Mamm Genome
DOI 10.1007/s00335-009-9227-0

Effect of IVF and laser zona dissection on DNA methylation pattern of mouse zygotes

Dominika D. Peters · Konstantin Lepikhov · Karsten Rodenacker · Susan Marschall · Auke Boersma · Peter Hutzler · Hagen Scherb · Jörn Walter · Martin Hrabé de Angelis

Received: 29 January 2009 / Accepted: 4 September 2009

Abstract In vitro fertilization (IVF) and zona pellucida laser microdissection-facilitated IVF (Laser-IVF) are presently routine procedures in human assisted reproduction. The safety of these methods at the epigenetic level is not fully understood. Studies on mouse Laser-IVF embryos provide evidence that the use of Laser-IVF leads to reduced birth rate, indicating a potential harm of this technique for the embryo. Hence, the aim of this study was to examine the difference in DNA methylation pattern between IVF- and Laser-IVF-derived mouse zygotes. We examined two experimental groups of C3HeB/FeJ oocytes: (1) zona-intact and (2) laser-microdissected oocytes that were fertilized in vitro with freshly collected spermatozoa. Zygotes were fixed 5, 8, and 12 h after fertilization, and indirect immunofluorescence staining was studied using an anti-5-methylcytidine (5-MeC) antibody. The fluorescence intensities of paternal and maternal pronuclei were evaluated using the computer-assisted analysis of digital images. In addition, we performed a semiquantitative RT-PCR analysis of the presence of transcripts of three developmental marker genes, *Oct4*, *Dab2*, and *Dnmt3b*, in IVF- and Laser-IVF-derived blastocysts. We observed no significant differences in methylation status of the paternal genome and in the transcripts of the developmental marker genes after IVF and Laser-IVF. In conclusion, epigenetic patterns and early embryonic development are not altered by laser-assisted IVF techniques and another explanation must be sought for the poor implantation rates observed in mice.

D. D. Peters · S. Marschall · A. Boersma · M. Hrabé de Angelis (✉)
Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
e-mail: hrabe@helmholtz-muenchen.de

K. Rodenacker · H. Scherb
Institute of Biomathematics and Biometry, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

K. Lepikhov · J. Walter
Department of Natural Sciences – Technical Faculty III FR 8.3, Biological Sciences, Institute of Genetics/Epigenetics, University of Saarland, Saarbrücken, Germany

P. Hutzler
Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

Introduction

Since the initiation of large-scale mouse mutagenesis projects (Hrabe de Angelis and Balling 1998; Hrabe de Angelis et al. 2000; Nolan et al. 2000), numerous mouse lines have been generated and cryopreserved (Marschall and Hrabe de Angelis 1999; Nakagata 2000; Thornton et al. 1999). Cryopreservation of mouse germ cells generally provides a means for long-term storage of valuable mouse lines and facilitates the management of animal facilities. Subsequently, the cryopreserved mouse lines have to be recovered using thawed spermatozoa and in vitro fertilization or thawed embryos and embryo transfer (Marschall et al. 1999).

In vitro fertilization has been frequently used to overcome defects in the reproductive ability of mutant or aged mice (Marschall et al. 1999; Nakagata 2000; Sztein et al.

Published online: 13 October 2009

2000; Thornton et al. 1999). Inbred mouse strains have defined reproductive parameters, which are characteristic of the strain. Also, differences in the ability of spermatozoa to survive cryopreservation and to fertilize oocytes of the same genetic background were described in several studies (Nakagata 2000; Nakagata and Takeshima 1993; Sztein et al. 2000). For example, for mutant mouse lines on a C57BL/6 J background, embryo freezing after in vitro fertilization using fresh sperm of a mutant male and wild-type oocytes (Cryo-IVF) is the routine cryopreservation method, because frozen-thawed sperm of these strains has poor fertilizing ability (Landel 2005).

To increase the efficiency of fertilization the routinely used rederivation methods can be enhanced by additional assisted reproductive technologies (ART) such as zona pellucida removal (Ribas et al. 2006), partial zona dissection (PZD) (Depypere et al. 1988; Nakagata et al. 1997; Payne 1995), zona incision by a piezomanipulator (ZIP) (Kawase et al. 2002, 2004), subzonal insemination (SUZI) (Enginsu et al. 1995), or intracytoplasmic sperm injection (ICSI) (Szczygiel et al. 2002; Yanagimachi 1998). Since the 1990s laser-assisted systems have been used for zona microdissection of murine and human embryos (Enginsu et al. 1995; Germond et al. 1995, 1996; Hollis et al. 1997; Liow et al. 1996; Peters et al. 2006). The purpose of these laser systems was to improve the fertilization rate (el-Danasouri et al. 1993) or the hatching rate of the blastocysts (Obruca et al. 1994). The safety of the procedure was tested for some systems with respect to the potential damage at the ultrastructural (scanning and transmission electron microscopy) and biological (number of mice born, morphologic examination, and reproductive capacity of offspring) levels (Germond et al. 1995, 1996; Liow et al. 1996), but epigenetic safety of the procedure has not been studied yet. One study that used the same laser system (Octax laser) as in this study reported a significantly higher cleavage rate but, at the same time, a reduced birth rate of laser-dissected compared to normal IVF embryos (Boersma et al. 2007).

Normal embryonic development in mammals requires differential imprinting in both male and female genomes (McGrath and Solter 1984). After the oocyte is activated by sperm and completes meiosis, haploid oocyte chromosomes transform into a maternal pronucleus and the haploid sperm nucleus transforms into a paternal pronucleus. When sperm chromatin protamines are replaced with oocyte cytoplasmic histones, the extensive demethylation of paternal DNA occurs; this has been described as "active demethylation" (Mayer et al. 2000; Oswald et al. 2000). After completion of the first cell cycle the demethylation of the maternal genome proceeds and is referred to as the "passive phase" of demethylation (Howlett and Reik 1991). This DNA methylation pattern is important in the sex-specific germline "marking" of parental alleles and contributes to the appropriate expression of many imprinted and developmentally important genes (Reik and Walter 2001; Reik et al. 2001).

