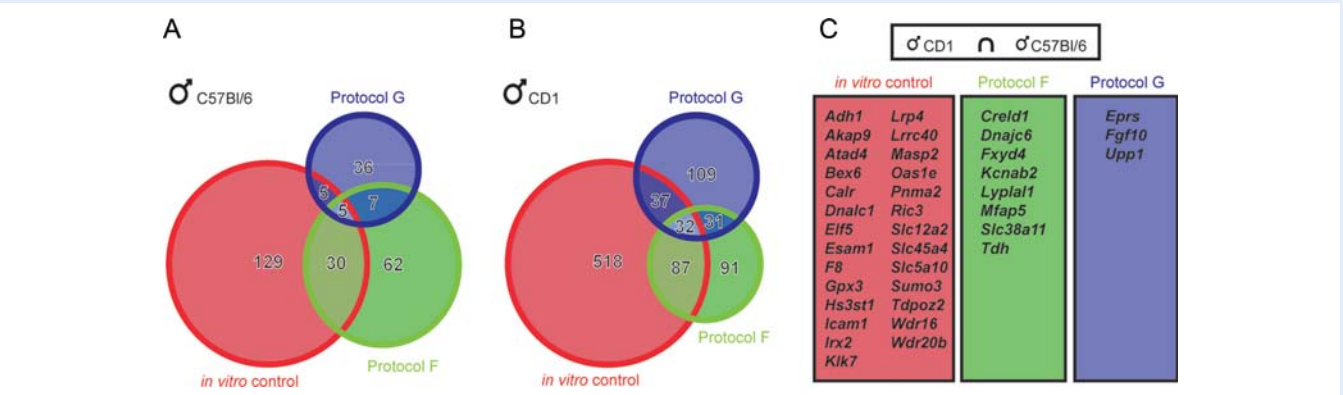

Figure 6 Venn diagrams showing the intersection of the gene sets that are differently expressed (4-fold threshold) in blastocysts from protocol F, G and *in vitro* control, relative to *in vivo* counterparts. (**A**) Gene sets of blastocysts with C57Bl/6 father. (**B**) Gene sets of blastocysts with CD1 father. (**C**) Genes that are higher or lower expressed (4-fold) simultaneously in both genetic backgrounds, in culture medium relative to *in vivo* counterparts.

