Transcriptional signatures throughout development: the effects of mouse embryo manipulation in vitro

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Abstract

Stressful environmental exposures incurred early in development can affect postnatal metabolic health and susceptibility to non-communicable diseases in adulthood, although the molecular mechanisms by which this occurs have yet to be elucidated. Here, we use a mouse model to investigate how assorted in vitro exposures restricted exclusively to the preimplantation period affect transcription both acutely in embryos and long term in subsequent offspring adult tissues, to determine if reliable transcriptional markers of in vitro stress are present at specific developmental time points and throughout development. Each in vitro fertilization or embryo culture environment led to a specific and unique blastocyst transcriptional profile, but we identified a common 18-gene and 9-pathway signature of preimplantation embryo manipulation that was present in all in vitro embryos irrespective of culture condition or method of fertilization. This fingerprint did not persist throughout development, and there was no clear transcriptional cohesion between adult IVF offspring tissues or compared to their preceding embryos, indicating a tissue-specific impact of in vitro stress on gene expression. However, the transcriptional changes present in each IVF tissue were targeted by the same upstream transcriptional regulators, which provide insight as to how acute transcriptional responses to stressful environmental exposures might be preserved throughout development to influence adult gene expression.


Introduction

There is robust epidemiological and animal evidence that exposure to different environmental conditions during early development affects postnatal growth, metabolism and disease susceptibility in adulthood. As dictated by the Developmental Origins of Health and Disease (DOHaD) hypothesis, poor or suboptimal developmental experiences—including nutritional, oxidative or in vitro stress—can predispose chronic diseases, including hypertension and components of the metabolic syndrome (Gillman 2005).

Arguably the most pressing question in the DOHaD field is identifying how the molecular changes occurring in a developing organism secondary to adverse environmental or nutritional exposures alter the growth and metabolic trajectories across the life course. Interestingly, many models of DOHaD exhibit common long-term outcomes, including glucose intolerance, hypertension and vascular dysfunction, irrespective of both the form of developmental stress and the timing of exposure (Simmons et al. 2001, Jungheim et al. 2010, Watkins et al. 2011). This suggests that common mechanisms may be involved in both sensing and transducing the environment stimuli, leading to a reprogrammed epigenetic, transcriptional and/or metabolic state that could potentiate an increased susceptibility for metabolic disease throughout postnatal life. As a result, understanding the mechanisms by which the developing embryo and fetus assimilate environmental input into a programmed metabolic response is relevant across multiple DOHaD fields.

Appropriate to this discussion is the evidence that preimplantation development is an important period of environmental sensitivity and that stresses incurred within this window can affect glucose metabolism, β-cell function, blood pressure and fat deposition in adulthood (Kwong et al. 2000, Fernandez-Gonzalez et al. 2004, Watkins et al. 2010, Rexhaj et al. 2013, Donjacour et al. 2014). The preimplantation period is a particularly advantageous model for investigating DOHaD-related questions because exposures are limited to a time frame of 4–5 days and embryos exhibit cellular uniformity (only 2 cell types are formed after 4 days in culture: the...
inner cell mass and trophectoderm)—thereby providing an opportunity to connect the precise variations in exposure to outcome. In addition, embryo manipulation holds wide clinical significance due to the routine use of assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF), which has resulted in the birth of over 5 million children as of 2012 (ICMART 2012).

It is also clear that increasing the severity of a particular stressor leads to progressively worse phenotypes in adulthood. For example, mouse IVF and embryo culture performed under clinically optimized conditions affect the expression of nearly 300 genes in blastocysts but do not alter adult glucose tolerance. Conversely, increasing embryo culture stress with a higher oxygen tension (20% O$_2$) or an inferior culture medium exacerbates the transcriptional changes (over 2000 misexpressed genes in blastocysts) and results in significant glucose intolerance in adulthood (Fig. 1) (Donjacour et al. 2014, Feuer et al. 2014).

Over the past 15 years, the Rinaudo laboratory has performed microarray studies in mice to examine how the many different components of *in vitro* embryo manipulation (culture medium composition, oxygen tension and method of fertilization) have influenced transcriptional profiles in both blastocysts and adult offspring tissues (Rinaudo & Schultz 2004, Rinaudo et al. 2006, Giritharan et al. 2007, 2010, 2012, Feuer et al. 2014). These experiments have been conducted by several scientists in multiple locations, using various microarray platforms and analysis software. Further, there have been significant advancements in our knowledge of the genome over the past decade. This manuscript provides a systematic analysis and integration of the gene expression changes present in (1) blastocysts exposed to assorted culture conditions and methods of fertilization and (2) selected adult tissues from offspring generated by *in vitro* fertilization. The ultimate goal of this study was to compare the transcriptional profiles to identify common signatures of *in vitro* embryo manipulation and to determine if a relationship exists between the acute transcriptional response to *in vitro* stress and subsequent adult gene expression.

**Methods**

**Animals**

All animals were maintained according to institutional regulations and NIH guidelines, under a constant 12 h light/darkness cycle with *ad libitum* access to water and standard chow (PicoLab #5058). The strains used in this study included vasectomized CD-1 males, B6D2F1/J males, CF-1 females and C57Bl/6J males and females. Beginning at 24 weeks, the postnatal IVF and control inbred C57Bl/6J cohorts were placed on a high-fat diet (Research Diets, Inc. #D12492) until time of death at 29 weeks. The postnatal IVF and control outbred CF1 x B6D2F1 cohorts were maintained on a standard chow diet (20% protein, 9% fat, LabDiet) until time of death at 40 weeks.

**Embryo generation, culture and collection**

A detailed methodology of the embryo collection and transfer techniques has been published and may be found in the following: (Rinaudo & Schultz 2004, Rinaudo et al. 2006, Giritharan et al. 2007, 2010, 2012, Feuer et al. 2014). Briefly, CF-1 females aged 6–8 weeks were injected with 5 IU PMSG followed 46–68 h later by 5 IU hCG to induce superovulation. For *in vitro* culture (IVC) experiments, superovulated dams were mated overnight post-hCG injection with B6D2F1/J males. The next morning, fertilized zygotes were flushed from ampullae, washed and cultured to the blastocyst stage at 37°C under Ovoil (Vitrolife, #10029) with 5% CO$_2$ in a modular humidified chamber. At the two-cell stage, all unfertilized oocytes were removed. Culture conditions included either Whitten’s medium (WM (Whitten 1971); made in house) or potassium simplex optimization medium supplemented with amino acids (KAA (Ho et al. 1995); Millipore, MR-106-D), with 5% or 20% oxygen (4 possible conditions).