Previous studies have shown that preimplantation embryos are sensitive to the environment and manipulation can effect developmental potential and fetal growth. For example, the methylation pattern of imprinted genes has been shown to change in response to embryo culture conditions (Doherty et al. 2000; Khosla et al. 2001a, b). In addition, zona-free embryos in which the zona pellucida was removed between 1 and 8 h post fertilization show a significant reduction in the DNA methylation level at two-cell and four-cell stages (Ribas et al. 2006). These results indicate that changes in the environment of the embryo, such as in vitro culture and mechanical or chemical manipulation, can cause some epigenetic modifications. On the other hand, Fulka and Fulka (2006) found no differences in the DNA methylation pattern in mouse zygotes produced in vivo, in vitro, or by intracytoplasmic sperm injection. For this reason the analysis of very early changes occurring just after fertilization is an essential step in the assessment of the safety of given procedures. Thus, the safety of all ART methods with respect to epigenetic patterns should be evaluated. This is especially important because these techniques are widely applied for the archiving and rederivation of valuable mice mutant strains in international repositories such as the European Mouse Mutant Archive (EMMA; www.emmanet.org). Furthermore, the mouse serves as a model organism to estimate the safety of procedures before their application in human assisted reproduction.

The analysis of the expression of marker genes for some developmentally important genes using RT-PCR provides additional valuable information about correct embryonic development. Several genes have been shown to be indispensable for embryonic development, and the properly orchestrated gene expression profile accompanies the formation of two distinct cell lineages in blastocyst: trophectoderm and inner cell mass (ICM). For example, the pluripotency state of ICM cells is characterized by the expression of specific genes, including *Oct4* (Scholer et al. 1990), *Dab2* (Yang et al. 2002), and *Dnmt3b* (Hirasawa and Sasaki 2009).

In this study we investigated the effect of IVF and Laser-IVF on mouse zygotes. The formation of pronucleus (PN) stages, the DNA methylation pattern, the methylation status of the paternal genome at three different time points, and the expression of three developmentally important genes (*Oct4*, *Dab2*, *Dnmt3b*) after IVF and Laser-IVF are also examined.

Materials and methods

Animals

Inbred C3HeB/FeJ mice served as sperm (12-week-old) and oocyte (56-day-old) donors. Mice were bred at the Helmholtz Zentrum Munich. The animal facility was tested for microorganisms according to the FELASA recommendations for mouse health monitoring (Nicklas et al. 2002). The husbandry conditions were as follows: room temperature at 20–24°C, air humidity was 50–60%, there were 20 air changes per hour, and the lighting regimen was on a 12-h light/dark cycle. Wood shavings (Altromin GmbH, Lage; Germany) were used as bedding material. Mice were fed a standardized mouse diet (1314 Altromin) and drinking water was available ad libitum.

Collection of sperm samples

Mice were killed by cervical dislocation. Both caudae epididymides and vasa deferentia were dissected and placed in 220 µl of human tubal fluid (HTF) medium which was prepared from reagent-grade chemicals (Sigma-Aldrich, Taufkirchen, Germany) as Quinn et al. (1985) reported. The tissues were cut with a sterile injection needle (B. Braun, Melsungen, Germany) and the sperm were allowed to swim out and disperse for 10 min at 37°C and 5% CO_2. The dish was gently shaken until the sperm suspension was homogeneous and 6 µl of the sperm suspension was placed in 500 µl of HTF medium under mineral oil (embryo tested, Sigma-Aldrich, Taufkirchen, Germany). The HTF drops with spermatozoa were incubated at 37°C and 5% CO_2 and used for in vitro fertilization. The sperm were capacitated for 1.0 h. The sperm quality was determined using a Hamilton Thorn IVOS computerized semen analyzer (Hamilton Thorn, Beverly, MA) as previously reported by Sztein et al. (2000).

In vitro fertilization

Female mice were superovulated by intraperitoneal (i.p.) injection of 5 IU of eCG (Intergonan 1000, Intervet, Unterschleißheim, Germany) followed by 5 IU of hCG (Ovogest 1500, Intervet) 48 h later. The oocytes were collected 14 h after hCG injection according to standard procedures (Nagy et al. 2003). Mice were killed by cervical dislocation and the oviducts were dissected. The cumulus-oocytes complexes were isolated from oviducts in a 500-µl HTF drop under mineral oil. The oocytes were denuded of cumulus cells by washing in M2 medium containing 600 µg/ml of bovine testes hyaluronidase (Sigma-Aldrich, Taufkirchen, Germany). Four aliquots of 100 µl of M2 medium (Sigma-Aldrich) were placed into petri dishes under mineral oil and denuded oocytes were distributed into these drops. Oocytes from two drops of M2 medium were used for IVF without laser microdissection. Oocytes from two other drops of M2 medium were used for Laser-IVF. After laser microdissection all oocytes were added immediately to the appropriate fertilization dishes with capacitated sperm samples. After 5 h of incubation (5% CO_2, 37°C, and 95% humidity), the oocytes were washed four times through four medium changes (100 µl each) of KSOM (Powdered Media Kit, Metachem Diagnostics Ltd., Northampton, UK) and incubated in a CO_2 incubator in a fifth drop of 100 µl of KSOM until 8 or 12 h post fertilization.

Laser microdissection with Octax Laser

The Octax Laser Shot System with a 1.48-µm infrared diode laser (MTG Medical Technology Vertriebs-GmbH, Altdorf, Germany) was used for the microdissection of the zona pellucida. The laser system was adapted to an inverted microscope (Leica DM IRB, Leica Microsystems GmbH, Wetzlar, Germany), and images of the oocytes were transmitted by video camera (Octax Eye USB Camera) to the monitor system. Petri dishes were fixed on the microscope stage and holes of about 7-µm diameter were cut through the zona pellucida. Under visual control, the zona pellucida was partly, but not completely, penetrated. The success of the microdissection procedure was verified on the computer screen and oocytes with completely disrupted zona pellucida were discarded. After laser microdissection, the oocytes were placed into fertilization dishes with capacitated spermatozoa.

