Inhibition of Oct4 Expression in Mouse Preimplantation Embryos Using Morpholino Antisense Oligonucleotides

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Tsuji, I., Mitani, T., Mitsuhashi, A., Watanabe, Y., Hosoi, Y. and Hoshiai, H. Inhibition of Oct4 Expression in Mouse Preimplantation Embryos Using Morpholino Antisense Oligonucleotides. Tohoku J. Exp. Med., 2006, 208 (4), 333-342 —— Morpholino oligonucleotides (MO) can induce gene silencing by binding to a target mRNA and inhibiting its translation, and this technique has been especially successful in studies of embryonic development in various vertebrates. But in mice MO-induced downregulation of target genes has not been widely reported. In this study, we examined whether MO delivery using ethoxylated polyethylenimine (EPEI) delivery reagent is useful for silencing gene expression in the mouse preimplantation embryo, by targeting endogenous gene Oct4. To optimize the conditions for MO delivery, we examined the MO concentration, the EPEI concentration, the treatment time, and the number of MO treatments. The MO treatment was performed at the 2-cell, the morula, the blastocyst, and the hatched blastocyst stage. We first determined the optimal conditions for MO delivery into the nucleus using fluorescein isothiocianate (FITC)-labeled MO, and demonstrated that treatment with a combination of 20 μM MO and 0.56 μM EPEI for 3 hrs produced effective MO delivery. MO-induced downregulation of Oct4 was then examined. Two-step MO treatment at the 2-cell and blastocyst stages successfully suppressed Oct4 expression. This MO treatment resulted in marked reduction of Oct4 protein at the blastocyst stage. After cultivation of blastocysts for further 4 days, derivatives of embryos either differentiated to trophoblastic cells or showed developmental arrest at the blastocyst. This phenocopy is similar to Oct4-deficient embryos. Overall, our results indicate that MO delivery with EPEI is an effective tool for analyzing gene function in mouse preimplantation embryos. —— morpholino; antisense; EPEI; blastocyst; Oct4

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The fertilization of the oocyte by the sperm generates the zygote that begins to cleavage. After several cleavage divisions, the embryo develops to the compact ball of more cells called the morula. After several more cleavage divisions, the morula cells begin to specialize and form a hollow sphere of cells called the blastocyst. The outer layer of the blastocyst is named the trophectoderm (TE) and the cells inside inner cell mass (ICM). The cells of TE are restricted to generation of the trophoblast cells of the placenta. The cells of ICM are pluripotent stem cells that can give rise to all of the embryonic cells and the extraembryonic endoderm.

Oct4 is a member of the Pit-Oct-Unc (POU) transcription factor family and acts as a key regulator in maintaining pluripotency of ICM and embryonic stem cells (Nichols et al. 1998; Pesce and Scholer 2001). During embryonic development in mice, Oct4 shows a remarkable expression pattern. Oct4 protein accumulated in oocytes is present until the 2-cell stage and zygotic Oct4 expression begins at the 4-cell to 8-cell stages (Rosner et al. 1990; Yeom et al. 1996; Stewart 2000). Oct4 is also initially expressed in all blastomeres, but subsequently its expression becomes restricted to the ICM, with corresponding downregulation in trophoblast and primitive endodermal cells (Palmieri et al. 1994). Gene knock-out of Oct4 has shown that Oct4-deficient embryos develop to the blastocyst stage, but then the ICM cells lose their pluripotency and can only differentiate along the extraembryonic trophoblast lineage (Nichols et al. 1998), resulting in embryonic lethality before the egg-cylinder stage.

The decoding of the human and mouse genomes has accelerated understanding of the life sciences, and analysis of gene function in developmental biology is increasingly important in the field of regenerative medicine. To date, gene function in mouse has been studied using gene knockout technology, but this approach is time-consuming and laborious. As an alternative, novel gene-silencing methods, such as antisense DNA oligonucleotides and RNA interference (RNAi), have been developed. Such methods allow targeting of a specific gene in an organism, including at the developmental stage, but often also give rise to non-specific effects and embryonic toxicity.