For *in vitro* fertilization (IVF) experiments, 13–15 h after hCG administration, the cumulous-oocyte complexes were isolated from ampullae and incubated 4–6 h in HTF medium (Millipore, MR-070-D) with capacitated (1 h) cauda epididymal sperm from B6D2F1/J males. Fertilized zygotes were washed and cultured
to the blastocyst stage in WM and 20% oxygen as described previously. A second IVF cohort was generated using C57Bl/6j animals and cultured under KAA and 5% oxygen conditions.

For intracytoplasmic sperm injection (ICSI) experiments, oocytes were obtained the morning after superovulation as described for IVF. Cauda epididymides were isolated from B6D2F1/J males aged 10–11 weeks, punctured gently with 30-G needles and incubated 10 min in a microcentrifuge tube at 37°C in pre-warmed 1 mL EGTA Tris–HCl buffered solution (10 mM Tris–HCl pH 8.2, 50 mM EGTA and 50 mM NaCl). ICSI was performed using the top 800 μL sperm suspension, and oocytes containing the first polar body were microinjected using a Piezo drill (PMM Controller, Prime Tech) as described by Li and coworkers (2009). Fertilized zygotes were washed and cultured to the blastocyst stage in WM and humidified air.

To obtain late-cavitating blastocysts of similar morphology, embryos were harvested at different time points: after 96–100 h (IVC), 106–110 h (IVF) or 112 h (ICSI) and snap-frozen for microarray experiments. The in vivo-derived control group was represented by blastocysts isolated from naturally mated superovulated dams 96 h after hCG injection (CF-1 × B6D2F1/J, or C57Bl/6j, as indicated in the text). At all times, the experimental groups were compared to the same in vivo control breeds. All embryo generation experiments were performed ≥4 times.

We have previously shown that IVF and in vivo embryos derived by these protocols contain a similar number of inner cell mass (ICM) cells (12.8 ± 0.4 vs 13.8 ± 0.5, not significant) (Giritharan et al. 2012). ICM was isolated from CF-1 × B6D2F1 control and IVF blastocysts cultured in WM and 20% oxygen by immunosurgery (n = 3 times per treatment group). Briefly, trophectoderm cells were lysed by incubating embryos 30 min in WM containing 20 μg/mL anti-mouse rabbit antibodies (Sigma) and 30 min in WM with 5 μg/mL rabbit complement (Invitrogen) at 37°C. ICM samples were cleaned of destroyed TE via repeated pipetting using a glass pipette with a 30–40 μm diameter under a dissecting microscope. Upon collection, ICM samples were immediately transferred to cell lysis buffer provided in the PicoPure RNA Isolation Kit (Molecular Devices) and frozen at −80°C.

**Embryo transfer**

For postimplantation cohorts, pseudopregnancy was induced by mating naturally cycling CF-1 females to vasectomized CD-1 males, confirmed by the presence of a copulation plug the next morning (considered day 0.5). Late-cavitating blastocysts were transferred to the uterine horns of recipients on day 2.5 of pseudopregnancy. For control experiments, superovulated dams were mated overnight; embryonic day 3.5 blastocysts (96 h after hCG administration) were flushed from the uterine horns and transferred immediately to the uterine horns of CF-1 recipients. This experimental strategy controls for litter size and any effects of superovulation or the embryo transfer procedure, both of which can influence imprinted gene expression (Fortier et al. 2008, Rivera et al. 2008). Resulting pups were not cross-fostered, as this procedure may alter adult phenotypes and imprinted gene expression (Hager et al. 2009, Matthews et al. 2011).

**Microarray preparation**

Comprehensive descriptions of RNA extraction, amplification, fragmentation and hybridization to Affymetrix GeneChips for each microarray experiment may be found in the following: (Rinaudo & Schultz 2004, Rinaudo et al. 2006, Giritharan et al. 2007, 2010, 2012, Feuer et al. 2014). Table 1 outlines the individual microarrays presented or re-analyzed in this study.

For embryo studies, total RNA was extracted either with TRIZol containing 2 mL Pellet Paint (Novagen) or a PicoPure RNA Isolation Kit (Arcturus) from 3–4 pooled embryo replicates comprising 80 blastocysts (IVC), 10 blastocysts (IVF or ICSI) or 40 ICM samples. RNA samples were submitted for preparation and hybridization to the University of Pennsylvania Microarray

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**Table 1** Summary of the microarray experiments used in this study.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fertilization</th>
<th>Culture</th>
<th>Strain</th>
<th>Tissue</th>
<th>Platform</th>
<th>Location</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of embryo culture</td>
<td>Natural (IVC)</td>
<td>KAA 5% O₂, WM 5% O₂, KAA 20% O₂, WM 20% O₂</td>
<td>CF1 × B6D2F1/J</td>
<td>Blastocyst</td>
<td>Affymetrix MOE 430A (22,690 probes)</td>
<td>University of Pennsylvania</td>
<td>Rinaudo and Schultz (2004), Rinaudo et al. (2006)</td>
</tr>
<tr>
<td>Effect of fertilization</td>
<td>IVF</td>
<td>KAA 5% O₂, WM 5% O₂, KAA 20% O₂, WM 20% O₂</td>
<td>CF1 × B6D2F1/J</td>
<td>blastocyst</td>
<td>Affymetrix MOE 430.2 (45,101 probes)</td>
<td>University of California, San Francisco</td>
<td>Giritharan et al. (2007, 2010)</td>
</tr>
<tr>
<td>Long-term effects: adult tissues</td>
<td>IVF</td>
<td>KAA 5% O₂, C57Bl/6j, 29 week female</td>
<td>Liver, Gonadal fat, Skeletal muscle, Pancreatic islets</td>
<td></td>
<td>Affymetrix Mouse 1.0 ST array (&gt;770,000 probes)</td>
<td>University of California, San Francisco</td>
<td>Feuer et al. (2014)</td>
</tr>
<tr>
<td>Long-term effects: adult tissues</td>
<td>IVF</td>
<td>WM 20% O₂, CF1 × B6D2F1/J, 40 week male</td>
<td>Heart</td>
<td></td>
<td>Affymetrix Mouse 1.0 ST array (&gt;770,000 probes)</td>
<td>University of California, San Francisco</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Cell type specificity</td>
<td>IVF</td>
<td>WM 20% O₂, CF1 × B6D2F1/J</td>
<td>Inner cell mass (ICM)</td>
<td></td>
<td>Affymetrix MOE 430.2 (45,101 probes)</td>
<td>University of California, San Francisco</td>
<td>Giritharan et al. (2012)</td>
</tr>
</tbody>
</table>
Facility (IVC) or the Genomic Core Facility at the University of California, San Francisco (IVF, ICSI and ICM).