Fixation of mouse zygotes

All procedures were performed on microscope slides with cavities (Carl Roth, Karlsruhe, Germany), siliconized with 5% dimethyldichlorosilane in toluene (Sigma-Aldrich, Taufkirchen, Germany). The zona pellucida was removed by incubation for 1–2 min in 0.1 ml of acidic Tyrode's solution (Sigma-Aldrich). Directly after zona removal zygotes were fixed in 0.1 ml of 3.7% paraformaldehyde in 1× PBS for 30 min at room temperature (RT).

Immunofluorescence staining

All procedures were carried out at RT in a humidified chamber. The immunostainings were performed in a blocking solution (BS) which consisted of 1% BSA and 0.1% Triton X100 in 1× PBS. After fixation with 3.7% PFA the zygotes were permeabilized with 0.2% Triton X100 in 1× PBS for 20 min and DNA was denatured for 10 min with 4 M HCl. After denaturation embryos were neutralized for 10 min with 0.1 M Tris in 1× PBS (pH 8.0)

and post-fixed for 30 min with 3.7% PFA. After permeabilization with 0.2% Triton X100 in 1× PBS for 10 min, embryos were blocked for 3 h in BS. Subsequently, embryos were incubated with the primary anti-5-methylcytidine antibody (Eurogentec, Köln, Germany, Cat. No. BI-MECY-0100) for 3 h in BS. Washing was carried out three times with 100 µl of BS for 1 h. Incubation with the secondary antibody Alexa Fluor 488 goat anti-mouse IgG (Invitrogen GmbH, Karlsruhe, Germany, Cat. No. A11001) was performed for 2 h in BS followed by subsequent washes with BS. In addition, propidium iodide (PI) was added to BS for 15 min after a second wash with BS. Afterward embryos were equilibrated for 5 min in a small drop of mounting medium (Vectashield, Linaris, Germany). In the meantime clean microscope slides with a small drop (1–2 µl) of the mounting medium were prepared and up to 20 zygotes were put into one drop. A tiny drop of mounting solution was placed on the coverslip and gently (using fine forceps) put over the drop with the embryos. After 5 min the coverslips were sealed with nail polish.

Digital image microscopy and analysis

The slides were analyzed on a Zeiss Axiovert 200 M fluorescence microscope equipped with a 40 ×/0.75 Plan-Neofluar lens and the filter sets for Alexa Fluor 488 (with excitation 450–490 and emission 515–565) and PI (with excitation 540–552 and emission 575–640) and the digital black/white camera Axiocam HRm for imaging. The final images were generated using Zeiss Axiovision software 4.5.

As shown schematically in Fig. 1, the masks of maternal and paternal pronuclei were automatically estimated from PI and anti 5-MeC images. Results were interactively controlled and corrected if necessary. The larger pronucleus was considered as paternal. Masks of the whole cell (A), of the paternal (1) and maternal (2) pronuclei, and of the background of the cell (B) without pronuclei masks (1) and (2) and polar bodies were estimated. From each mask the area (area1 and area2), the mean value (M11 and M21), and standard deviation (M12 and M22) of underlying fluorescence intensities were calculated. Pronucleus masks could overlap. Intensities of overlapping areas were equal weight distributed to both pronuclei. Derived parameters per cell are the paternal to maternal ratios of area (ra = area1/area2), the mean fluorescence intensity (rif = M11/M21), and the total fluorescence intensity [rtf = (area1*M11)/(area2*M21)]. Background-corrected parameters were examined but did not add further information and were therefore not included in the results. The relative ratio of paternal to maternal methylation was shown as a percentage of zygotes with a hyper- (ratio >1.0) or hypomethylated (ratio <1.0) paternal genome. Image analysis was performed using Image J (W. S. Rasband, Image J, U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2007) and IDL (Ittvis, Boulder, CO).

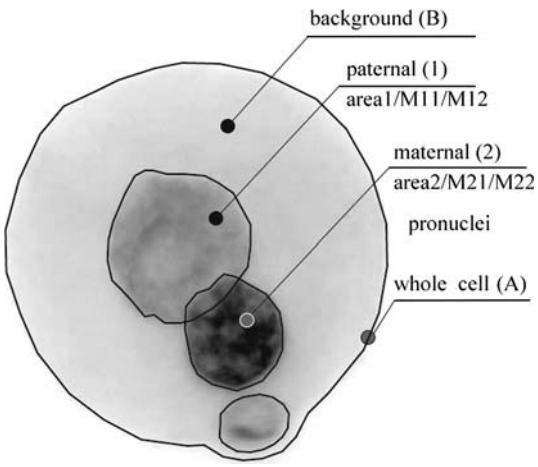

Fig. 1 Digital image analysis. Scheme illustrating the calculation of the fluorescence intensity of paternal and maternal pronuclei, using masks of the whole cell (*A*), the paternal (*1*) and maternal (*2*) pronuclei, and of the background of the cell (*B*). For details see text

Statistical analysis

The statistical package SAS v9.1 (SAS Institute, Cary, NC, USA) was used to analyze the difference between the anti-5-MeC fluorescence intensity patterns of paternal and maternal pronuclei seen in IVF- and Laser-IVF-derived mouse oocytes in order to assess the difference in DNA methylation patterns. Simple data descriptions and comparisons were set up by procedures UNIVARIATE and NPAR1-WAY. The SAS procedures REG and GLM were used to develop synoptic interactive models controlling for treatment of relative fluorescence intensities on time, paternal intensities on maternal intensities, and paternal area on maternal area of the pronuclei. In these models, gender of genome and treatment are dummy variables coding 0 for the male and 1 for the female genome, and coding 0 for IVF and 1 for Laser-IVF treatment. These models fit the data reasonably well. The SAS procedure CATMOD was used to develop a log linear model for the time pattern of the methylation states of pronuclei by treatment.

