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Methodology matters: IVF versus ICSI and embryonic gene expression

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Abstract

The use of assisted reproduction treatment, especially intracytoplasmic sperm injection (ICSI), is now linked to a range of adverse consequences, the aetiology of which remains largely undefined. The objective was to determine differences in gene expression of blastocysts generated by ICSI as well as ICSI with artificial oocyte activation (ICSI-A) versus the less manipulative IVF, providing fundamental genetic information that can be used to aid in the diagnosis or treatment of those adversely affected by assisted reproduction treatment, as well as stimulate research to further refine these techniques. Murine blastocysts were generated by ICSI, ICSI-A and IVF, and processed for a microarray-based analysis of gene expression. Ten blastocysts were pooled for each procedure and three independent replicates generated. The data were then processed to determine differential gene expression and to identify biological pathways affected by the procedures. In blastocysts derived by ICSI versus IVF, the expression of 197 genes differed ($P < 0.01$). In blastocysts derived by ICSI-A versus IVF and ICSI-A versus ICSI, the expression of 132 and 65 genes differed respectively ($P < 0.01$). Procedural-induced changes in genes regulating specific biological pathways revealed some consistency to known adverse consequences. Detailed investigation of procedure-specific dysfunction is therefore warranted.

Keywords

activation; blastocyst; gene expression; ICSI; IVF; mouse

Introduction

The use of assisted reproductive technology has increased dramatically over the last 30 years, providing an unprecedented opportunity for infertile couples to conceive a child. Unfortunately, the use of techniques such as intracytoplasmic sperm injection (ICSI) has become increasingly linked to adverse consequences that can affect both the mother and her child. In the mother, this includes an increased incidence of placental abruption, pre-eclampsia and stillbirths (Blumenfeld *et al.*, 1992; Aytoz *et al.*, 1998; Devroey and Van Steirteghem, 2004; Katalinic *et al.*, 2004; Lucifero *et al.*, 2004; Osmanagaoglu *et al.*, 2004; Unger *et al.*, 2004; Bonduelle *et al.*, 2005; Lidegaard *et al.*, 2005; Pinborg *et al.*, 2005; Woldringh, 2005; Buckett *et al.*, 2008; Arav *et al.*, 2010; Poret *et al.*, 2010). In children, dysfunction can range from the development of tumours and carcinomas (White *et al.*, 1990;

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Toren *et al.*, 1995; Odone-Filho *et al.*, 2002; Moll *et al.*, 2003a; Moll *et al.*, 2003b; Katalinic *et al.*, 2004; Lightfoot, 2004; Niemitz and Feinberg, 2004; Owen and Segars, 2009) to congenital anomalies such as septal heart defects and cleft lip or palate (Wennerholm *et al.*, 2000; Anthony *et al.*, 2002; Orstavik, 2003; Katalinic *et al.*, 2004; Rimm *et al.*, 2004; Bonduelle *et al.*, 2005; Hansen *et al.*, 2005; Kallen *et al.*, 2005; Karpman *et al.*, 2005; Olson *et al.*, 2005; Schieve *et al.*, 2005; Sutcliffe and Derom, 2006; Bertelsmann, 2008; Reefhuis *et al.*, 2009; Poret *et al.*, 2010; Williams *et al.*, 2010), as well as neurological problems that may result in an intellectual lag (Katalinic *et al.*, 2004; Bonduelle *et al.*, 2005; Lidegaard *et al.*, 2005). Unfortunately, the aetiology of these unwanted effects has proven difficult to determine, in part due to the patient-, clinic- and MD-specific practices that are associated with the use of these reproductive techniques.

The extent to which assisted reproduction treatment-induced defects can be traced back to an altered pattern of embryonic gene expression is unknown. However, aberrant gene expression within the blastocyst, the first differentiated stage of development that occurs after any manipulation of the gametes, could increase the incidence of, or predisposition to, the multitude of defects that have now been associated with the use of techniques such as intracytoplasmic sperm injection (ICSI). The objective of this study was to identify the genes and the biological pathways that they regulate, which differ in blastocysts generated by ICSI versus IVF. Because chemical activation of the oocyte is now included in some ICSI protocols, aiming to mimic the events within the oocyte that are induced by the penetrating spermatozoon, the experimental design was expanded to include ICSI with chemical activation (ICSI-A) as an independent procedure in itself. The hypothesis was that gene expression would differ in blastocysts derived by ICSI versus IVF and that these differences would be negated by the inclusion of the chemical activation procedure to the ICSI protocol. In effect, artificial activation of the oocyte would overcome some of the differences in gene expression induced by the ICSI procedure. Determining how these methodologies affect gene expression in the blastocyst should stimulate research that will translate to advances in the diagnosis, treatment and/or management of patients and offspring adversely affected by these technologies, as well in the refinement of these practices that benefit so many. To facilitate this, the data generated is provided in a manner as complete as possible, relying heavily upon the use of supplementary tables (available online only) that will allow the identification of specific gene targets for further investigation by others.