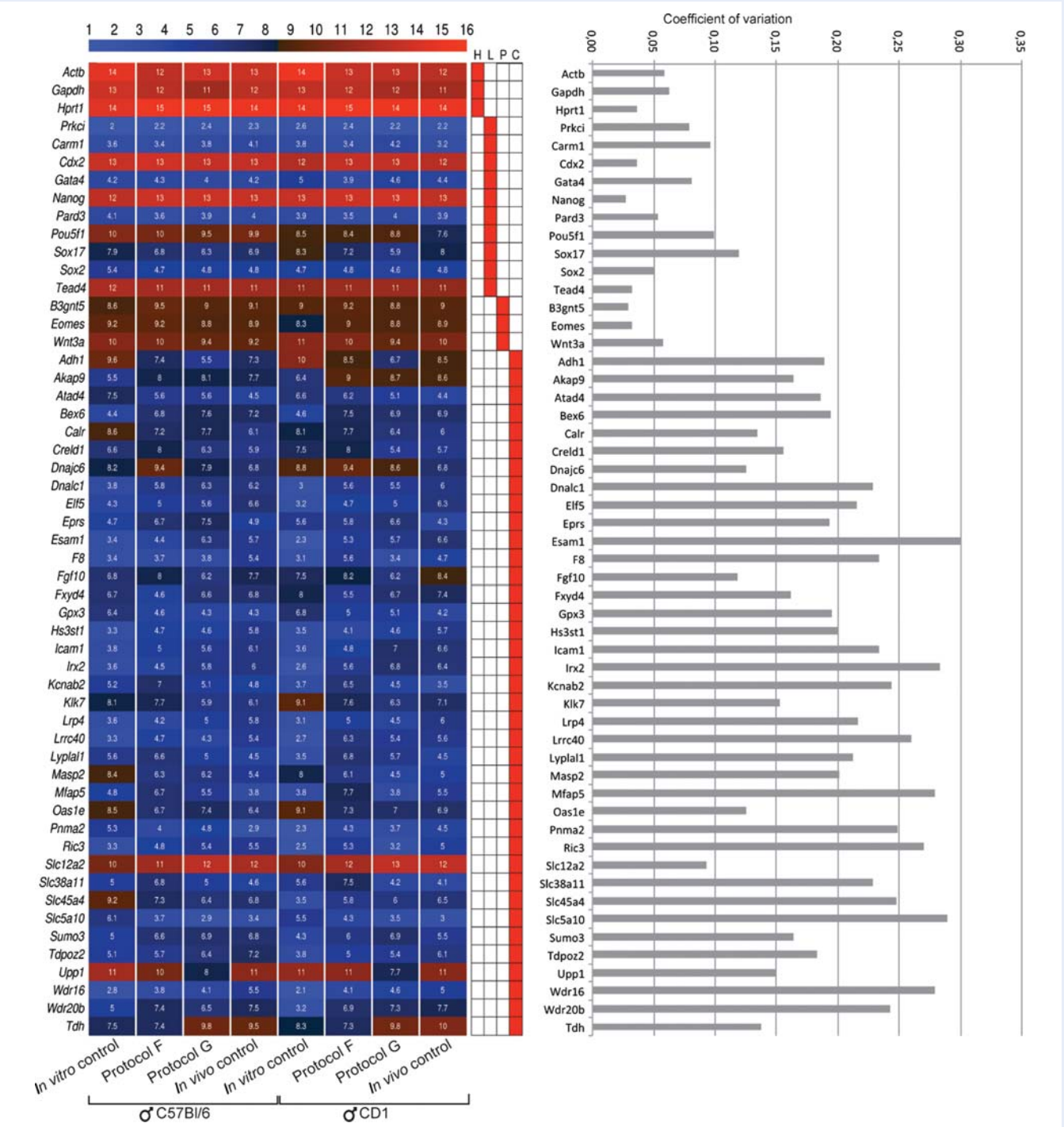

Figure 7 Heat map of gene expression levels in blastocysts from protocol F, G as well as *in vitro* and *in vivo* controls. The string of red rectangles indicates housekeeping genes (H), established lineage regulator genes (L), genes with value that is prognostic of fetal development (P) and genes belonging to the 'core of 38' described in this study (C). The color bar at the top codifies the gene expression in $\log_2$ scale. The higher the gene expression level, the more red the color. The histogram to the right shows the CVs for each gene.

(Xella *et al.*, 2010). However, comparable differences were not observed in independent studies in which the zygotes had been divided initially into two groups and cultured in parallel in two media types (Aoki *et al.*, 2005; Hambiliki *et al.*, 2011). It may be noted that all these culture media have also been included in our study, but they produced contrary results in mice compared with humans, namely: ART media reported to perform differently in humans belong to the same cluster in mice (Fig. 1B), while ART media reported to perform similarly in humans belong to different clusters. All of these observations render it unlikely that a definite

conclusion on the effects of ART culture protocols on human development can ultimately be achieved, since it is difficult to resolve the effects of embryo culture *per se* from those of other factors that relate to the genotype and life history of ART patients. At present, these sources of variability can be minimized only in an animal model. Using a mouse model of human reproduction, we examined 13 ART culture protocols and we determined that they prepare embryos differently for post-implantation development—the question being how.

We tested the hypothesis that ART protocols influence the fate decisions that commit the blastomeres to the blastocyst's cell lineages. We examined the number of TE, pEct and pEnd cells in ART blastocysts of two genetic backgrounds (B6C3F1xCD1 and B6C3F1xC57Bl/6), and found that blastocysts that developed under the opposite protocols F (high fetal rates) and G (low fetal rates) and in the control environments (*in vivo*, *in vitro*) had different total numbers of cells in both genetic backgrounds examined. However, the linear correlation between total cell numbers and fetal rates was low ($R^2 \leq 0.303$). Fine analysis of cell lineage allocation in ART blastocysts affirmed the same total number of cells, 40 ± 10, to rule out that asynchrony of fertilization and consequently, variation in the number of cell cycles completed, caused the observed effect on the proportions of TE, pEct and pEnd cells. When we sampled the subset of blastocysts composed of 40 ± 10 total cells, we were surprised to observe different proportions of TE, pEct and pEnd cells (χ^2 test, $P < 0.05$). In particular, ART blastocysts with a CD1 father have pEct/pEnd ratios that vary across the environments of protocols F and G and the two controls, while ICM/TE ratios are conserved. In contrast, ART blastocysts with a C57Bl/6 father differ in the ICM/TE cell number ratio but not in the pEct/pEnd ratio, across the four environments. Given the remarkable ability of mouse embryos to survive after bisection, which disproportionally reduces the number of ICM cells to less than half the normal number (Papaioannou and Ebert, 1995), it may not surprise us that the ART blastocysts of the present study could develop further despite 10% imbalance of the founder cell lineages. Surprising is that, for the first time, such effects were seen in the context of ART.