Semiquantitative RT-PCR analysis

Total mRNA was isolated from blastocysts derived by in vitro cultivation of fertilized normal and laser-treated oocytes in modified KSOM medium (Millipore, Germany) in the atmosphere of 5% CO_2, 37°C and 95% humidity for 80 h. Polyadenylated mRNA was isolated as described (Wrenzycki et al. 1999) with some modifications. Briefly, five to ten blastocysts were rapidly lysed in 30 µl of lysis

Table 1 Primers used for PCR

Genes	Primer sequences 5′ → 3′	Annealing temperature (°C)	Fragment size (bp)
Pol2a			
Forw:	ACCAAAGAGAAGGGCCATGGCG	58	467
Rev:	TTCTGCATGCGACGGGGTAAGC		
Oct4			
Forw:	GGCGTTCTCTTTGGAAAGGTGTTC	58	312
Rev:	CTCGAACCACATCCTTCTCT		
Dab2			
Forw:	CAAACCAGGAAGAGGCAGAAGGACT	53	276
Rev:	ACTTATTATGGCTCCTGGGACCACA		
Dnmt3b			
Forw:	TGGGTACAGTGGTTTGGTGATGGCA	63.5	287
Rev:	TGGTTGCTTCTTGTTGGGTTTGAGG		

solution [100 mM Tris HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA, 0.5% sodium dodecylsulfate, 5 mM dithiothreitol] and vortexed for 10 s. After incubation at room temperature for 10 min, 5 µl of oligo-dT Dynabeads suspension (Dynal, Norway) was added and the sample was then incubated for 10 min at room temperature to allow binding of poly(A) mRNA to the Dynabeads. The tubes were then placed in the magnetic separator for 2 min, and after the removal of the supernatant, the beads were washed once with 40 µl of wash buffer 1 [10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA, 0.1% sodium dodecylsulfate] and 2 times with 100 µl of wash buffer 2 [10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA]. The RNA was eluted from the beads with 11 µl of sterile water, heated at 65°C for 2 min, and used directly for reverse transcription. The reverse transcription was done using M-MLV RNase H minus reverse transcriptase and random primers (Promega, Madison, WI, USA) according to the manufacturer's protocol. Reverse transcription reaction (0.5–1 µl) was used as a template for the subsequent PCR amplification in 20 µl of solution with HOT FIREPol®DNA Polymerase (Solis BioDyne, Estonia). The primers used for the amplification are listed in Table 1. The PCR program employed an initial denaturation step at 95°C for 5 min and then 35 cycles of 45-sec denaturation at 95°C, annealing at primer-specific temperature (indicated in Table 1), and a 1-min elongation step at 72°C.

Results

Formation of pronucleus stages after IVF and Laser-IVF

The global pattern of pronuclear morphology and the relative positions of pronuclei after fertilization have been assigned according to the nomenclature of Adenot et al. (1997) and Santos et al. (2002). Corresponding to changes in size, morphology, and positioning of both pronuclei (PN) the fertilized oocytes were subdivided into five groups (PN1–PN5) and analyzed at defined time points after fertilization. Figure 2 demonstrates the pronuclear stage distributions after IVF and Laser-IVF in mouse zygotes at 5, 8, and 12 h post fertilization.

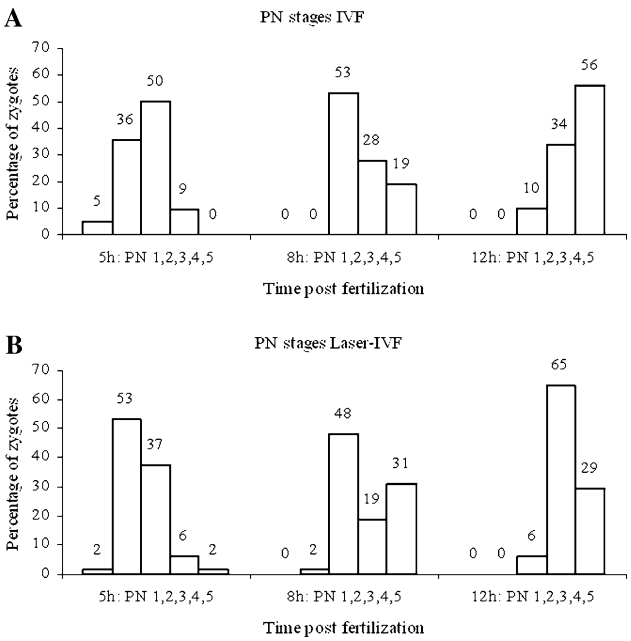

Fig. 2 Distribution of pronuclear (PN) stages after IVF (**a**) and Laser-IVF (**b**). PN stages were assigned according to the following morphological changes in the mouse zygote: *PN1* small pronuclei located at the periphery of the embryo, *PN2* pronuclei increased in size located at the periphery of the embryo, *PN3* large pronuclei migrated toward the center of embryo, *PN4* large pronuclei were close to each other in the center of embryo, *PN5* large pronuclei partially overlapped in the center of embryo. The total number of oocytes scored was for IVF: 5 h (42), 8 h (58), 12 h (41), and for Laser-IVF: 5 h (64), 8 h (58), 12 h (34). The PN stages are described in terms of methylation status in Fig. 3

Five hours after fertilization most of the IVF and Laser-IVF zygotes reached PN stage 2 or 3 (86 and 91%, respectively). The majority of zygotes in the IVF group were already at PN3 (50% at PN3 vs. 36% at PN2), while for Laser-IVF most of the zygotes were at PN2 (53% at PN2 vs. 38% at PN3). Eight hours after fertilization the highest percentage of IVF and Laser-IVF zygotes demonstrated PN3 (53 and 48%, respectively). Twelve hours after fertilization the obtained zygotes were mostly at PN5 in the IVF group (56%) and at PN4 in the Laser-IVF group (65%).