Materials and methods

Animals

All mice were purchased from Samtaco (IcrTacSam; Samtaco, Seoul, Korea), housed under a 12:12 h light/dark cycle in a temperature- and humidity-controlled room and provided with food and water *ad libitum*. The protocol for the use of these animals was approved by the Institutional Animal Care and Use Committee of CHA University, Seoul, Korea.

Sperm collection and preparation

Epididymal spermatozoa were obtained from male BDF1 mice at 8–10 weeks of age. Spermatozoa used for IVF were collected from the cauda epididymis in 200 μl drops of Quinn's Advantage Medium with Hepes (SAGE In-Vitro Fertilization; Pasadena, CA, USA) and capacitated by incubation for 1.5 h at 37°C under 5% CO_2 in air. Spermatozoa used for ICSI and ICSI-A were squeezed from the cauda epididymis and placed in the bottom of 1.5-ml tubes containing 500 μl of the same Quinn's Advantage medium with Hepes. Spermatozoa were then allowed to ‘swim up’ for 5 min at room temperature before being collected for the intracytoplasmic injections.

Oocyte collection

Six-week old female B6D2F1 mice were treated with 5 IU pregnant mare's serum gonadotrophin (PMSG; Sigma-Aldrich, St Louis, MO, USA) to induce follicular development and 48 h later with 5 IU human chorionic gonadotrophin (Sigma-Aldrich) to induce ovulation. At 12 to 15 h after human chorionic gonadotrophin, ovulated cumulus–oocyte–complexes were retrieved from the ampullary region of each oviduct and placed in Quinn's Advantage Medium with Hepes. Cumulus–oocyte–complexes to be used for IVF were then transferred into fertilization drops of the same medium. Cumulus–oocyte–complexes to be used for ICSI and ICSI-A were first incubated in Quinn's medium with the addition of 0.1% bovine testicular hyaluronidase (Tokyo Kasei Kogyo, Tokyo, Japan), effectively dispersing cumulus cells. The cumulus-free oocytes were then washed in fresh Quinn's Advantage Medium with Hepes and used immediately for ICSI.

IVF

Cumulus-intact oocytes in 200 μl drops of Quinn's Advantage Fertilization medium were incubated with capacitated spermatozoa at a final concentration of 100 spermatozoa/μl medium. Gametes were co-incubated for 6 h at 37°C under 5% CO_2 in air. After the 6 h co-incubation, oocytes were washed several times with fresh Quinn's Advantage Medium with Hepes. This protocol is based on the well-established methodology of Hogan *et al.* (1994).

Intracytoplasmic sperm injection

A small volume of the sperm suspension was mixed with Quinn's Advantage Medium with Hepes containing 12% polyvinyl pyrrolidone. Injections were performed immediately thereafter using a micromanipulator with a Piezo-electric actuator (PMM Controller, model PMAS-CT150; Prima Tech, Tsukuba, Japan). The head of each spermatozoa was separated from the tail by applying pulses to the head-tail junction by means of the Piezo-driven pipette. Only the head of one spermatozoon was injected into the cytoplasm of each metaphase II-stage oocyte and the oocytes were injected in groups of 10–15 (on a particular day), overall taking less than 10 min per group. A photographic time-course of the procedure is provided as Figure 1. Spermatozoa-injected oocytes were then transferred into KSOM medium and cultured at 37°C, 5% CO_2 in air. To activate the oocytes after ICSI (ICSI-A treatment), injected oocytes were placed in Ca^{2+}-free CZB medium containing 10 mmol/l $SrCl_2$ at 60 min after the injection and cultured for 1 h. ICSI-A oocytes were then returned to culture in KSOM medium at 37°C, 5% CO_2 in air. This protocol is based on the original work of Kimura and Yanagimachi (1995).

Embryo culture

Following ICSI, ICSI-A and IVF, oocytes were placed in 50 μl drops of KSOM medium and incubated at 37°C, 5% CO_2 in air. The culture drops were contained in plastic culture dishes and overlaid with mineral oil. Cultured embryos were then evaluated for developmental progress after 24 and 96 h. Embryos were generated by the hands of a single experienced embryologist, with the rates of fertilization and development shown in Table 1. A comparison of cell numbers (total, inner cell mass and trophectoderm) for blastocysts generated by ICSI, ICSI-A and IVF is presented as Table 2.