In adult offspring, microarray analysis was performed on two postnatal cohorts: (1) 29-week-old C57Bl/6j female gonadal fat, liver, skeletal muscle and pancreatic islets derived from IVF KAA-5% oxygen or naturally (n=3 per condition, with each animal providing the 4 tissues to minimize variation), and (2) 40-week-old CF1 × B6D2F1/J male cardiac tissue derived from IVF WM-20% oxygen or naturally (n=3 per condition). Animals contributing to these analyses were selected from at least two separate litters of 5–10 pups per condition. Total RNA was extracted and purified from previously frozen tissues using the RNeasy mini kit (Qiagen), then submitted to the Gladstone Genomics Core Facility at the University of California, San Francisco for labeling, hybridization and scanning of the microarrays. Whole frozen islets were also sent to the core facility for RNA isolation and amplification prior to microarray processing.

Microarray data analysis

All microarray data were analyzed with GeneSpring GX 13.1 software (Agilent Technologies). Scanned arrays were uploaded into GeneSpring for background adjustment, summarization, log transformation and baseline transformation. Samples were interpreted by culture medium, oxygen, method of fertilization and cell type, via individual microarray platform. In addition to β-actin, Gapdh signal is used as an internal quality control in both Affymetrix MOE 430A and 430.2 arrays, and its intensity level can lead to the inclusion vs exclusion of specific samples as outliers in a dataset during a quality control analysis. We recently discovered that Gapdh is altered in IVF blastocysts compared to naturally derived control blastocysts (data not shown), suggesting that previous analysis of these microarrays may have led to the incorrect inclusion/exclusion of data. As a result, these experiments were re-analyzed excluding Gapdh signal as a quality control influence. The principal component analysis (PCA) algorithm in GeneSpring was applied to all specimens grouped by individual ART condition (including culture medium, oxygen percentage and method of fertilization) using all genes and expressed sequence tags, separated by culture medium, oxygen percentage and method of fertilization) using all genes and expressed sequence tags, separated by microarray platform, to evaluate for patterns in gene expression and underlying cluster structures. Fold-changes were calculated based on the normalized scores instead of the raw expression data. To identify differentiates genes between two groups (e.g. ART vs in vivo), an un-paired t-test with a significance threshold of P value <0.05 was applied to compare ART to control samples. The Effect-of-Culture and Cell-Type-Specificity experiments were analyzed using a 30% cutoff with Benjamin–Hochberg correction; the Effect-of-Fertilization experiments were analyzed using a 50% cutoff with Benjamin–Hochberg correction; and the long-term adult tissue data were analyzed using a 30% cutoff without correction.

Gene functional analysis and pathway analysis

Post-processing of the resulting gene lists was conducted using Ingenuity Pathway Analysis (IPA, June 2015 release), a structured knowledge repository that identifies biologically significant relationships and networks based on previously characterized functional associations of genes (http://ingenuity.com). The analysis includes canonical pathways overrepresented in gene lists, how genes and pathways integrate into broader biological networks, as well as predicted upstream regulators responsible for the cascade of gene expression changes. Only data experimentally observed in animal tissues were considered, and fold-change thresholds were set such that the number of entities contributing to each analysis remained between the recommended 100 and 2000.

Heatmaps were generated using GENE-E software developed by the Broad Institute (available at http://www.broadinstitute.org/cancer/software/GENE-E/).

Microarray validation by real-time quantitative RT-qPCR

To validate the microarray data, blastocysts derived in vivo or cultured from the zygotic stage (IVC, WM with 20% oxygen or KAA with 5% oxygen) were collected as described previously, and real-time quantitative RT-qPCR was conducted on 3–4 independent biological replicates containing 10 or more pooled blastocysts. Total RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems) and reverse transcribed to cDNA using an iScript CDNA synthesis kit (Bio-Rad Laboratories). Quantification of gene transcripts was performed in duplicate with SyBr Green PCR Supermix using 0.2 embryo equivalents of cDNA from each treatment group per reaction. Amounts of Arrd4 (Forward: 5′-CCC TTA TTG ACT CCT ATG CT and reverse: 3′-CTT CTC TAC AGT TGT CTA TGT TTG and reverse: 3′-AGT GCC GTT CTG GTG TCT C (Bloise et al. 2012)); Socs3 (Forward: 5′-GCA GGA GAG CGG ATT CTA CT and reverse: 3′-AGC CTC AAC GTG AAG TTG CCG CTC ACT AT (Rappolee et al. 1994)) transcripts were normalized to levels of histone 2A (H2A) transcript (Forward: 5′-ACA TGG CGG CGG TGC TGG AGT A and reverse: 3′-GGG GAT GCG GCT CTT CTG GTT). H2A was selected as a reference because it is stably and reliably expressed across preimplantation embryo development (Jeong et al. 2005, Kuijik et al. 2007).

Primers were designed using PerlPrimer software (http://perlprimer.sourceforge.net/) unless otherwise indicated. ART blastocyst transcriptional results are reported as normalized to gene expression in vivo.