Maximum-likelihood analysis of variance showed that the second-order effect pronuclear state * time is dependent on the type of treatment (IVF vs. Laser-IVF, $P = 0.0187$).

DNA methylation pattern after IVF and Laser-IVF

After classification to PN stages, the timing for active demethylation of paternal pronuclei in the IVF and Laser-IVF groups was determined. Figure 3 demonstrates representative PN stages and anti-5-MeC labeling for maternal and paternal pronuclei at 5, 8, and 12 h post fertilization.

After 5 h of fertilization, most of IVF zygotes reached PN3 (50%) and DNA methylation was observed in both pronuclei in 100% of cases. At 8 h post fertilization, 19% of IVF zygotes showed demethylation of paternal pronuclei and 53% of the zygotes reached PN3. At 12 h post fertilization, the IVF zygotes demonstrated 100% demethylation of paternal pronuclei and 56% of the zygotes were at PN5.

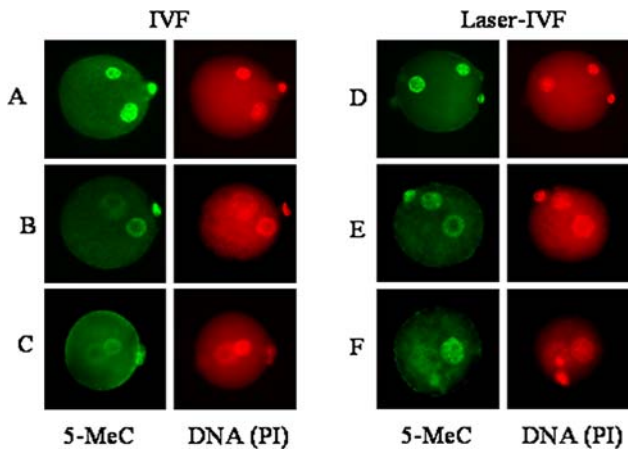

Fig. 3 Methylation status of representative PN stages after IVF (**a**–**c**) and Laser-IVF (**d**–**f**). Indirect immunofluorescence staining with anti-5-methylcytidine (5-MeC) antibody was applied. Pronuclei and polar bodies were stained with propidium iodide (PI). IVF: **a** 5 h after fertilization, PN3 (50%), both pronuclei methylated (100%); **b** 8 h after fertilization, PN3 (53%), demethylation of paternal pronuclei (19%); **c** 12 h after fertilization, PN5 (56%), demethylation of paternal pronuclei (100%). Laser-IVF: **d** 5 h after fertilization, PN2 (53%), demethylation of paternal pronuclei (12%); **e** 8 h after fertilization, PN3 (48%), demethylation of paternal pronuclei (7%); **f** 12 h after fertilization, PN4 (65%), demethylation of paternal pronuclei (100%)

The Laser-IVF zygotes, fixed 5 h after fertilization, presented the demethylation of paternal pronuclei in 12% of the cases and the majority of zygotes were at PN2 (53%). At 8 h post fertilization, 7% of Laser-IVF zygotes showed demethylation of paternal pronuclei and most of the zygotes reached PN3 (48%). At 12 h post fertilization, Laser-IVF zygotes demonstrated 100% demethylation of paternal pronuclei and reached PN4 in 65% of the cases.

Quantitative estimation of the methylation status of paternal genome after IVF and Laser-IVF

Methylation states of the paternal genome after IVF and Laser-IVF were examined 5, 8, and 12 h post fertilization. Figure 4 demonstrates the relative ratio of paternal to maternal methylation as a percentage of zygotes with hyper- (ratio >1.0) or hypomethylated (ratio <1.0) paternal genome.

At 5 h after IVF, the paternal genome in 62% of zygotes was found to be hypomethylated, similar to the paternal genome after Laser-IVF (55%). At 8 h after fertilization, the paternal genome was hypomethylated in 79% of zygotes of both IVF and Laser-VF groups. At 12 h after

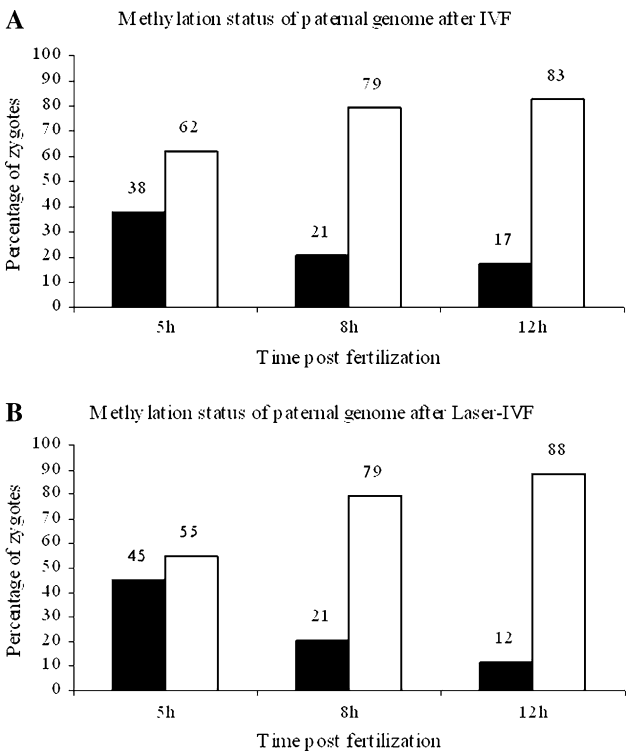

Fig. 4 Relative ratio of paternal to maternal DNA methylation after IVF (**a**) and Laser-IVF (**b**). After indirect immunofluorescence staining with anti-5-methylcytidine (5-MeC), analysis of images was performed using Image J and IDL. The relative ratio of paternal to maternal methylation was shown as a percentage of zygotes with hyper- (ratio >1.0, depicted in *black*) or hypomethylated (ratio <1.0, depicted in *white*) paternal genome