Sample processing and microarray hybridization

Embryos that developed to the blastocyst stage *in vitro* were collected for the microarray analysis, chosen because at that stage of early development, blastocysts have differentiated to have an inner cell mass and trophectoderm and have a high chance of continuing in their development. Blastocysts were not collected from naturally bred mice *in vivo* as embryo-oviductal interactions that affect embryonic gene expression would confound the results.

Ten blastocysts were pooled for each treatment group (ICSI, ICSI-A and IVF) and three independent replicates for each treatment procedure were collected. Pooling was required in order to obtain sufficient RNA for the analysis and also to reduce the overall chance of generating errors. Total RNA was extracted using Trizol and purified using an RNeasy kit (Qiagen, Valencia, CA, USA). Total RNA was then amplified and labelled using the Nugen WT Ovation One-Direct RNA Amplification and Nugen FL-Ovation cDNA Biotin Module V2 labelling kits, respectively (NuGen Technologies, San Carlos, CA, USA). Microarray hybridization was then performed using Affymetrix Mouse 430–2.0 whole genome arrays (Affymetrix, Santa Clara, CA, USA) by the Microarray Core Facility at the University of Kentucky, as previously described (Jo *et al.*, 2004; Jeoung *et al.*, 2010). Three independent microarray chips were hybridized for each treatment procedure.

Data analysis

Data from the microarray hybridization were sorted for each analysis, excluding probe sets that consistently exhibited absent or marginal detection calls (i.e. at least two of the three detection calls for both treatment groups in a particular analysis were absent or marginal). The resultant datasets were then processed using Pathways Studio 7.1 software (Ariadne Genomics, Rockville, MD, USA) to: (i) identify probe sets that differed among two treatment groups; and (ii) sort the data into biological pathways regulated by treatment. We have successfully used this software to analyse microarray datasets in the past (Jeoung *et al.*, 2010). For the analysis to identify probe sets that differed among two treatment groups, differential expression was defined as those transcripts that exhibited a difference in expression of 2-fold or greater and were statistically different ($P < 0.01$), as determined by the software package that utilizes a statistical algorithm with a one-sided Mann–Whitney *U*-test to determine *P*-values indicating significance. To sort the data into biological processes regulated by treatment, the statistical algorithm was relaxed ($P < 0.05$) and a Gene Set Enrichment performed by the software package. The comparisons performed were ICSI versus IVF, ICSI-A versus IVF and ICSI-A versus ICSI, with only biological processes that exhibited at least five overlapping entities (genes) presented in the results.

Results

Gene expression I: ICSI versus IVF

Analysis of the microarray dataset identified 236 probes reflecting 197 known genes that differed in blastocysts generated by ICSI versus IVF ($P < 0.01$). For each of these genes, its probe set and Entrez gene identification number, fold-change in level of expression and *P*-value comparing the two treatment techniques is listed in Supplementary Table 1 (available online only). The dataset was also processed to reveal biological pathways that differed when blastocysts were generated by these two techniques (Table 3). Consistent with some of the developmental anomalies that have been associated with the use of assisted reproduction treatment (Wennerholm *et al.*, 2000; Anthony *et al.*, 2002; Orstavik, 2003; Katalinic *et al.*, 2004; Rimm *et al.*, 2004; Bonduelle *et al.*, 2005; Hansen *et al.*, 2005; Kallen *et al.*, 2005; Karpman *et al.*, 2005; Olson *et al.*, 2005; Schieve *et al.*, 2005; Sutcliffe and Derom, 2006; Bertelsmann, 2008; Reefhuis *et al.*, 2009; Poret *et al.*, 2010; Williams *et al.*, 2010), the analysis identified development as a primary biological process affected by technique (ICSI versus IVF). Inclusive to this were several genes regulating structural and organ-specific processes, as well as those that were classified into more general cellular categories. Several metabolic pathways were identified to differ by procedure as well as a variety of response processes, signalling and transport mechanisms. As the purpose of this study is to provide the background genetic information required to stimulate further research, the results are presented without overt explanation or likely bias to any one physiological mechanism. The

complete list of individual genes that were grouped into each biological function is also presented as an expanded version of Table 3 (Supplementary Table 2, available online only).

Gene expression II: ICSI-A versus IVF

A total of 146 probe sets representing 132 known genes were found to differ in blastocysts generated by ICSI-A versus IVF ($P < 0.01$). Again, the individual probes and associated information are listed in full (Supplementary Table 3, available online only). The biological process classification for differentially expressed genes in blastocysts derived by ICSI-A versus IVF ($P < 0.05$) is presented in Table 4 and expanded to include gene listings in Supplementary Table 4 (available online only). Interestingly, while differences in developmental processes were still found to differ in blastocysts generated by ICSI-A versus IVF, the number of classes identified was approximately halved. A large number of response-type pathways were identified that ranged from the specific (e.g. the regulation of ossification) to the general (e.g. immune response) that should be evaluated in the future in concert with the specific probes listed in the supplementary tables.