Morpholino oligonucleotides (MO) offer a simple and effective gene-silencing method that allows transient inhibition of a specific gene while minimizing the side effects seen in other antisense technologies. MO are a novel class of non-ionic synthetic DNA analogues, which were designed to overcome many of the limitations of conventional antisense oligonucleotides (ODNs). MO have high affinity for RNA, high specificity, resistance to nuclease degradation, and minimal toxicity, which collectively give high efficacy in the downregulation of gene expression (Summerton and Weller 1997; Ghosh and Iversen 2000; Corey and Abrams 2001; Ekker and Larson 2001; Heasman 2002). MO are designed to be complementary to 5´ leader sequences, or to the first 25 bases 3´ to the AUG translational start site, and it is thought that they act by preventing ribosome binding and blocking translation of the target mRNA (Heasman 2002). Given their advantageous properties, MO have been used in a variety of cell types and have been demonstrated to be effective in gene silencing in mouse oocytes and preimplantation embryos (Coonrod et al. 2001; Siddall et al. 2002; Cheng et al. 2004).

In antisense approaches, poor uptake and inadequate intracellular compartmentalization are major problems. To overcome these problems, various delivery systems based on physical (microinjection, scrape-loading, syringe-loading, osmotic-loading) and chemical (ethoxylated polyethyleneimine [EPEI], lipofectin, lipofectamine) methods have been used to introduce ODNs into cultured cells (Partridge et al. 1996; Ghosh and Iversen 2000; Marcos 2000, 2001; Coonrod et al. 2001; Ekker and Larson 2001; Siddall et al. 2002; Cheng et al. 2004). Wagner (1994) demonstrated that ODNs accumulate in the nucleus if introduced directly into the cytoplasm, but that they accumulate in endosomes and lysosomes, and not in the nucleus, when added to the culture medium. For effective biological activity, it is important to introduce ODNs into the nucleus efficiently, because the antisense agent must enter the cellular
compartments (specifically the cytosol and nucleus) where the target mRNA is located (Summerton and Weller 1997). Among the many delivery methods, the MO delivery system with EPEI described by Morcos (2001) has several unique features. In this system, a non-ionic MO is paired to a complementary DNA carrier and the DNA is then bound electrostatically to a partially ionized, weakly-basic EPEI, giving an MO/DNA/EPEI complex that is efficiently endocytosed. Subsequently, ionization of EPEI increases at the lower pH in the endosome, and the fully ionized EPEI induces permeabilization of the endosomal membrane and releases the MO into the cytosol.

RNAi technology has revealed developmental phenocopies of various genes in mice (Svoboda et al. 2000; Wianny and Goetz 2000; Kim et al. 2002; Mellitzer et al. 2002; Haraguchi et al. 2004), but MO-induced downregulation of target genes has not been widely reported, although effects on genes such as c-mos, α-catenin and leukemia inhibitory factor (LIF), have been demonstrated. Thus, to determine whether the EPEI-based MO delivery system is useful for silencing genes in the mouse preimplantation embryo, the expression level of endogenous Oct4 was examined as a model target gene.

In this study, we have developed a simple and efficient gene-silencing method using MO in mouse preimplantation embryos. Our results demonstrate that EPEI-based MO delivery can facilitate the analysis of gene function in early development of mouse embryos.

**MATERIALS AND METHODS**

**Oligonucleotides**

MO were provided by Gene Tools, LLC (Philomath, OR, USA). The sequences of Oct4 morpholino antisense oligonucleotide (Oct4MO), inverted Oct4 morpholino non-sense oligonucleotide (InvMO) and FITC-labeled morpholino oligonucleotide (FITC-MO) were 5´-AGCT TAGCCAGGTTCGAGGATCCAT-3´, 5´-TACCTAGGAGCTGACCGAATTGCA-3´ and 5´-CCTCTTACCTCA GTTACAATTATATA-3´, respectively. Oct4MO was designed to be complementary to the first 25 bases of the Oct4 gene, including the translational start site. InvMO was used as a control to assess non-specific effects, and FITC-MO was used to assess uptake into embryos.