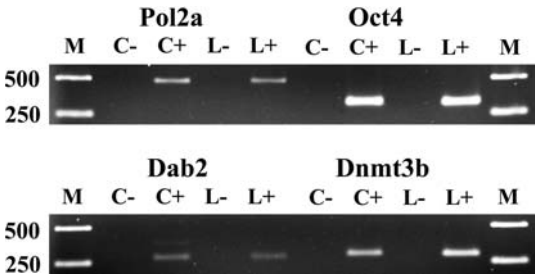

Fig. 5 Semiquantitative analysis of the relative abundance of *Oct4*, *Dab2*, and *Dnmt3b* transcripts in IVF- and Laser-IVF-derived blastocysts. Total mRNA was isolated from blastocysts developed in vitro from fertilized control IVF group oocytes and oocytes that had undergone Laser-IVF. The isolated mRNAs were reverse transcribed and used as templates for PCR with primers amplifying fragments of *Pol2a*, *Oct4*, *Dab2*, and *Dnmt3b* cDNAs. *M* DNA size marker, *C*− reverse transcriptase minus control IVF group, *C*+ reverse transcriptase plus control IVF group, *L*− reverse transcriptase minus Laser-IVF-treated group, *L*+ reverse transcriptase plus Laser-IVF-treated group

IVF and Laser-IVF, hypomethylation of the paternal genome was observed in 83 and 88% of zygotes, respectively.

Maximum-likelihood analysis of variance showed no statistical significance of all tested variables dependent on time and treatment (IVF vs. Laser-IVF, $P = 0.9602$).

RT-PCR analysis of some developmentally important gene transcripts

To estimate the presence of mRNAs encoding some developmentally important proteins in IVF-and Laser-IVF-derived blastocysts, we performed semiquantitative reverse transcriptase PCR analysis on total mRNA, isolated with the help of oligo-dT-covered magnetic beads. As marker genes we chose the transcription factor *Oct4*, which is predominantly expressed in inner cell mass; *Dab2*, a mitogen-responsive phosphoprotein expressed in ovaries and early embryos; and *Dnmt3b*, DNA methyltransferase participating in establishing *de novo* DNA methylation. *Pol2a* served as the internal normalization control. As shown in Fig. 5, there is no significant difference in the expressions of *Oct4*, *Dab2*, and *Dnmt3b* mRNAs.

Discussion

In this study we have demonstrated the quantitative analysis of the DNA methylation pattern of IVF- and Laser-IVF-derived mouse embryos. Using indirect immune fluorescence staining with anti-5-MeC antibodies, we compared the methylation status of the paternal genome in mouse zygotes 5, 8, and 12 h post fertilization. In addition, we calculated the PN stage distribution of zygotes in the IVF and Laser-IVF groups, collected at 5, 8, and 12 h post fertilization.

We observed slower development of Laser-IVF zygotes to PN2 5 h after fertilization. Most IVF zygotes reached PN3 during the same time. This delay of development was compensated for by 8 h post fertilization at which time we observed similar development of most zygotes to PN3 in the IVF and Laser-IVF groups. However, at 12 h post fertilization we observed again slower development of the Laser-IVF zygotes to PN4. At this time after fertilization most IVF zygotes reached PN5.

Our experiment's setup (hyaluronidase treatment of both IVF and Laser-IVF oocytes; fertilization in both groups at the same time) allowed us to attribute the observed delay in development of Laser-IVF zygotes to the laser manipulation of the zona pellucida. In a previous study (Peters et al. 2006) we reported a significantly higher cleavage rate of inbred C3HeB/FeJ oocytes after Laser-IVF; however, the birth rate after Laser-IVF was significantly lower compared to IVF. Boersma et al. (2007) reported a similarly significantly higher cleavage rate of Laser-IVF inbred C57BL/6 J oocytes and significantly lower birth rate after Laser-IVF compared to IVF. When culturing two-cell-stage inbred C3HeB/FeJ embryos through to the blastocyst stage after IVF and Laser-IVF, 84% of the Laser-IVF but only 67% of the IVF embryos developed into blastocysts (unpublished data). These results indicate that further in vitro development of embryos is not delayed after laser zona dissection, but the birth rate after Laser-IVF is significantly lower compared to that after IVF.

Second, we followed the course of paternal pronuclei demethylation in IVF and Laser-IVF zygotes. The active demethylation of paternal pronuclei was observed at 8 h post fertilization in 19% of IVF zygotes (50% at PN3). At 5 and 8 h post fertilization, 12 and 7% of Laser-IVF zygotes, respectively, demonstrated demethylation of paternal pronuclei (53% at PN2 and 48% at PN3, respectively). At 12 h post fertilization, 100% of the IVF and Laser-IVF zygotes demonstrated demethylation of paternal pronuclei (56% at PN5 and 65% at PN4, respectively). Our findings were compared with previous results obtained in mice (Fulka and Fulka 2006; Santos et al. 2002). Santos et al. (2002) demonstrated methylation of both pronuclei in 94–100% of zygotes at PN1 and PN2 during the whole development after fertilization. Zygotes at PN3, PN4, and PN5 presented methylation of both pronuclei in 27, 15, and 8% of cases, respectively. Fulka and Fulka (2006) observed that 12 h after fertilization 91% of PN4 zygotes had undergone demethylation of paternal pronuclei. Our IVF and Laser-IVF zygotes showed methylation of both pronuclei at PN3 in 81 and 93% of cases, respectively. Zygotes at PN4 and PN5 demonstrated 100% demethylation of paternal pronuclei. The relatively high number of IVF and Laser-IVF