Gene expression III: ICSI or ICSI-A versus IVF

From the 197 genes that were found to differ when blastocysts were derived by ICSI versus IVF, only 18 probe sets representing 17 genes were common to those identified when ICSI-A and IVF were compared. These 17 genes, their average intensities, fold changes and individual *P*-values are identified in Table 5.

Gene expression IV: ICSI versus ICSI-A

The analysis identified 74 probe sets representing 65 known genes that differed between the ICSI and ICSI-A treatment groups ($P < 0.01$). Each of these 65 genes, its identity, fold-change in level of expression and *P*-value comparing the two treatment techniques is listed in Supplementary Table 5 (available online only). The method of ICSI (i.e. with or without artificial activation of the oocytes) used to generate blastocysts was also found to affect developmental, metabolic and response processes in the biological pathway analysis ($P < 0.05$; Table 6) with this information expanded to include gene information in Supplementary Table 6 (available online only).

Discussion

Recent data from the US Centres for Disease Control and Prevention indicate that ~7.3 million US couples suffer from infertility and that ~140,000 assisted reproduction cycles are performed per year (Wright *et al.*, 2007; http://www.cdc.gov/ART/). The benefit from the development and use of these techniques is unquestionable; however assisted reproduction treatment and especially ICSI have become increasingly linked to a broad range of unwanted and often serious consequences to the mother and/or her offspring. The objective of this study was to determine, in the developing mouse embryo, genetic pathways affected by ICSI versus the less manipulative IVF. In addition to this, this study evaluated whether artificial activation of oocytes would overcome some of the differences in gene expression attributed specifically to the ICSI procedure. Mice were used to generate the embryos as they represent a population of fertile, genetically homogenous and healthy subjects. Determination of procedural-specific changes in embryonic gene expression should stimulate research that will translate into advances in clinical practice, both in the management of prior treatment-induced effects, as well as in the refinement of these techniques in the future. Immediate advances could include identifying an array of treatment-induced genes as genetic markers, signalling the need for planned intervention and management of certain diseases in the newborn, or increasing the breadth of preimplantation genetic diagnosis for the most serious consequences associated with these treatments for infertility. The first live births after IVF

and ICSI were reported in 1978 (Steptoe and Edwards, 1978) and 1992 (Palermo *et al.*, 1992), respectively. The field of assisted reproduction treatment has grown at an unprecedented pace, making this analysis of treatment-induced gene function a timely and necessary report.

The majority of treatment cycles performed in the USA utilize IVF and/or ICSI (>50% ICSI) with the other treatment types, gamete and zygote intra-Fallopian transfer (GIFT and ZIFT) accounting for <1% of all procedures (Wright *et al.*, 2007; http://www.cdc.gov/ART/). However, the data from these reports also indicate that ICSI is proportionally increasing in its use and routinely prescribed over IVF, regardless of the aetiology of infertility; i.e. even when the cause of infertility would indicate success with IVF alone. This is extremely pertinent given the data presented herein; 236 probe sets accounting for 197 genes differed in blastocysts generated by ICSI versus IVF, suggesting procedural-driven changes in embryonic gene expression and, potentially, the unwanted consequences that have been associated with assisted reproduction treatment.

Of the birth defects related to the use of these techniques, the manifestation of aberrant growth and development appears a common consequence (Wennerholm *et al.*, 2000; Anthony *et al.*, 2002; Orstavik, 2003; Katalinic *et al.*, 2004; Rimm *et al.*, 2004; Bonduelle *et al.*, 2005; Hansen *et al.*, 2005; Kallen *et al.*, 2005; Karpman *et al.*, 2005; Olson *et al.*, 2005; Schieve *et al.*, 2005; Sutcliffe and Derom, 2006; Bertelsmann, 2008; Reefhuis *et al.*, 2009; Poret *et al.*, 2010; Williams *et al.*, 2010). One of the most readily recognizable of these defects includes cleft lip and palate, condition(s) reflecting an asymmetry in development. A septal heart defect is less overtly recognizable, although a serious defect that is also related to symmetry and assisted reproduction treatment. Interestingly, this study identified the differential expression of 14 genes (ICSI versus IVF, Table 3 and Supplementary Table 2) that were categorized to regulate proximal–distal pattern formation (i.e. symmetry) in these early embryos. That being said, developmental problems associated with assisted reproduction treatment are not confined to those manifested from asymmetry. Several specific syndromes that include Beckwith–Wiedemann (Filippi and McKusick, 1970; Neelanjana and Sabaratnam, 2008) and Silver–Russell (Abu-Amero *et al.*, 2008; Neelanjana and Sabaratnam, 2008), which are characterized by overgrowth and intrauterine growth retardation, respectively, are also well recognized (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Gosden *et al.*, 2003; Maher *et al.*, 2003a; Maher *et al.*, 2003b; Halliday *et al.*, 2004; Katalinic *et al.*, 2004; Lucifero *et al.*, 2004; Niemitz *et al.*, 2004; Niemitz and Feinberg, 2004; Lidegaard *et al.*, 2005; Sutcliffe *et al.*, 2006; Owen and Segars, 2009). In addition to this, aberrant development extends to the manifestation of neurological defects (Katalinic *et al.*, 2004; Bonduelle *et al.*, 2005; Lidegaard *et al.*, 2005) and the present study identified procedural-induced changes in several genes regulating neural development when the expression profiles of blastocysts derived from IVF and ICSI were compared.