**Embryo preparation and in vitro cultivation**

Preimplantation mouse embryos were prepared by in vitro fertilization (IVF). ICR female mice (6-8 weeks old) were superovulated by intraperitoneal injection of 5 IU pregnant mare’s serum gonadotropin (PMSG) and 5 IU hCG 48 hrs later. The mice were sacrificed 14 hrs after hCG injection. Oocytes were collected from the oviducts and were placed in 50-μl culture drops of TYH (Toyoda, Yokoyama, and Hoshi) medium (Mitsubishi Kagaku Iatron, Inc., Tokyo) under mineral oil. ICR male mice were sacrificed and sperm were extracted from the cauda epididymides. The sperm were capacitated in 100 μl drops of TYH medium under mineral oil in an atmosphere of 5% CO2 at 37°C for 1.5 hrs, and then inseminated at a concentration of 1 × 106 sperm/ml in 50 μl drops of TYH medium containing oocytes. Oocytes and sperm were then incubated in an atmosphere of 5% CO2 at 37°C for 4 hrs, followed by incubation of oocytes in 50 μl drops of modified Whitten’s medium (mWM) (Mitsubishi Kagaku Iatron, Inc.) in an atmosphere of 5% CO2 at 37°C. For MO treatment, 2-cell stage embryos, morula and blastocysts were collected after 24, 72 and 96 hrs of insemination, respectively. In some experiments, blastocysts were transferred to feeder layers of mouse embryonic fibroblasts inactivated with mitomycin C in knockout Dullbecco’s Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 15% knockout serum replacement (KSR, Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 584 mg/L L-glutamine (Sigma, St. Louis, MO, USA) and 1,000 U/ml leukemia inhibitory factor (LIF) (Invitrogen) in an atmosphere of 5% CO2 at 37°C for further 4 days. Animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals of Kinki University.

**Delivery of morpholino oligonucleotides**

Delivery of MO into the embryos was performed using the EPEI delivery reagent, according to the published method (Marcos 2001). This protocol was designed to facilitate MO uptake in HeLa cells and involved treatment with a combination of 1 μM MO and 0.56 μM EPEI for 3 hrs. Briefly, MO and EPEI were combined in water to form an MO/EPEI complex, which was allowed to stand at room temperature for 20 min. The MO/EPEI complex solution was then diluted with mWM and vortexed to prepare the MO delivery solution.
Embryos were placed in 50 μl drops of the delivery solution under mineral oil and incubated in an atmosphere of 5% CO₂ at 37°C.

To optimize the conditions for MO delivery into the mouse preimplantation embryos, we investigated the MO and EPEI concentrations, the MO:EPEI ratio, the treatment time (3 hrs, 24 hrs), and the number of MO treatments (once, twice). MO concentrations from 1 to 20 μM (1, 2, 5, 10, 20 μM) and EPEI concentrations from 0.14 to 0.56 μM (0.14, 0.28, 0.56 μM) were examined, and the MO treatment was performed after 24, 72, 96 and 144 hrs of insemination. Development of treated embryos was assessed every 24 hrs after insemination, and the number of embryos developed to the blastocyst stage was examined after 96 hrs of insemination. After cultivation of blastocysts for 4 days on feeder layers, the derivatives were classified as follows: type I, derivative outgrowth containing ICM-derived cell mass; type II, outgrowth consisting of trophoblast cells and lacking ICM-derived cells; and type III, mortal embryo (developmental arrest) (Haraguchi et al. 2004) (Fig. 1).

Assessment of FITC-MO uptake into mouse preimplanta
tion embryos

Cellular uptake of FITC-MO was assessed after 96 hrs of insemination. Blastocysts treated with FITC-MO and control embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and mounted with glycerol. The fluorescence level was examined using a confocal laser-scanning microscope (LSM, BH-2, Olympus, Tokyo).

Indirect immunofluorescence

Indirect immunofluorescence of whole embryos was performed according to the published method (Kim et al. 2002). After 96 hrs of insemination, blastocysts were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed samples were permeabilized in a storage solution (3% fetal calf serum [FCS] and 0.3% Triton X-100 in PBS) for 10 min and washed three times in PBS containing 0.3% bovine serum albumin (BSA) and 0.2% Triton X-100. Immediately before staining, the samples were incubated for 30 min in blocking buffer (0.5% BSA and 0.2% Triton X-100 in PBS) at 37°C. They were then incubated with goat anti-Oct4 polyclonal antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1/100 dilution for 1 hrs at 37°C, and subsequently washed 3 times in PBS for 10 min per washing. The samples were then incubated with Alexa Fluor 488 conjugated donkey anti-goat IgG antibody (A-11055, Molecular Probes, Carlsbad, CA, USA) as the secondary antibody at a 1/200 dilution. Immunostained samples were subsequently incubated with propidium iodide (PI, 0.5 μg/ml) for 5 min at room temperature for DNA staining before mounting (Vectashield H-1000, Vector laboratories, Burlingame, CA, USA). The subcellular localization of Oct4 protein in the embryos was analyzed using a confocal laser-scanning microscope. Using an argon-ion laser, excitation wavelengths of 488 nm and 529 nm were selected for the Alexa Fluor conjugated secondary antibody for PI, respectively. Images of serial optical sections were recorded every 1.5 to 2 μm in vertical steps along the Z-axis of each embryo.