zygotes presenting methylation of both pronuclei at PN3 at 8 h post fertilization could result from hyaluronidase treatment of zygotes before fertilization in contrast to cumulus-oocyte complexes routinely used for IVF. Also, the time between preparation of oocytes and fertilization was lengthened due to the laser microdissection of zona pellucida compared to the routinely performed IVF. A similar study was performed by Ribas et al. (2006) for zona pellucida removal in early mouse embryos. They observed that the time of zona removal influenced the levels of DNA methylation. When zona removal was delayed for 8 h, there was no difference in DNA methylation levels between zona-free and zona-intact two-cell embryos, indicating that the critical time is early on, between 1 and 8 h post fertilization. They suggested that the period immediately after the fertilization is the most sensitive. At this time the male DNA is even very close to the oolemma, which may make it more vulnerable to changes during zona pellucida removal and may possibly affect DNA methylation as well.

Another reason for the relatively high number of IVF and Laser-IVF zygotes presenting methylation of both pronuclei at PN3 could be the difference in mouse strain and IVF protocol used in our study compared with those used in previously published studies. In our study we used hyaluronidase denuded oocytes from inbred C3HeB/FeJ mice and HTF medium, while Santos et al. (2002) used cumulus-enclosed oocytes from hybrid B6CBAF1 mice and performed fertilization in modified KSOM medium. According to their protocol, the oocytes were incubated with spermatozoa for only 15–30 min, and after extensive washes with KSOM, oocytes were cultured for an additional 4, 6.5, or 9.5 h without spermatozoa. In our IVF procedure, the oocytes were incubated with spermatozoa for 5 h in HTF medium and then washed with KSOM and incubated for another 3 or 7 h. After incubation with spermatozoa for 5 h, we could potentially get more zygotes at different PN stages. This effect was, however, partly compensated by the use of cumulus-denuded oocytes which allowed a more simultaneous fertilization. Fulka and Fulka (2006) used inbred ICR oocytes and after 3–4 h of fertilization in HTF only viable-looking zygotes with a second polar body were chosen for further incubation without spermatozoa. In our study all oocytes were incubated until 12 h post fertilization and no selection in any step of preparation or immunocytochemistry was performed.

Finally, we showed that there were no significant differences in methylation status of the paternal genome after IVF and Laser-IVF. We measured the relative ratio of paternal to maternal methylation using Image J and IDL software and calculated the percentage of zygotes with hyper- (ratio >1.0) or hypomethylated (ratio <1.0) paternal genome at 5, 8, and 12 h post fertilization. At 5, 8, and 12 h post fertilization, the paternal genome was found to be similarly hypomethylated after IVF and Laser-IVF. Similar quantitative analysis of pronuclear methylation levels was performed by Kishigami et al. (2006). Using Image J the authors investigated the differences in DNA methylation of paternal genomes after intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI). At 10 h after ROSI, the sperm- and spermatid-derived paternal genomes exhibited different methylation states. Methylation of the spermatid-derived genome was higher than that of the sperm-derived paternal genome. This difference in DNA methylation patterns after ICSI and ROSI was evident in 90% of the zygotes. These results indicated that the DNA hypermethylation of spermatid-derived paternal genomes was caused by remethylation occurring irrespective of demethylation after fertilization.

Our data show no significant differences in methylation status of the paternal genome after IVF and Laser-IVF. Analysis of *Oct4*, *Dab2*, and *Dnmt3b* gene expression also suggests that laser drilling of oocytes' zona pellucida does not alter the expression profiles of some developmentally important genes in derived blastocysts. In conclusion, epigenetic and early embryonic development patterns are not altered by laser-assisted IVF techniques and another explanation must be found for the poor implantation rates observed in mice. The study of other genetic, epigenetic, and developmental characteristics of the embryo to verify the safety of new artificial reproduction techniques and to clarify, for example, the reason for the reduced birth rate after Laser-IVF should be pursued in further studies.

Acknowledgments This work was supported by the European Commission under FP6, by EMMA inf (RII3-CT-2004-506455) to MHdA and by a grant from the National Genome Research Network, NGFN + (01GS0850), to MHdA. We thank Stefanie Dunst, Monika Beschorner, Andrea Bäßler, Alexander Huber, and Bernhard Rey for excellent technical assistance. We are grateful to John Favor for valuable comments on this manuscript.

References

Adenot PG, Mercier Y, Renard JP, Thompson EM (1997) Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. Development 124:4615–4625

Boersma A, Marschall S, Hrabe de Angelis M (2007) Laser-assisted IVF—an alternative approach for successful cryopreservation of mutant mouse lines on C57BL/6 background. International Mouse Genome Conference, Kyoto, Japan, October 28 - November 1, 2007, P77

Depypere HT, McLaughlin KJ, Seamark RF, Warnes GM, Matthews CD (1988) Comparison of zona cutting and zona drilling as techniques for assisted fertilization in the mouse. J Reprod Fertil 84:205–211

Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM (2000) Differential effects of culture on imprinted H19