Similar to the wide scope of developmental disorders implicated with assisted reproduction treatment, a range of metabolic consequences have been described including neonatal hypoglycaemia (Beckwith–Wiedemann syndrome (Hussain *et al.*, 2005; Kapoor *et al.*, 2009; Palladino *et al.*, 2009)) and childhood-onset obesity (Prader–Willi and MatUPD14 syndromes, (O'Neill *et al.*, 2005; Bouchard, 2009; Butler *et al.*, 2009)). Analysis of the present data indicated differences in the expression of genes regulating several metabolic pathways, including cholesterol and lipid metabolism/catabolism, in blastocysts derived from ICSI versus IVF. The concomitant change in genes regulating organ morphogenesis, as an example, pancreatic development, is consistent with a metabolic consequence to assisted reproduction treatment and suggests an underlying genetic cause for the development of certain metabolic diseases. Overall, the changes in gene expression that this study has identified between ICSI and IVF indicate that further investigation into these pathways is

vital. Although the refinement of techniques to circumvent adverse consequences is obviously a primary goal for the future, the development of genetic markers indicating susceptibility to disease could have a dramatic impact on the diagnosis and management of several of the adverse consequences, including those of a metabolic origin, that are associated with assisted reproduction treatment.

A more recent modification to the procedure of ICSI is the inclusion of a chemical activation step (Dozortsev *et al.*, 1995), reviewed by Nasr-Esfahani *et al.*, (2010), which aims to mimic the events within the oocyte that occur during the normal fertilization process; i.e. under normal conditions, spermatozoon–oocyte fusion is followed by the incorporation of, and interaction between, a demembranated sperm nucleus and the cytoplasm of the oocyte. The nucleus of the spermatozoa is readily accessible to ooplasmic factors, with fusion of the gametes stimulating pivotal intracellular calcium oscillations. Modifications to the zona pellucida and release from meiotic arrest ensue. Artificial methods to activate the oocyte have now been developed, that improve the rate of fertilization (especially in cases of low fertility) and can be performed using chemical agents such as strontium chloride (Kumagai, 2006) and calcium ionophores (Murase *et al.*, 2004; Borges *et al.*, 2009) or by mechanical (Dirican *et al.*, 2008) or electrical (Yanagida *et al.*, 1999) stimulation. In the experiments described herein, strontium chloride was used. Although this technique has proven very effective (Suttner *et al.*, 2000; Murase *et al.*, 2004), the utilization of activation after ICSI (ICSI-A) appears to be clinic-specific with only a very limited number of clinics activating oocytes after microinjection.

It is very interesting that when compared with IVF, the inclusion of this chemical activation step to the ICSI protocol brought the sample clustering of the probe sets closer together. i.e. ICSI-A clustered closer to IVF than to ICSI alone, suggesting that this artificial activation step effectively mimics, at the genetic level, a proportion of the events initiated by sperm penetration. When gene expression in blastocysts derived by ICSI-A and IVF were analysed independently, 146 probe sets were identified to differ, 90 fewer than the 236 probes identified to differ in the ICSI versus IVF analysis. In itself, this is a striking reduction in differential gene expression and further illustrative of the alignment of IVF and ICSI-A observed in the clustering analysis. When gene expression in blastocysts derived by ICSI was compared directly to those generated with the optional activation step (ICSI-A), 74 probe sets reflecting the differential expression of 65 known genes was revealed. The classification of development was highlighted by the biological pathway grouping and differences in the expression of genes regulating structural and neural developmental pathways uncovered, consistent with known treatment-induced dysfunction.

Overall, the expression of genes in blastocysts was affected by the procedure used to generate the embryo and classification of differentially expressed genes into biological pathways revealed consistency to known treatment-induced adverse consequences. Obviously, further investigation is needed before these findings can be translated into clinical advances and the reader must remain cognizant that these results were generated using blastocysts of a murine origin, and not those obtained from an assisted reproduction clinic. However, with the use of ICSI now appearing to be dominant over IVF, regardless of the aetiology of infertility, and ICSI-A not yet standardized into the operating procedure of the majority of assisted reproduction clinics, deadlines for teasing out procedure-specific consequences cannot be delayed.