Immunocytochemistry

Blastocysts cultured on feeder layers for 4 days were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Samples were washed in PBS for 10 min, permeabilized by three treatments in 0.1% Triton X-100, and then incubated with 0.5% BSA and 0.2% Triton X-100 in PBS for 30 min at room temperature. The fixed samples were incubated with goat anti-Oct4 polyclonal antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1/100 dilution for 1 hrs at 37°C, and subsequently washed 3 times in PBS for 10 min per washing. The samples were then incubated with Alexa Fluor 488 conjugated donkey anti-goat IgG antibody (A-11055, Molecular Probes, Carlsbad, CA, USA) as the secondary antibody at a 1/200 dilution. Immunostained samples were subsequently incubated with propidium iodide (PI, 0.5 μg/ml) for 5 min at room temperature for DNA staining before mounting (Vectashield H-1000, Vector laboratories, Burlingame, CA, USA). The subcellular localization of Oct4 protein in the embryos was analyzed using a confocal laser-scanning microscope. Using an argon-ion laser, excitation wavelengths of 488 nm and 529 nm were selected for the Alexa Fluor conjugated secondary antibody for PI, respectively. Images of serial optical sections were recorded every 1.5 to 2 μm in vertical steps along the Z-axis of each embryo.
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X-100 in PBS (0.1% Triton X-100 in PBS [PBT]) for 10 min each, and then quenched in 4% H_{2}O_{2} in cold methanol and washed three times in 0.1% PBT for 10 min. They were then incubated for 60 min in a blocking solution containing 5% BSA in 0.1% PBT at room temperature and washed three times in 0.1% PBT for 10 min. The samples were then incubated with goat anti-Oct4 polyclonal antibody (N-19, Santa Cruz Biotechnology) at a 1:100 dilution for 1 hr at room temperature. After washing three times in 0.1% PBT for 15 min, samples were incubated with Horse radish peroxidase (HRP) conjugated donkey anti-goat IgG polyclonal antibody overnight at 4°C, washed three times in 0.1% PBT for 15 min, and stained using a HRP staining kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA).

Statistical analysis

All experiments were performed at least three times. Differences in blastocyst formation and between derivatives classified as type I were compared using one-way factorial analysis of variance (ANOVA). The percentages of type I, type II and type III derivatives after a 4-day culture of blastocysts on feeder layers were compared using a chi-square test. A value of < 0.05 was considered to be statistically significant.

RESULTS

Effects of EPEI-based MO delivery in preimplantation embryos

We examined the efficacy of EPEI-based MO delivery into the nucleus using fluorescein isothiocyanate (FITC)-MO, and determined the effect of the MO on the development of embryos. In the first series of experiments, the embryos were treated with FITC-MO for 3 hrs at the morula stage (72 hrs after insemination). To define an optimal EPEI concentration for MO delivery, the embryos were first treated with 1.0 μM of FITC-MO pre-mixed with EPEI at concentrations of 0.14, 0.28 and 0.56 μM. Embryos treated with 0.14, 0.28, or 0.56 μM EPEI developed similarly to the blastocyst stage, at rates of 72.0%, 69.4% or 68.0%, respectively. As a control, 94.6% of untreated embryos developed to the blastocyst stage. Embryos treated with 0.14, 0.28, or 0.56 μM EPEI also developed similarly to type I outgrowth, at rates of 57.1%, 54.1% or 52.7%, respectively. Eighty four percent of untreated embryos developed to type I outgrowth. The percentages of blastocyst formation and type I outgrowth were clearly lower in the treated groups than in the control group, but there were no significant differences among the treated groups. Delivery of FITC-MO into the nucleus was observed in each group using LSM, and the fluorescence intensity strengthened as the EPEI concentration was increased. Treatment with EPEI at 0.56 μM resulted in stronger fluorescence intensity than at 0.14 μM and 0.28 μM (Fig. 2). Thus, to define an optimal MO concentration, the embryos were treated with FITC-MO at concentrations of 1, 2, 5, 10 and 20 μM pre-mixed with 0.56 μM EPEI. There were no significant differences in the percentages of blastocyst formation and type I derivative outgrowths among the MO concentrations.