Results

More severe preimplantation manipulation increases the levels of blastocyst transcriptional alteration

We examined the impact of in vitro manipulation on blastocyst gene expression by testing either the specific effects of different preimplantation embryo culture conditions or by varying the method of fertilization and maintaining the culture conditions constant (Fig. 2). First, superovulated and naturally fertilized zygotes were cultured in either WM or KAA, and 5% or 20% oxygen (4 conditions total) and compared to in vivo-
Effect-of-Culture Experiments

Common fertilization method, different embryo culture conditions

Figure 2 Effect of different embryo culture conditions and types of fertilization on blastocyst gene expression. Microarrays were performed on blastocysts either generated naturally and cultured in WM or KAA with 5% or 20% oxygen (Effect-of-Culture experiments, A, B, C and D) or produced by IVF, ICSI or naturally and cultured in WM and 20% oxygen (Effect-of-Fertilization experiments, E, F, G and H), with naturally derived blastocysts as controls. (A) Number of transcripts altered after each culture condition shows a strong impact of suboptimal WM and high oxygen on transcription. (B) Principal component analysis (PCA) indicates that oxygen concentration has a more robust effect on gene expression than culture medium composition. (C) Venn diagram of concordant gene misexpression across the 4 culture conditions. (D) Ingenuity pathway analysis (IPA) highlighting the predominant networks (network score ≥30) associated with the gene expression changes. (E, F, G and H) Same as A, B, C and D for the Effect-of-Fertilization experiments. (E) There was a severe effect of fertilization by ICSI on blastocyst gene expression, which (F) contributed significantly to PCA clustering. (G) Venn diagram comparing the concordance of gene misexpression after each fertilization type, with (H) top IPA networks associated with the gene lists. ICSI, intracytoplasmic sperm injection; IVC, in vitro culture; IVF, in vitro fertilization; KAA, potassium simplex optimization medium with amino acids; WM, Whitten’s medium.
generated blastocysts from superovulated dams (flushed blastocyst control). Both WM and higher oxygen tensions are considered stressful; conversely, KAA and 5% oxygen are optimal for mouse embryo culture and represent current IVF clinical practices (Schwarzer et al. 2012, Chronopoulou & Harper 2015). This gradation in stress was reflected in the number of transcripts altered by each culture condition, with WM and higher oxygen levels increasingly perturbing blastocyst gene expression (Fig. 2A). Principal component analysis revealed a marked effect of oxygen on transcription, significantly more pronounced than the relative influence culture medium composition (Fig. 2B, red circle). Each condition begat a unique blastocyst transcriptome, with gene expression perturbations associated with distinct pathways and gene networks (Fig. 2C and D). 77 genes were commonly misexpressed in all four in vitro culture (IVC) conditions and were enriched for stress response pathways, cell cycle control, cancer and pluripotency signaling (Supplementary Fig. 1, Supplementary Table 1, see section on supplementary data given at the end of this article).

We next evaluated the influence of different fertilization techniques by comparing blastocyst transcriptomes among embryos fertilized via ICSI, IVF or naturally (in vivo culture only, IVC) and cultured from zygote to blastocyst in WM and 20% oxygen, vs in vivo. ICSI—a more mechanically intrusive procedure—had a dramatic effect on transcription, altering the expression of over 7000 transcripts corresponding to 5832 genes (Fig. 2E, F and G). Unexpectedly, the IVC condition had a more prominent effect on gene expression than IVF. As with the effect-of-culture experiments, each fertilization method had a separate impact on blastocyst gene expression, with altered gene lists associated with unique pathways and networks (Fig. 2H and Supplementary Table 2). 104 genes showed concordant misexpression in each fertilization condition and were associated with a wide range of cellular processes including cytoskeletal dynamics, metabolite biosynthesis, the cell cycle and growth (Supplementary Fig. 2).

**Blastocysts exhibit a common gene signature of in vitro embryo manipulation**

Following the observation that each fertilization method or culture condition resulted in unique gene expression changes, we searched for evidence of a ubiquitous transcriptomic fingerprint indicative of a shared effect of embryo manipulation. We identified a list of 18 genes concordantly misexpressed in all in vitro conditions compared with in vivo blastocyst gene expression (Fig. 3A). Importantly, all of the genes were similarly increased or decreased compared to in vivo embryos except for Camk1 (increased in the ICSI microarray only), suggesting a common effect of in vitro manipulation on blastocyst gene expression. We chose 4 genes (Arrdc4, Fgi4, Slc7a3 and Socs3) for validation in naturally-derived blastocysts cultured from the zygotic stage (IVC) in either KAA-5% oxygen or WM-20% oxygen, which confirmed the microarray data for all conditions except for Slc7a3 expression in IVC KAA-5% O₂ blastocysts (Fig. 3B). Further, 9 pathways associated with growth, cancer, pluripotency and response to stress (Fig. 3C) were collectively enriched in the in vitro manipulated embryos.

**The impact of IVF on gene expression is cell type-specific**

Because blastocysts are composed of both trophectoderm and the inner cell mass (ICM), we further probed our embryo data by comparing the blastocyst IVF WM-20% oxygen transcriptome to blastocyst ICM derived from the same conditions (Supplementary Table 3). Of the 293 genes and 42 pathways affected in the blastocysts, only 39 genes and 2 pathways were similarly altered in the ICM (Fig. 3D), confirming that controlling for different cell types is highly relevant when analyzing the influence of in vitro embryo manipulation. Only 6 of the 39 genes, as well as both pathways (cell cycle: G1/S checkpoint regulation and p53 signaling) were also highlighted in the ‘Common Fingerprint’ of in vitro embryo manipulation (Fig. 3E and F, red text).