Imbalances of cell lineage composition were previously observed as the consequence of major metabolic perturbation (Fraser *et al*., 2007), forced activation of gene expression pathways (Plusa *et al*., 2005) or use of mechanical cues (Motosugi *et al*., 2005; Kurotaki *et al*., 2007), but not as the consequence of the mere choice of ART culture medium. For example, Fraser *et al*. (2007) observed that CD1 mouse embryos subjected to 'hyperglycemia' in culture media of the KSOM family formed blastocysts with a disproportionate reduction of cell number in the ICM compared with TE, resulting in a drop of ICM/TE ratio from 0.43 to 0.27. Interestingly, ICM-derived mouse ES cells seem to benefit from increased glucose supply (Fernandes *et al*., 2010), raising the question of whether an ES cell test (Marx-Stoelting *et al*., 2009) could possibly model the responses of pre- and peri-implantation embryos to culture conditions and thereby replace the MEA. When Plusa *et al*. (2005) manipulated the expression of *Pard3* and *aPKC* (*Prkci*) via RNA interference and dominant negative protein, respectively, they observed that the cell progeny of the manipulated blastomeres had been directed toward the ICM, although the proportion of ICM cells over the total cells had not changed. Mechanical forces and space constraints exerted by zona pellucida on blastomeres may also bias their allocation to the blastocyst lineages (Motosugi *et al*., 2005; Kurotaki *et al*., 2007). In contrast to metabolism, cell fate and physical manipulation, the death of cells preferentially in one lineage does not explain observed imbalances of cell lineage, as shown by our YOPRO1-PI staining data. At any rate, the prevalence of apoptosis would be low in mouse blastocysts (Brison and Schultz, 1997), and high only in media that were not originally intended for embryos, such as Ham's F10 (Xie *et al*., 2006), and would become substantial only in the haploid genome condition (Liu *et al*., 2004).

We pursued the processes and genes that underlie the biological response of mouse embryos to ART culture protocols. We reasoned that genes accounting for the imbalance of lineage composition as well as for the different fetal rates of ART blastocysts would exhibit a broader variation in mRNA levels compared with genes that do not play a significant role in this process. The transcriptomes of blastocysts cultured under ART protocols F and G were highly similar, with ≈1% (i.e. $n \leq 241$) of the total transcripts being differently expressed compared with *in vivo* counterparts, based on 4-fold thresholds. The high transcriptome similarity extends to imprinted genes. Likewise, Rinaudo and Schultz (2004) found 114 genes mis-expressed after culture in the suboptimal Whitten's medium, as compared with 29 genes mis-expressed in the optimized medium KSOM(aa). At face value, these similar findings lend support to the view that *in vitro* culture is less consequential for gene expression than the method of fertilization (Giritharan *et al*., 2010). While it may well be so, Morgan *et al*. (2008) found that culturing embryos in Sydney IVF Cleavage/Blastocyst medium (protocol L in our study) caused the epigenetically labile allele 'Agouti Viable Yellow' to be differently expressed compared with natural matings or ET alone, resulting in a different coat color of these mice after birth. Therefore, in spite of minimal transcriptome variation, ART culture protocols may elicit highly specific and consequential changes of gene expression that result in overt phenotypes. We searched the transcriptomes of ART blastocysts for significant differences of mRNA level compared with *in vivo* control. We pursued these genes by performing Venn diagram intersection of the differently expressed genes based on 4-fold thresholds. Only 8, 3 and 27 genes are differently expressed in ART protocols F and G and in the *in vitro* control relative to *in vivo* control, respectively. Expression levels of these 38 genes have higher CVs than those of housekeeping genes. The CVs were also higher than those of genes with a proved role in cell lineage regulation [*Pou5f1*, *Sox2*, *Pard3* and *Carm1* (Plusa *et al*., 2005; Parfitt and Zernicka-Goetz, 2010) for the ICM; *Tead4* and *Cdx2* for the TE; *Sox17* and *Gata4* for the pEnd; *Nanog* for the pEct] as well as genes proposed to be prognostic of successful implantation and healthy fetal development (*B3gnt5*, *Wnt3a* and *Eomes*; Parks *et al*., 2011). GO analysis of these 38 genes reveals that GO terms related to metabolism and sterol biosynthesis prevail among the top five terms of the statistically significant enriched GO categories MF, BP and CC. An MGI database query (http://www.informatics.jax.org) for the mouse phenotypes associated with these 38 genes reveals that dysregulation of these genes would not impair development to term. Phenotypes are related to inflammatory and immune response (*Icam1*), growth retardation (*Slc12a2*), hypotension (*Slc12a2*), decreased cardiac cell mass (*Calr*) and reduced kidney Selenium levels (*Gpx3*).