![Fig. 2. Fluorescent image of embryos.](image)

Embryos were treated with 1.0 μM FITC-MO and EPEI (0.14 [a], 0.28 [b], or 0.56 μM [c]). Fluorescence was observed in the nuclei of the blastocysts. The fluorescence intensity strengthened as the EPEI concentration was increased. Treatment with EPEI at 0.56 μM resulted in notably stronger fluorescence intensity than at 0.14 μM and 0.28 μM. Scale bar = 50 μm.
concentrations (data not shown). Strong fluorescence intensity was observed in each group, but treatment with 20 μM of FITC-MO showed the strongest fluorescence intensity. An increase in the MO concentration enhanced the uptake of FITC-MO into the nucleus of treated embryos without causing harmful effects to the blastulation of the embryos. Thus, we examined the effects of extending the MO treatment time on embryo development and delivery of FITC-MO into the nucleus. MO treatment was performed for 24 hrs with 0.56 μM EPEI pre-mixed with MO concentrations of 1, 2, 5, 10 and 20 μM. No significant differences were observed in the percentages of blastocyst formation and type I derivative outgrowths among the MO concentrations. However, MO treatment for 24 hrs decreased blastocyst formation and development of type I derivatives in culture, compared to MO treatment for 3 hrs, but did not enhance uptake of FITC-MO into the nucleus (data not shown).

Effects of Oct4MO treatment at the morula stage

Based on the above experiments, 20 μM of Oct4MO or InvMO pre-mixed with 0.56 μM EPEI was incubated with embryos at the morula stage for 3 hrs. Embryos treated with Oct4MO and InvMO developed similarly to the blastocyst stage, at rates of 76.3% and 69.3%, respectively. The ratio of type I, type II, and type III derivatives in Oct4MO- treated group were 79.3%, 3.4%, and 17.2%, respectively. The ratio of type I, type II, and type III derivatives in InvMO-treated group were 78.8%, 1.9%, and 19.2%, respectively. There was no significant difference in the ratio of type I, type II and type III derivatives after blastocysts from Oct4MO- and InvMO-treated embryos were cultured on a feeder layer for 4 days. Thus, under these conditions, we did not observe the phenotypic effect anticipated with Oct4MO treatment, which may have been due to poor uptake of Oct4MO into the nucleus, an insufficient antisense effect induced by the MO, selec-

![Fig. 3. Immunocytochemical analysis of Oct4MO- and InvMO-treated embryos developed to the blastocyst stage after 3 days of in vitro culture. Embryos at the 2-cell stage were treated with a mixture of 0.56 μM EPEI and 20 μM Oct4MO or InvMO for 3 hrs. Individual embryos were stained with anti-Oct4 antibody (green) and with PI for visualization of nuclei (red). Confocal images taken by LSM showed that treatment with Oct4MO resulted in a clear reduction in the intensity of Oct4 immunofluorescence (green) in the inner cell mass (lower panel), compared to control embryos treated with InvMO (upper panel). Scale bar = 50 μm.](image-url)
tion of an embryonic stage that was unsuitable for induction of an antisense effect, or no fundamental effect of Oct4MO on target gene (Oct4) expression. Since delivery of FITC-MO into the nucleus was confirmed by the strong fluorescence intensity in laser scanning microscopy, as shown in Fig. 2, poor uptake of MO into the embryos is unlikely. To examine the second possibility, the embryos were treated twice with Oct4MO 72 hrs after insemination (morula stage) for 3 hrs and additionally at 144 hrs after insemination (hatched blastocyst stage). However, Oct4MO treatment had no effect on the morphology (data not shown). We therefore assumed that choice of an embryonic stage unsuitable for MO treatment was responsible for the lack of an effect, and Oct4MO treatment was subsequently performed at the 2-cell stage.