The field of assisted reproduction treatment has grown exponentially over the last 30 years, providing an unprecedented opportunity for infertile couples to conceive a child. This genetic analysis was performed to stimulate research in this field with the overall goal of advancing understanding (and performance) of these most valuable treatment options for

infertility. It is hoped that future investigation into the genes and pathways uncovered in this report will prove fruitful and that advances made will be timely.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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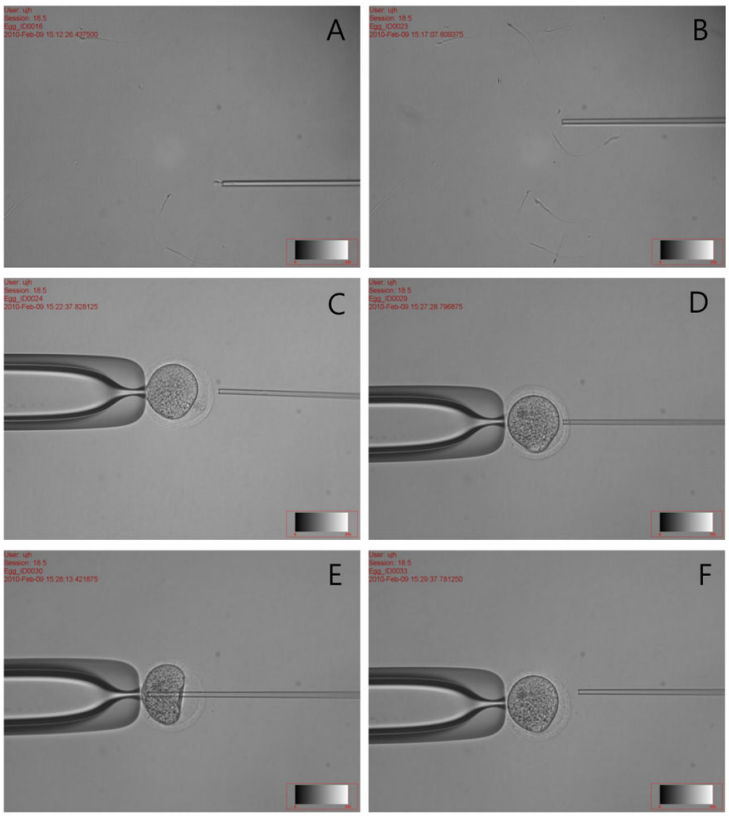

Figure 1.
Representative images taken during intracytoplasmic sperm injection. (**A**) Loading of an epididymal spermatozoa. (**B**) Separation of head and tail. (**C**) The metaphase II-staged oocyte ready for injection. (**D**) Penetration of the zona pellucida. (**E**) Injection of the spermatozoa into the oocyte. (**F**) The injected oocyte.

Table 1

Development of embryos obtained by IVF, intracytoplasmic sperm injection (ICSI) and ICSI with artificial oocyte activation (ICSI-A).

Technique	Fertilized oocytes cultured	2-cell embryos	Blastocysts
IVF	108	101 (93.52)	79 (78.22)
ICSI	130	111 (85.38)	57 (51.35)
ICSI-A	80	74 (92.50)	40 (54.05)

Data are the sum results from four independent experiments. Values are *n* or *n* (%).

Table 2

Comparison of cell numbers in blastocysts derived by IVF, intracytoplasmic sperm injection (ICSI) and ICSI with chemical activation (ICSI-A).

Technique (no. of blastocysts)	Mean cells/blastocyst (n)	Cells in trophectoderm		Cells in inner cell mass	
		n	%	n	%
IVF (*n* = 10)	39.7	25.9 ± 1.2[a]	65.3 ± 2.1[b]	13.8 ± 1.0[b]	34.7 ± 2.1[b]
ICSI (*n* = 10)	39.4	29.2 ± 1.4[a]	74.2 ± 1.7[a]	10.2 ± 0.9[a]	25.8 ± 1.7[a]
ICSI-A (*n* = 9)	38.7	28.3 ± 3.5[a]	72.6 ± 2.7[a]	10.3 ± 1.1[a]	28.0 ± 2.7[a]

Values are mean ± SD.

Values in columns with different superscripts differ ($P < 0.05$).

Table 3

Functional classification of genes that differed in blastocysts derived by intracytoplasmic sperm injection (ICSI) versus IVF ($P < 0.05$).