How can minute differences of blastocyst gene expression, in terms of number of genes concerned, result in marked differences of fetal

development? We envision a post-transcriptional scenario. We propose that when it comes to genes coding for metabolic enzymes, cells have the additional possibility of changing their molecular phenotype without significantly changing gene expression, depending on the availability and concentration of metabolic substrates and cofactors. Enzymes like lactate dehydrogenase have higher or lower activity depending on the concentration of nicotinamide adenine dinucleotide^{+} (Lane and Gardner, 2000) and obviously of lactate. More mouse fetuses have been obtained from embryos cultured for 3 days in high levels of lactate, although blastocyst formation was rather similar (Gardner and Sakkas, 1993). Examples of effects of medium compositions on mouse development can be extended. Culture of (C57Bl/6 x CBA)F1 embryos in Whitten medium resulted in blastocysts with a reduced mitotic index in the ICM and an increased mitotic index in the TE, compared with *in vivo* blastocysts (reduction of ICM/TE ratio; Harlow and Quinn, 1982). No study, to date, has analyzed the effects of culture medium composition on pEct and pEnd, which together form most of the egg cylinder. Wales *et al.* (1995) analyzed the metabolism of glucose by ectoplacental cone and egg cylinder of E6.5 mouse concepti. Authors concluded that glucose metabolism of ectoplacental cone and egg cylinder reflects the difference existing between precursor tissues in the blastocyst. In mouse ES cells, L-proline and L-ornitine force transition toward an epiblast (pEct) stem cell-like phenotype (Casalino *et al.*, 2011). It follows that ART culture protocols entailing different concentrations of energy substrates or amino acids could shift the ICM/TE and the pEct/pEnd balance, respectively. Unfortunately, the proprietary nature of ART media (Biggers, 2000) often prohibits the solute concentrations from being disclosed, thereby preventing the effects of these and other medium components from being tackled experimentally.

To summarize, our data show unequivocally that the choice of the ART culture protocol prepares mouse embryos differently for post-implantation development through defined cellular and molecular responses to culture media. Even though the mouse responses to ART are not transferable 1-to-1 to human due to structural and physiologic differences between oocytes of the two species (Hardy *et al.*, 1989; Gott *et al.*, 1990; Leese and Ferguson, 1999; Menezo and Herubel, 2002; Roberts, 2005; Benkhalifa *et al.*, 2010), our data promote awareness that culture media may not be considered interchangeable let alone neutral toward embryo development, in the context of ART. Although cases of medium-dependent ART outcomes have been reported in humans (Dumoulin *et al.*, 2010; Xella *et al.*, 2010), they remain difficult to investigate due to the broad genotypic variation of our species and the unique life histories of ART patients.

In conclusion, we subscribe to an emerging view that *in vitro* culture may provide embryos with an opportunity to exploit resources of mammalian development that are not used during the natural (i.e. *in vivo*) process (Vajta *et al.*, 2010). Accordingly, resultant newborns should not be considered 'superior' or 'inferior', but simply 'different' when compared with natural concepti. A question remains open: by designing appropriate animal experiments, shall we ever be able to reach any firm conclusion about the effects of ART culture protocols, if any, on human development? Probably not, but we may be able to perform cautious extrapolation from model species such as the mouse or other mammals. Despite the obvious 'distance' between the mammalian species, evolutionarily acquired differences become more and more prominent as development unfolds, as exemplified in the placenta (Knox and Baker, 2008); that is, early developmental stages share more similarities than differences even across distantly related taxa.

Supplementary data

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

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Authors' roles

C.S. and T.C.E. performed the experiments and collected the data. M.B. designed the study, analyzed the data except the microrray data and wrote the manuscript with input from C.S. and the other coauthors. M.J.A.B. performed the microarray data analysis. V.N. supplied the ART culture media, advised on how to use them and helped designing the *in vitro* culture experiments. S.L.G. and S.S. co-designed the study with M.B.

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Conflict of interest

None financial or professional. V.N. is an Associate Editor of *Human Reproduction*.

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