Effects of Oct4MO treatment at the 2-cell stage

Either 20 μM Oct4MO or InvMO was pre-mixed with 0.56 μM EPEI and incubated for 3 hrs with embryos at the 2-cell stage (after 24 hrs of insemination). Embryos treated with Oct4MO and InvMO developed similarly to the blastocyst stage, at rates of 37.6% and 41.3%, respectively. Although similar blastulation occurred in the Oct4MO- and InvMO-treated groups, immunocytochemistry using anti-Oct4 antibody revealed that the Oct4MO-treated embryos lacked Oct4-positive cells, whereas InvMO-treated embryos contained an inner cell mass with Oct4-positive cells (Fig. 3). Thus, MO treatment of the embryos at the 2-cell stage induced suppression of the target gene at the blastocyst stage.

The blastocysts from the MO-treated embryos were cultured on feeder layers for 4 days. The ratio of type I, type II, and type III derivatives in Oct4MO-treated groups were 65.5%, 6.9%, and 27.9%, respectively. The ratio of type I, type II, and type III derivatives in InvMO-treated group were 69.7%, 6.1%, and 24.2%, respectively. No

Fig. 4. Effect of 2-step treatment of Oct4MO exposure at the 2-cell and blastocyst stages on the outgrowth of treated embryos. The embryos were exposed to MO/EPEI in combination with 0.56 μM EPEI and 20 μM Oct4MO or InvMO at the 2-cell and blastocyst stages for 3 hrs respectively, and cultured for 4 days further in vitro. (a) Oct4MO-treated embryos significantly impaired in the outgrowth classified as type I compared to the control embryos untreated or treated with InvMO. 
Significantly different to controls (p < 0.01). (b) Immunocytochemical analysis of Oct4MO and InvMO treated embryos cultured for 4 days in vitro. Derivatives of embryos treated with InvMO consisted of Oct4-positive ICM-derived cells and Oct4-negative trophoblastic cells, whereas morphology of the outgrowth derived from Oct4MO-treated embryos lacked the small, Oct4-positive cells supposed to be derived from the ICM cells and resulted in a large spread derivative with weakly Oct4-positive cells instead. The embryo treated with InvMO is corresponding to type I and the embryo treated with Oct4 type II. Scale bar = 100 μm.
significant difference in the percentages of type I, type II and type III derivatives or in morphology were observed for blastocysts derived from embryos originally treated with Oct4MO and InvMO. To maintain the antisense effect of Oct4MO, blastocysts (derived from embryos treated with the MO at the 2-cell stage) were re-treated with the MO for 3 hrs, and subsequently cultivated for 4 days on feeder layers. As a control, embryos developed to the 2-cell stage after 24 hrs of insemination were cultured for another 3 days. Significant morphological and immunocytochemical changes were found in derivatives formed from embryos treated with Oct4MO at both the 2-cell and blastocyst stages, and the percentage of type I outgrowths significantly decreased (Fig. 4a). On the fourth day of the culture, immunocytochemistry demonstrated that derivatives of embryos treated with InvMO consisted of Oct4-positive ICM-derived cells and Oct4-negative trophoblastic cells (Fig. 4b), whereas those treated with Oct4MO differentiated to trophoblastic cells and Oct4-negative cells that may have been derived from ICM cells (Fig. 4b).

DISCUSSION

The objective of this study was to investigate whether EPEI-facilitated MO delivery is useful for the analysis of gene function during early development in mice. Thus, it was necessary to determine the optimal conditions (that is, efficient delivery with minimal toxicity) for MO delivery to mouse preimplantation embryos using EPEI. To have an effect on biological activity, MO must enter the cytosol and nucleus, in which the target RNA sequence is synthesized, processed and translated (Summerton and Weller 1997). Therefore, we first examined antisense MO uptake using confocal laser scanning microscopy to visualize the delivery of MO to the nucleus, with variation of the MO and EPEI concentrations and the treatment time.