Type		Name	Entities	P-value
Development	Cellular	Multicellular organismal development	550	0.00062
		Cell differentiation	332	0.00160
		Ureteric bud branching	17	0.00264
		Cell development	11	0.00627
		Metanephros development	10	0.00767
		Morphogenesis of a branching structure	5	0.00911
	Growth	Growth	18	0.00091
	Neural	Nervous system development	219	0.00419
	Organ	Organ morphogenesis	95	0.00008
		Pancreas development	12	0.00142
		Sex differentiation	11	0.00427
		Endocrine pancreas development	9	0.00898
		Inner ear morphogenesis	30	0.00905
		Forebrain development	44	0.00979
		Sex determination	9	0.00993
	Structural	Anatomical structure development	9	0.00047
		Embryonic gut development	7	0.00061
		Proximal–distal pattern formation	14	0.00119
	Other	Pattern specification process	27	0.00294
Metabolism		Lipid catabolic process	57	0.00018
		Xenobiotic metabolic process	14	0.00059
		Lipid glycosylation	6	0.00155
		Cholesterol metabolic process	42	0.00581
		Taurine metabolic process	5	0.00958
Response	Cellular	Negative regulation of chondrocyte differentiation	5	0.00442
		Positive regulation of ossification	10	0.00781
		Negative regulation of erythrocyte differentiation	5	0.00854
		Positive regulation of cell proliferation	209	0.00065
		Negative regulation of angiogenesis	17	0.00089
		Positive regulation of vasodilation	10	0.00223
		Chemotaxis	57	0.00309
		Proteoglycan biosynthetic process	6	0.00315
		Positive regulation of survival gene product expression	7	0.00521
		Neurotransmitter secretion	21	0.00580
		Positive regulation of mitosis	11	0.00942
		Response to glucocorticoid stimulus	58	0.01000
	Immune	Inflammatory response	111	0.00060
		Immune response	202	0.00263

Type		Name	Entities	P-value
		Response to wounding	31	0.00630
		Positive regulation of T cell-mediated cytotoxicity	8	0.00637
	Mechanical	Response to mechanical stimulus	30	0.00023
		Sperm motility	12	0.00102
		Ciliary or flagellar motility	5	0.00812
		Positive regulation of smooth muscle contraction	8	0.00868
	Other	Visual perception	93	0.00095
		Response to stimulus	80	0.00139
		Digestion	18	0.00204
		Synaptic transmission	93	0.00014
		Response to external stimulus	5	0.00568
Signalling		G-protein coupled receptor protein signalling pathway	210	0.00000
		Signal transduction	789	0.00022
		Cell–cell signalling	104	0.00069
		Inositol phosphate-mediated signalling	5	0.00399
		Wnt receptor signalling pathway, calcium modulating pathway	5	0.00455
		Cytokine-mediated signalling pathway	41	0.00590
		Elevation of cytosolic calcium ion concentration	38	0.00867
Transport		Iron ion transport	22	0.00460
		Ion transport	281	0.00902
Miscellaneous		Feeding behaviour	11	0.00016

Table 4

Functional classification of genes that differed in blastocysts derived by ICSI with chemical activation (ICSI-A) versus IVF ($P < 0.05$). The number of entities and level of statistical significance are indicated for each biological function.

Type		Name	Entities	P-value
Development	Cellular	Ureteric bud branching	16	0.00469
	Neural	Peripheral nervous system development	14	0.00398
		Neural crest cell development	5	0.00798
	Organ	Organ morphogenesis	98	0.00007
		Thyroid gland development	5	0.00147
		Inner ear morphogenesis	29	0.00197
		Middle ear morphogenesis	6	0.00324
		Embryonic gut development	5	0.00792
		Pattern specification process	28	0.00890
	Structural	Cartilage development	28	0.00659
		Embryonic skeletal system morphogenesis	26	0.00720
Metabolism		Lipid glycosylation	7	0.00147
		Retinol metabolic process	10	0.00297
Response	Cellular	Glial cell differentiation	10	0.00072
		Elevation of cytosolic calcium ion concentration	40	0.00005
		Cell adhesion	265	0.00036
		Chemotaxis	59	0.00217
		Regulation of ossification	7	0.00378
		Positive regulation of cellular protein metabolic process	5	0.00605
		Positive regulation of mitosis	12	0.00678
		NAD biosynthetic process	6	0.00710
		Cell–cell adhesion	52	0.00812
		Cellular calcium ion homeostasis	36	0.00877
		Cholesterol biosynthetic process	26	0.00889
		Protein amino acid N-linked glycosylation	18	0.00963
	Immune	Inflammatory response	112	0.00000
		Immune response	203	0.00002
		Defence response to bacterium	26	0.00055
		Antigen processing and presentation	18	0.00128
		Complement activation, alternative pathway	5	0.00259
		Complement activation, classical pathway	15	0.00723
	Mechanical	Response to mechanical stimulus	33	0.00154
		Vasoconstriction	5	0.00188
	Neural	Axon guidance	59	0.00678
		Synaptic transmission	98	0.00005
	Other	Detection of chemical stimulus involved in sensory perception of smell	5	0.00647
		Visual perception	93	0.00001
		Response to stimulus	84	0.00006