expression in the preimplantation mouse embryo. Biol Reprod 62:1526–1535
el-Danasouri I, Westphal LM, Neev Y, Gebhardt J, Louie D et al (1993) Zona opening with 308 nm XeCl excimer laser improves fertilization by spermatozoa from long-term vasectomized mice. Hum Reprod 8:464–466
Enginsu ME, Schutze K, Bellanca S, Pensis M, Campo R et al (1995) Micromanipulation of mouse gametes with laser microbeam and optical tweezers. Hum Reprod 10:1761–1764
Fulka H, Fulka J (2006) No differences in the DNA methylation pattern in mouse zygotes produced in vivo, in vitro, or by intracytoplasmic sperm injection. Fertil Steril 86:1534–1536
Germond M, Nocera D, Senn A, Rink K, Delacretaz G et al (1995) Microdissection of mouse and human zona pellucida using a 1.48-microns diode laser beam: efficacy and safety of the procedure. Fertil Steril 64:604–611
Germond M, Nocera D, Senn A, Rink K, Delacretaz G et al (1996) Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 microm diode laser beam. Hum Reprod 11:1043–1048
Hirasawa R, Sasaki H (2009) Dynamic transition of *Dnmt3b* expression in mouse pre- and early post-implantation embryos. Gene Expr Patterns 9:27–30
Hollis A, Rastegar S, Descloux L, Delacretaz G, Rink K (1997) Zona pellucida microdrilling with a 1.48-micron diode laser. IEEE Eng Med Biol Mag 16:43–47
Howlett SK, Reik W (1991) Methylation levels of maternal and paternal genomes during preimplantation development. Development 113:119–127
Hrabe de Angelis M, Balling R (1998) Large scale ENU screens in the mouse: genetics meets genomics. Mutat Res 400:25–32
Hrabe de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D et al (2000) Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet 25:444–447
Kawase Y, Iwata T, Ueda O, Kamada N, Tachibe T et al (2002) Effect of partial incision of the zona pellucida by piezo-micromanipulator for in vitro fertilization using frozen-thawed mouse spermatozoa on the developmental rate of embryos transferred at the 2-cell stage. Biol Reprod 66:381–385
Kawase Y, Aoki Y, Kamada N, Jishage K, Suzuki H (2004) Comparison of fertility between intracytoplasmic sperm injection and in vitro fertilization with a partial zona pellucida incision by using a piezo-micromanipulator in cryopreserved inbred mouse spermatozoa. Contemp Top Lab Anim Sci 43:21–25
Khosla S, Dean W, Brown D, Reik W, Feil R (2001a) Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. Biol Reprod 64:918–926
Khosla S, Dean W, Reik W, Feil R (2001b) Culture of preimplantation embryos and its long-term effects on gene expression and phenotype. Hum Reprod Update 7:419–427
Kishigami S, Van Thuan N, Hikichi T, Ohta H, Wakayama S et al (2006) Epigenetic abnormalities of the mouse paternal zygotic genome associated with microinsemination of round spermatids. Dev Biol 289:195–205
Landel CP (2005) Archiving mouse strains by cryopreservation. Lab Anim 34:50–57
Liow SL, Bongso A, Ng SC (1996) Fertilization, embryonic development and implantation of mouse oocytes with one or two laser-drilled holes in the zona, and inseminated at different sperm concentrations. Hum Reprod 11:1273–1280
Marschall S, Hrabe de Angelis M (1999) Cryopreservation of mouse spermatozoa: double your mouse space. Trends Genet 15: 128–131
Marschall S, Huffstadt U, Balling R, Hrabe de Angelis M (1999) Reliable recovery of inbred mouse lines using cryopreserved spermatozoa. Mamm Genome 10:773–776
Mayer W, Niveleau A, Walter J, Fundele R, Haaf T (2000) Demethylation of the zygotic paternal genome. Nature 403: 501–502
McGrath J, Solter D (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 37: 179–183
Nagy A, Gertsenstein M, Vintersten K, Behringer R (2003) Manipulating the mouse embryo. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Nakagata N (2000) Cryopreservation of mouse spermatozoa. Mamm Genome 11:572–576
Nakagata N, Takeshima T (1993) Cryopreservation of mouse spermatozoa from inbred and F1 hybrid strains. Jikken Dobutsu 42:317–320
Nakagata N, Okamoto M, Ueda O, Suzuki H (1997) Positive effect of partial zona-pellucida dissection on the in vitro fertilizing capacity of cryopreserved C57BL/6 J transgenic mouse spermatozoa of low motility. Biol Reprod 57:1050–1055
Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA (2002) Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim 36:20–42
Nolan PM, Peters J, Strivens M, Rogers D, Hagan J et al (2000) A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. Nat Genet 25:440–443
Obruca A, Strohmer H, Sakkas D, Menezo Y, Kogosowski A et al (1994) Use of lasers in assisted fertilization and hatching. Hum Reprod 9:1723–1726
Oswald J, Engemann S, Lane N, Mayer W, Olek A et al (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475–478
Payne D (1995) Micro-assisted fertilization. Reprod Fertil Dev 7: 831–839
Peters DD, Marschall S, Mahabir E, Boersma A, Heinzmann U et al (2006) Risk assessment of mouse hepatitis virus infection via in vitro fertilization and embryo transfer by the use of zona-intact and laser-microdissected oocytes. Biol Reprod 74:246–252
Quinn P, Kerin JF, Warnes GM (1985) Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 44:493–498
Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2:21–32
Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089–1093
Ribas RC, Taylor JE, McCorquodale C, Mauricio AC, Sousa M et al (2006) Effect of zona pellucida removal on DNA methylation in early mouse embryos. Biol Reprod 74:307–313
Santos F, Hendrich B, Reik W, Dean W (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241:172–182
Scholer HR, Dressler GR, Balling R, Rohdewohld H, Gruss P (1990) Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. EMBO J 9:2185–2195
Szczygiel MA, Kusakabe H, Yanagimachi R, Whittingham DG (2002) Intracytoplasmic sperm injection is more efficient than in vitro fertilization for generating mouse embryos from cryopreserved spermatozoa. Biol Reprod 67:1278–1284
Sztein JM, Farley JS, Mobraaten LE (2000) In vitro fertilization with cryopreserved inbred mouse sperm. Biol Reprod 63:1774–1780
Thornton CE, Brown SD, Glenister PH (1999) Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. Mamm Genome 10:987–992

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H (1999) Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. Mol Reprod Dev 53(1): 8–18

Yanagimachi R (1998) Intracytoplasmic sperm injection experiments using the mouse as a model. Hum Reprod 13(Suppl 1):87–98

Yang DH, Smith ER, Roland IH, Sheng Z, He J et al (2002) Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. Dev Biol 251:27–44