**There is no common gene signature of in vitro embryo manipulation in adult tissues**

Previously, we examined the long-term effects of IVF (KAA and 5% O₂) on adult offspring liver, skeletal muscle, gonadal fat and pancreatic islet transcriptomes in an inbred C57Bl/6j mouse model and found no concordant changes or common biological themes (Feuer et al. 2014). These particular tissues were selected for their involvement in either secretion of or response to insulin, as these IVF offspring show altered growth curves, pancreatic beta cell hyperinsulinemia, and are predisposed to glucose intolerance (Fig. 1). The gene expression changes were subtle (>95% under 2-fold change), and re-analysis with updated software confirmed that only one gene, Gm14403, and no pathways were altered in all four IVF tissues compared to naturally conceived controls (Fig. 4 and Supplementary Table 4), indicating a tissue- and/or cell type-specific impact of IVF on gene expression. This was corroborated by the fact that removal of the pancreatic islet data (a highly complex tissue with many cell types) led to modest overlap (38 genes and 3 pathways) among fat, liver and muscle IVF transcriptomes (Fig. 4C and E). Interestingly, pathway analysis revealed that the 38 concordantly misexpressed genes were largely associated with glucose metabolic flux (through glycolysis, the TCA cycle and

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Figure 3 A common, but cell type-specific, fingerprint of in vitro embryo manipulation. (A) A core list of 18 genes were altered by in vitro embryo manipulation, regardless of the culture conditions or the method of fertilization. (B) Real-time qPCR verification of the microarray data. Four of the 18 genes were selected for analysis in IVC blastocysts cultured in KAA-5% O₂ (green) or WM-20% O₂ (red), and the graph shows their expression relative to controls (normalized to 1) from both the microarray (MA) and PCR data. (C) 9 pathways were collectively enriched in association with the individual microarray experiments evaluating the impacts of fertilization conditions and culture conditions on blastocyst transcription. (D, E and F) A cell type-specific effect of in vitro fertilization. (D) Venn diagram showing the differential effects of IVF WM-20% oxygen culture conditions on blastocyst vs inner cell mass (ICM) gene expression and pathway association. (E) Only 39 genes and (F) two pathways were concordantly affected in both blastocysts and ICM, in spite of similar conditions of fertilization and culture in vitro. Genes and pathways also present in the ‘Common Fingerprint’ are highlighted in red. Legends (fold-changes and P value) are applicable to all heatmaps in the figure.
mitochondrial function, glucogenesiosis and UDP-N-acetylglucosamine biosynthesis, Fig. 4F).

**There is no obvious transcriptional link between in vitro embryos and their corresponding adult tissues**

Given that in vitro embryo manipulation results in transcriptional changes in both embryos and adult tissues, we next inquired if the gene misexpression in adult tissues could be attributed to or explained by the transcriptional alterations in blastocysts. We compared the KAA-5% oxygen microarray data between IVC embryos and their analogous adult IVF liver, skeletal muscle, gonadal fat and pancreatic islets to determine if different tissues were vulnerable to retaining the in vitro embryo signature (Fig. 5 and Supplementary Table 5). There were minimal commonalities between the two time points, with fewer than 2% of the genes affected in each adult IVF tissue similarly altered in the blastocyst. Further, the shared gene misexpression between embryos and adult tissues was frequently altered in opposite directions. Pathway analysis demonstrated that although the
Transcriptome of embryo correlates to manipulation and gene misexpression was highly tissue specific, some pathways were consistently enriched in both KAA-5% O₂ blastocysts and adult tissues, including cell cycle G1/S checkpoint regulation, molecular mechanisms of cancer, protein ubiquitination pathway and Huntington’s disease signaling.

Although this initial comparison found negligible transcriptional linkage between in vitro embryos and adult tissues, the findings could have been influenced by differences in mouse strain and conception condition (CF-1 × B6D2F1/J IVF blastocysts, C57Bl/6J IVF adult tissues). We therefore expanded our analysis by investigating whether more stringent and homogenous conditions would reveal a relationship between the acute transcriptional response to in vitro stress and subsequent gene expression in adulthood. Microarray was performed on 40-week-old IVF and control cardiac tissue and compared to data from ICM or whole blastocysts derived from analogous conditions (outbred CF-1 × B6D2F1 tissues conceived by IVF with WM and 20% oxygen). We selected the heart because in addition to glucose intolerance, these mice exhibit cardiac left ventricular hypertrophy (Donjacour et al., 2014), a condition that has also been reported in IVF/ICSI infants (Valenzuela-Alcaraz et al., 2013). Relative to controls, 1,443 transcripts corresponding to 1,361 genes were altered in the IVF hearts, and only 16 genes exhibited fold-changes greater than ±2-fold (Fig. 6A). 99 pathways were significantly enriched in the transcriptional profiles, with overrepresentation in the inflammatory response, hematological system development and function, cancer, cellular assembly and organization, as well as cell-to-cell signaling, interaction and movement (Fig. 6B, C and Supplementary Table 6).

When we compared the IVF WM-20% oxygen transcriptional profiles between the early embryo and adult heart, there was again minimal concordance in gene misexpression: 22 genes and 4 pathways were commonly altered in blastocysts and heart, whereas 28 genes and 1 pathway were shared between ICM and heart (Fig. 6D, E, F and Supplementary Table 5). Only 4 genes (Ccng1, Rsu1 Runx1t1 and Trp53inp1) were misexpressed in blastocysts, ICM and heart tissue (Fig. 4E, red asterisks). Surprisingly, more than half of the genes with shared misexpression were altered in opposite ways between the embryos and heart.

A set of common upstream regulators are predicted to drive the transcriptional changes in embryos and adult tissues

Ingenuity pathway analysis offers an upstream regulator prediction algorithm that evaluates gene lists and microarray fold-change to identify molecules (transcriptional regulators, growth factors, receptors, transporters, etc.) with direct actions on the differentially expressed genes. Based on fold-change directionality of the downstream target genes, IPA predicts whether upstream regulator inhibition or activation might govern the differences observed. Given the significant cell type specificity observed in each IVF tissue transcriptome, we hypothesized that common regulators might control the gene expression changes, but based on tissue or cell type, this would manifest in unique transcriptional signatures (Fig. 7 and Supplementary Tables 1–6).