Type	Name	Entities	P-value
	Digestion	17	0.00629
	Acute-phase response	15	0.00840
Signalling	G-protein coupled receptor protein signalling pathway	216	0.00000
	Signal transduction	805	0.00008
	Cell surface receptor linked signal transduction	115	0.00014
	G-protein signalling, coupled to cyclic nucleotide second messenger	14	0.00026
	Cell–cell signalling	103	0.00115
	Inositol phosphate-mediated signalling	5	0.00651
	G-protein signalling, coupled to cAMP nucleotide second messenger	10	0.00783
Transport	Ion transport	286	0.00006
	Calcium ion transport	68	0.00078
	Iron ion transport	22	0.00197
Miscellaneous	Feeding behaviour	11	0.00024
	Axonal fasciculation	7	0.00188
	Memory	21	0.00749

Table 5

Identification of the 17 genes that differed when blastocysts were derived by IVF versus intracytoplasmic sperm injection (ICSI) and IVF versus ICSI with chemical activation (ICSI-A).

Name	IVF versus ICSI		IVF versus ICSI-A		Probe set ID	Intensity average		
	Fold change	P-value	Fold change	P-value		IVF	ICSI	ICSI-A
Unknown	−3.5516	0.0020	−2.334	0.0035	1439599_at	598.6	172.9	259.6
ABHD14B	−2.2566	0.0087	−2.0973	0.0055	1451326_at	1313.4	582.5	619.9
C10orf32	2.3728	0.0013	2.4465	0.0013	1419299_at	137.2	327.6	337.9
CA12	−6.8079	0.0026	−3.9913	0.0059	1428485_at	186.6	29.0	48.9
CUBN	−2.1933	0.0041	−2.0282	0.0018	1452270_s_at	1921.2	886.1	949.9
DDA1	−12.5618	0.0100	−12.0678	0.0019	1429039_s_at	239.0	22.1	19.6
EPAS1	3.2595	0.0005	2.5005	0.0011	1449888_at	173.2	567.6	434.7
FNTB	4.7982	0.0061	4.1234	0.0057	1459043_at	82.3	381.3	321.4
GRB10	−5.5633	0.0004	−4.8841	0.0005	1425457_a_at	755.8	135.6	154.3
GYLTL1B	−2.162	0.0065	−2.2273	0.0027	1434007_at	671.5	315.6	304.4
HAS2	−14.8595	0.0064	−24.3663	0.0051	1449169_at	272.3	20.7	13.0
HMGA2	2.2213	0.0022	2.1215	0.0048	1450780_s_at	1068.8	2346.3	2250.7
IL6	−3.569	0.0017	−4.072	0.0054	1450297_at	1257.0	355.5	321.3
LARGE	−2.0839	0.0075	−4.2598	0.0027	1417435_at	1017.6	478.2	240.3
NR3C1	2.8113	0.0007	2.1535	0.0007	1460303_at	414.5	1174.6	895.2
RAB17	2.6639	0.0005	2.1843	0.0010	1422178_a_at	702.5	1868.9	1531.4
Sco2	−5.5218	0.0026	−3.4909	0.0046	1432181_s_at	450.7	81.4	126.5
TBX20	3.6076	0.0000	3.5279	0.0004	1453351_at	686.4	2477.4	2451.8

Table 6

Functional classification of genes that differed in blastocysts derived by intracytoplasmic sperm injection (ICSI) versus ICSI with chemical activation (ICSI-A) ($P < 0.05$).

Type		Name	Entities	P-value
Development	Cellular	Somitogenesis	25	0.00686
	Neural	Peripheral nervous system development	12	0.00474
		Central nervous system development	72	0.00824
		Hindbrain development	28	0.00687
	Structural	Embryonic forelimb morphogenesis	6	0.00478
		Negative regulation of striated muscle development	10	0.00266
		Anatomical structure development	8	0.00717
Metabolism		Insulin secretion	15	0.00180
		Fucose metabolic process	6	0.00192
Response	Cellular	Receptor clustering	10	0.00151
		Fertilization	19	0.00266
		Protein localization	49	0.00039
		Synaptic vesicle endocytosis	7	0.00847
		Vesicle organization	7	0.00901
	Immune	Cytokine production	15	0.00822
	Neural	Synaptonemal complex assembly	8	0.00598

The number of entities and level of statistical significance are indicated for each biological function.