Ghosh et al. (2000) found that release of ODNs from the endosome is a slow process, and hence in vitro antisense experiments usually require a long exposure time and a high ODN concentration. However, for MO delivery our results suggest that the EPEI concentration and the MO concentration enhanced the delivery of MO into the mouse embryo in a dose-dependent manner without causing harmful effects to the embryo development. But the extension of the treatment time did not enhance uptake of MO into the mouse embryo, and affected the embryo development. It seems that the MO concentration and the EPEI concentration are the most important key factors for MO delivery. Using confocal laser scanning microscopy, we confirmed that even a low EPEI concentration facilitated FITC-MO delivery to the nucleus, although the fluorescence intensity under these conditions was rather weak. The efficacy of MO delivery might be enhanced further by higher EPEI concentrations than those used in our study, but Siddall et al. (2002) showed that the mouse embryo is very sensitive to EPEI, with MO treatment over 6 hrs at reduced EPEI concentrations showing embryonic toxicity. Morcos (2001) showed EPEI toxicity for HeLa cells, with MO treatment 3 hrs at 0.7 μM EPEI showing cellular deformity. It will be necessary to investigate the treatment time and optimal MO:EPEI ratio to minimize EPEI toxicity and enhance MO delivery in embryos of mice.

Next, we investigated the gene silencing activity of an antisense MO using our defined conditions. Embryos at the morula stage were treated with Oct4MO, but an antisense effect was not observed. This result may have been caused by poor uptake of Oct4MO into the nucleus, an insufficient antisense effect induced by the MO, selection of an embryonic stage that was unsuitable for induction of an antisense effect, or no fundamental effect of Oct4MO on target gene expression. The possibility of poor uptake of MO into the embryo was ruled out by the strong fluorescence intensity observed by laser scanning microscopy following introduction of FITC-MO into the nucleus. Since dilution of the MO due to cell division might lead to a diminished antisense effect, we repeated the MO treatment for 3 hrs after 144 hrs of insemination, but the phenotypic antisense effect of Oct4MO was still not observed. The importance of the stage of the pre-implantation embryo at which MO treatment is
performed has been demonstrated (Jones et al. 1997; Siddall et al. 2002), and MO treatment prior to the 8-cell stage is requisite for occurrence of an antisense effect at later stages. Indeed, in our study, MO treatment at the 2-cell stage was effective in exerting an antisense effect. This might be strongly related to the activation of the embryonic genome around the 2-cell stage, which leads to the generation of newly synthesized embryonic transcripts.

By treating the embryos with Oct4MO at the 2-cell stage, an antisense effect on the Oct4 gene was observed at the blastocyst stage by immuno-cytochemistry using anti-Oct4 antibody. However, the antisense effect was not maintained after additional cultivation for 4 days. Although this may be due to an inability of MO to exert prolonged effects, Cheng et al. (2004) have shown a decline of LIF protein expression after 4 days when a mouse embryo at the pronuclear stage is microinjected with an MO for LIF. In contrast, in examining the effect of an MO for α-catenin, using lipofectin as a delivery reagent, Siddall et al. (2002) showed that embryos treated with MO at the 2-cell stage were able to develop to blastocysts, and therefore additional MO treatment was necessary for extending the antisense effect. The difference in the prolongation effects in these studies might be related to the amount of MO delivered to the nucleus: the additional treatment required with the α-catenin MO may have been due to an insufficient amount of MO reaching the nucleus, rather than a short half life of the MO. Wagner (1994) has demonstrated nuclear localization of a fluorescent ODN after introduction into the cytoplasm of African green monkey kidney cells by microinjection, and in the current study, immunofluorescence analysis showed a similar result. Furthermore, EPEI-based MO delivery at the 2-cell stage gave an antisense effect on Oct4 for at least 3 days until the blastocyst stage. This result indicates that either the antisense effect can be extended for a longer period under optimal conditions, or EPEI has superior properties for MO delivery to the nucleus, compared to lipofectin. Although we successfully induced an MO antisense effect on Oct4 at the blastocyst stage, this effect did not last for 4 days of culture, and Oct4-positive cells were present in the outgrowth. This result may reflect the limit of prolongation of the antisense effect following the EPEI-based MO delivery. However, re-treatment with the MO at the blastocyst stage restored the antisense effect for at least further 4 days during in vitro cultivation, and reproduced the phenocopy of Oct4 deficient cells previously described by Nichols et al. (1998).

CONCLUSIONS

The effectiveness of antisense studies depends on the successful delivery of ODNs to the nucleus. Our results show that the delivery reagent, EPEI, has favorable characteristics for introduction of MO into the nucleus, leading to a sustained antisense effect. Using this approach, we have demonstrated that antisense MO are useful for analysis of gene function in early development in mice, as a simple and efficient gene-silencing method.

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