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Analysis of the transcriptional alterations present in blastocysts after each condition of in vitro manipulation identified a core group of 15 regulators—comprising kinases, enzymes, growth factors, phosphatases, ligand-dependent nuclear receptors and transcriptional regulators—that collectively function directly upstream of the altered genes, 10 of which additionally target the misregulated ICM genes (Fig. 7A). The predicted activation or inhibition of these regulators was largely concordant across all of the in vitro embryo conditions.

Focusing on the embryo-to-adult comparisons (Fig. 7B and 7C), there were 7 putative regulators of the genes altered in blastocysts, ICM and adult male heart derived from the IVF WM-20% oxygen conditions, compared to in vivo controls (Fig. 7B). Based on the directionality of the gene expression changes, the majority of these putative regulators were predictably activated (e.g. functionally increased) in blastocysts and heart, but inhibited in the ICM.

Regarding the KAA-5% oxygen blastocysts and their corresponding adult female fat, liver, and muscle, 21 regulators operate upstream of the misexpressed genes at both time points (Fig. 7C). Interestingly, these molecules showed predicted inhibition in blastocyst and liver profiles, but activation in fat and muscle, suggesting that differential mediation of gene expression changes by common regulators is tissue-specific. Supplementary Figure 3 summarizes the predicted upstream regulators shared by the ART embryo expression profiles and the condition-specific embryo vs adult comparisons.

**Discussion**

This study used a mouse model to determine if there are common mechanisms involved in the preimplantation embryo’s response to different in vitro exposures frequently used in assisted reproductive technologies, and if there exists a relationship between these acute transcriptional responses and gene expression in subsequent offspring adult tissues. This work has wide clinical relevance not only because well over 5 million children have been born from ART (ESHRE 2014) but also because it remains unclear how environmentally induced developmental reprogramming can predispose to abnormal metabolic phenotypes in adulthood. The main findings of the manuscript are that (1) mouse blastocysts exhibit a common gene signature of in vitro embryo manipulation; (2) blastocyst transcriptional alterations are increased with greater preimplantation manipulation or stress; (3) the impact of embryo manipulation on gene expression is cell type specific; (4) there is minimal overlap between IVF transcriptional profiles in different adult tissues and (5) there is no obvious transcriptional link between in vitro embryos and subsequent adult tissues derived from similar in vitro conditions. Finally, (6) we identified a set of...
Predicted upstream regulators of ART transcriptional profiles

common upstream regulators that could drive the transcriptional changes present in IVF embryos and adult tissues.

The first notable finding is that although each in vitro condition led to a specific and unique blastocyst transcriptional profile, we identified a common 18-gene and 9-pathway signature of preimplantation embryo manipulation that was present in all in vitro embryos irrespective of culture condition or method of fertilization. Coupled with the variation in time, location, scientist and microarray platform for these experiments, the emergence of a core fingerprint of in vitro embryo manipulation is particularly robust. Further, the 18 genes were altered in the same direction except for Camk1 expression. Camk1 operates within the calcium-triggered signaling cascade; a hallmark of mammalian fertilization is the presence of repetitive Ca$^{2+}$ oscillations from the site of sperm penetration. The unique increase in Camk1 expression in ICSI blastocysts compared to other ART conditions might be explained by data showing that ICSI induces subtle changes in oocyte Ca$^{2+}$ oscillatory patterns compared to conventional insemination (Sato et al. 1999, Miao et al. 2012).

In addition to several key transcriptional regulators with crucial roles in development and cellular differentiation (Eomes, Fgf4, Grhl1, Hif2a), many of the genes commonly affected by in vitro manipulation reflect an increase in reactive oxygen species (ROS). Indeed, there is abundant evidence that embryo culture induces an increase in ROS (Goto et al. 1993, Cebal et al. 2007, Martin-Romero et al. 2008). In our data set, oxidative stress is exemplified by changes in the hypoxia-inducible transcription factor Hif2α, the expression of genes involved in ubiquitination and protein turnover (Ube2a, Ube2l3, Lap3, Socs3, Arrdc4, (Shang & Taylor 2011)), and in particular Arrdc4 transcription.

Hif2α is a well-known transcriptional regulator of cellular and organismal responses to oxygen deprivation (Semenza 2016). Oxygen deprivation is sensed by mitochondria and converted to a reactive oxygen species-dependent signal that results in Hifα subunit stabilization (Chandel 2010). Although transcriptional induction of Hifα subunit gene expression under hypoxic conditions is less widely reported, it has been demonstrated in hematopoietic stem cells that redox stress can trigger Hif2α expression to help modulate redox balance via the homeodomain transcription factor Meis1 (Kocabas et al. 2012, Miller et al. 2016). Along these lines, the arrestin family member Arrdc4 is of interest because it also plays roles in cell metabolism and is related to molecules such as Tnixp, which can influence cellular redox status in response to glucose availability (Zhou & Chng 2013) and thus integrate cellular oxidative and metabolic states (Patwari & Lee 2012). Tnixp expression is upregulated in IVF embryos (Feuer et al. 2014), and other models have correlated this increase with impaired glucose tolerance (Parikh et al. 2007), oxidative stress (Schulze et al. 2004), apoptosis (Chen et al. 2008) and diabetes pathogenesis (Shalev 2008). The induction of these transcripts suggests a perturbation of the metabolic and redox axes in early embryos conceived and cultured in vitro. In further
support of this, GADD45 and p53 signaling—identified as one of the most significantly misregulated pathways in our dataset—are implicated in cellular and organismal responses to perturbations in cell metabolism or redox status (Zhuang et al. 2012, Salvador et al. 2013).

The remaining conserved transcripts were repressed in embryos after in vitro manipulation. Many of these, including Eomes, Fgf4, Socs3 and Dusp9 are implicated in placental development or are critical for trophoblast stem cell function (Maltepe & Fisher 2015). We have documented changes in mouse IVF placentae in prior studies (Delle Piane et al. 2010, Bloise et al. 2012, 2014), which suggest that dysregulation of critical placental genes is conserved across IVF techniques and could potentially contribute to alterations to placentation and development and function in this setting. Further, ART-induced remodeling of the placental landscape could contribute to the adult metabolic phenotypes observed in these mice. Another noteworthy gene in this common fingerprint is Ube2a, which catalyzes the monoubiquitination of histone H2B at Lys-120 to form H2BK120ub1; this marks epigenetic transcriptional activation, elongation by RNA pol II, telomeric silencing and is a requirement for H3K4 me and H3K79me formation. Together, these genes suggest a common fingerprint of in vitro embryo manipulation that links cellular differentiation, oxidative stress, glucose metabolism and insulin resistance with epigenetic changes.

The pathways with significant overrepresentation after any in vitro manipulation are also implicated in cellular development and differentiation, proliferation and the cell cycle (including cancer signaling), as well as stress response signaling via p53 and GADD45. Given that DOHaD-related phenotypes are often metabolic, it is notable that there was no enrichment for any particular metabolic pathways. However, many of the pathways with enrichment reveal interesting connections between the acute responses to in vitro manipulation and long-term metabolic phenotypes. First, oxygen homeostasis is important for cardiovascular system patterning during embryogenesis, including adaptive responses in signaling and redox homeostasis (reviewed in Simon et al. 2008); adult mice generated by IVF WM-20% O2 conditions exhibit cardiac hypertrophy (Donjacour et al. 2014). GADD45 and p53 signaling reflect cell stress and DNA damage, with known roles in the regulation of the cell cycle, senescence, survival and apoptosis. The link between GADD45 and apoptosis is significant, as GADD45 induction is known to downregulate pro-apoptotic JNK signaling and therefore suggests a pro-survival function in these embryos (De Smaele et al. 2001). Further, GADD45 proteins promote active DNA demethylation and thus mediate gene activation, which is particularly important for cell differentiation and transcriptional regulation during development (Schaler 2013). Given GADD45 function in the stress response and role in age-related disorders such as insulin resistance (Moskalev et al. 2012), alterations in this signaling pathway during an epigenetically vulnerable period might have lasting consequences for metabolic health.

This is highly relevant, as it is widely believed that epigenetic changes mediate developmental plasticity and contribute significantly to the programming of environmental signals (Gluckman et al. 2011). Given that both DNA methylation and histone modifications are extensively remodeled in the preimplantation embryo (Reik 2007) and because culture conditions can affect chromatin marks (Doherty et al. 2000), it is possible that IVF-induced changes in transcriptional and epigenetic regulation are responsible for propagating the adult DOHaD phenotypes.

Although each in vitro condition exerted a marked effect on blastocyst transcription, we additionally found that more severe transcriptional changes occurred with increased preimplantation stress. In particular, culture of embryos using atmospheric oxygen had a dramatic effect on gene expression (Fig. 2A and B). This was compounded by the addition of a suboptimal culture medium (WM), whereas the relative influence of culture medium composition alone on transcriptional profiles was small in comparison. Further, the Effect-of-Fertilization experiments (Fig. 2E, F, G and H) showed an outstanding effect of ICSI compared to IVC or IVF. The severity of the stress (oxygen and ICSI) was also reflected in the degree of fold-change for the 18-gene signature of in vitro manipulation in embryos. Interestingly, embryo culture of naturally fertilized zygotes had a stronger impact on gene expression than in vitro fertilization and culture in the same conditions (Fig. 2G, 883 vs 293 altered genes). This highlights the remarkable vulnerability of zygotes to the stresses incurred through the embryo isolation process, and may be related to the timing of zygotic genome activation. Indeed, transfer of mouse zygotes after in vitro exposure to different nutritional milieu during the pronuclear stage alters birth weight in a condition-specific manner (Banrezes et al. 2011). A notable physiological feature that separates the IVC group is that these in vivo-generated embryos are exposed to seminal fluid, which can elicit unique gene expression changes (Schjenken & Robertson 2015).

In addition to gene expression, protocol-specific effects have also been described for blastocyst cell number and lineage ratio, implantation efficiency, fetal and placental growth, postnatal growth and adult glucose metabolism (Scott et al. 2010, Kohda et al. 2011, Schwarzer et al. 2012). Along these lines, it is likely that additional ART procedures such as in vitro or in vivo oocyte maturation can distinctly modify embryo, fetal and postnatal phenotypes. Moreover, this would suggest that the recipient uterine environment could facilitate supplementary changes to transcriptional signatures of transferred embryos, further compounding the effects of ART techniques on gene expression. Overall, these findings are in agreement...
with the ‘quiet embryo’ hypothesis (Leese et al. 2008) and argue strongly for avoiding extensive embryo manipulation whenever possible.

Another key finding of this analysis is that significant cell-type transcriptional specificity of IVF is already observable at the blastocyst stage, where only 2 cell types are present. In fact, comparison of blastocyst vs ICM profiles derived from the same in vitro conditions showed an overlap of only 39 genes, 6 of which are present in the common fingerprint. Such nominal concordance demonstrates that individual cell types are primed to differentially respond to environmental perturbation, and that pooling multiple cell types may obscure investigations into DOHaD mechanisms. The relevance of cell type is further supported by the lack of a collective IVF fingerprint across the adult tissues, and the evidence that pancreatic islets—a highly differentiated and complex tissue—exhibited fewer transcriptional and pathway changes after IVF than other tissues. Interestingly, the adult transcriptional changes in IVF offspring were minimal (over 95% of the gene misexpression was ≤2 fold-change different). This was true for tissues derived from optimized IVF KAA-5% oxygen conditions (islets, liver, fat and muscle) as well as for cardiac tissue from suboptimal IVF WM-20% O₂ offspring. Other models of developmental environmental perturbation have similarly reported only subtle and tissue-distinct transcriptional profiles. For example, investigation of the transgenerational effects of gestational vinclozolin exposure demonstrated that F3 generation rats exhibited unique, tissue-specific changes to adult expression, with no common pathway overrepresentation (Skinner et al. 2012). Another study showed gene expression changes in ICSI but not IVF mouse neonates and no transcriptional or phenotypic differences by 8 weeks of age (Kohda et al. 2011). Given the lack of a common signature in adults, it is not surprising that the transcriptional changes observed in manipulated embryos did not persist through development.

The reasons for the tissue-specific variation and the lack of stability of the IVF-induced transcriptional changes throughout in utero and postnatal development are unknown. Because only 4–8 epiblast cells contribute to the adult body (Soriano & Jaenisch 1986, Morris & Zernicka-Goetz 2012), persistence and penetrance of early gene expression patterns into adulthood could depend on which particular founder cells contributed to organogenesis. Separately, the necessity of pooling blastocysts to assess gene expression could have captured embryos capable of adapting to a stressful in vitro environment but not necessarily able to develop beyond the blastocyst stage. This would introduce a strong transcriptional bias in embryos compared to postnatal tissues, although given the robust concordance in gene expression among embryos derived from different in vitro conditions, we believe this is an unlikely possibility. It is also possible that some ART-induced changes are resolved during development. However, the presence of distinct adult phenotypes (e.g. the manifestation of glucose intolerance in mouse IVF offspring) makes this scenario unlikely. Another explanation is that the molecular changes present in blastocysts after preimplantation disturbance are differentially affected by organogenesis, growth factors or sexually dimorphic signals occurring during later stages of development. As a result, cell physiology and metabolism within each developing tissue is altered in accordance with new, tissue-specific developmental cues.

Fitting within this framework, we have previously investigated whether specific genes altered in IVF embryos exhibit tissue-specific maintenance of transcriptional and epigenetic changes throughout development. We showed that expression of the glucose-sensitive gene Txnip was significantly increased in IVF blastocysts, selectively increased in female IVF fat and muscle tissues but not liver, and that this dysregulation was associated with enriched H4 acetylation at the Txnip promoter (Feuer et al. 2014). In the current study, Txnip is upregulated in all ART blastocysts except for the ICSI and the IVC KAA-20% oxygen conditions. In another study, we reported that glucocorticoid receptor (GR) expression is increased in IVF KAA-5% O₂ embryos and male offspring fat, but not liver or muscle (Simbulan et al. 2015).

Finally, it is possible that the gene expression changes in adult IVF tissues occur secondary to an altered function of key upstream transcriptional regulators, which would not necessarily be identifiable in a microarray profile. Transcriptional regulators are able to control diverse processes with cell type and temporal specificity through combinatorial interplay with other transcriptional factors and modulators. In this manner, altering the function of a transcriptional regulator would exert a variety of different effects depending upon the cell type, which could explain the stark tissue specificity observed in the ART expression profiles. Several of the proposed upstream regulators are highly relevant to DOHaD phenotypes and provide grounds for directly testable hypotheses in future investigations. CEBPA/B play essential and redundant functions during early embryogenesis and placentation (Begay et al. 2004), and CEBPA enhances iPS cell reprogramming efficiency (Di Stefano et al. 2014). CEBPA is crucial for liver and lung development, adipocyte terminal differentiation, the establishment and maintenance of energy homeostasis, lipid storage and gluconeogenesis—all through combinatorial interactions with other transcription factors such as Myc or members of the PPAR family (Wang et al. 1995, Flooby et al. 1996, Wu et al. 1999). Context-specific interactions might explain why some transcriptional regulators exhibited predicted activation in certain tissues and inhibition in others. Further, the Cebpa locus encodes a functional RNA that sequesters DMNT1 to ensure robust Cebpa transcription through local inhibition of Cepba gene methylation, thus
directly linking Cepba activity to site-specific genomic methylation (Di Ruscio et al. 2013).

Separately, NFE2L2 (also known as NRF2) is involved in antioxidant defense mechanisms and binds DNA antioxidant response elements (AREs) to regulate the adaptive response to oxidative stress (reviewed in Ma 2013). NFE2L2 was predictedly inhibited in our ART datasets, which is particularly relevant because mice deficient in NFE2L2 exhibit increased sensitivity to oxidative stress. There is ample evidence in the literature that embryo culture induces an increase in reactive oxygen species (ROS) (Goto et al. 1993, Cebral et al. 2007, Martin-Romero et al. 2008), and ROS induction is a central event precipitating diabetes pathogenesis (Nishikawa et al. 2000, Sakai et al. 2003). ROS can also impair telomerase activity, with telomere attrition being a predictive marker for cardiovascular dysfunction, metabolic syndrome and other age- and DOHaD-related pathologies (Hallows et al. 2012). It is therefore possible that an increase in ROS levels is the initial stimulus that reprograms the ART embryo, which employs transcription factors like NFE2L2, CEBPs (Manea et al. 2014) and p53 to exert acute transcriptional responses and subsequent chromatin remodeling that persists throughout development, leading to different cell type-contextual expression patterns.

Among the potential caveats of this paper is the fact that the results presented are specific to data from one laboratory and one species. Although this enables direct comparisons between datasets, small technical discrepancies between different laboratories can affect the results and subsequent conclusions. Similarly, choice of mouse strain(s) can influence gene expression signatures (Turk et al. 2004). The mouse data provided may not apply to other species and thus should be confirmed in, for example, bovine (Smith et al. 2009) or sheep (Wei et al. 2016) models. Finally, given the progressive maturation of translation machinery in the early embryo, changes in RNA may not be reflected by changes in protein abundance (Seydoux et al. 1996).

To conclude, we have provided a systematic, integrated analysis of the acute and long-term transcriptional effects of different mechanisms of in vitro embryo manipulation commonly used in assisted reproduction. The results of this study indicate that the expression of 18 genes may be used to evaluate ART embryo health and could be developed into a new tool for identifying embryos with the greatest implantation potential. Because fertilization and embryo culture practices vary worldwide, this list of 18 genes is valuable for its robustness and potential application across the entire ART field. This in vitro fingerprint does not persist throughout development, and there is no clear transcriptional cohesion between adult IVF offspring tissues compared to their preceding embryos. However, the unique transcriptional signatures present in each IVF tissue are collectively targeted by the same upstream transcriptional regulators, which provides insight into DOHaD mechanisms regarding how acute transcriptional responses to stressful environmental exposures might be preserved throughout development to influence adult gene expression and manifest metabolic phenotypes. Our results suggest that the activity of these regulators may be reliable markers of in vitro stress present at specific developmental points extending into adulthood.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-16-0473.

**Declaration of interest**

The 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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