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Genetic Approach to Identify Critical Factors for Mouse Early Embryogenesis

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Abstract: Development of the mammalian pre-implantation embryos has unique features, such as a slow and unsynchronized cell division, compaction, and eventual formation of blastocysts with inner cell mass and trophectoderm. In order to have a clue on molecular mechanisms that reside in mouse early development, we suppressed expression of early embryo-specific genes with RNAi and observed their development in vitro. We observed developmental defects in embryos microinjected with dsRNAs for *Oct4* or *Nanog* among the tested genes. Careful examinations revealed that development of the most of the *Oct4*- or *Nanog*-suppressed embryos were arrested at the morula stage. These results suggest that the Oct4 and Nanog activities are also required for embryogenesis earlier than the blastocyst stage.

Key words: Embryogenesis, Nanog, Oct4, Sox2

The early development of mammalian embryos has unique features that cannot be observed in other animals. Cleavage in mammalian eggs is among the slowest in the animal kingdom so that it takes 12-24 hours per cell division. Zygotic gene expression starts from the 2-cell stage. Mammalian blastomeres lack synchrony of early division. At 8-cell stage, the mammalian embryos undergo compaction at which tight interactions among blastomeres are created. The cells of the compacted 8-cell embryo divide to produce 16-cell morula. Initially, the morula does not have an internal cavity. However, during a process called cavitation, the trophoblast cells secret fluid into the morula to create a blastocoel. The morula consists of a small group of internal cells surrounded by a larger group of external cells. Most of the descendants of the external cells become the trophectoderm cells from which extraembryonic tissues such as chorion and placenta are originated. The descendents of the internal cells become inner cell mass (ICM) which will form embryonic tissues later.

Molecular mechanisms that reside in mammalian early development have not been studied extensively, largely due to limitation in the amount of available materials. Perhaps Oct4 and Nanog, two homeodomain transcription factors, may be the most well-known regulators of mammalian early embryogenesis (Nichols et al., 1998; Mitsui et al., 2003). Disruption of Oct4 and Nanog results in the inappropriate differentiation of ICM and embryonic stem (ES) cells to trophectoderm and extra-embryonic endoderm, respectively (Nichols et al., 1998; Mitsui et al., 2003; Chambers et al., 2003). Oct4 is known to interact with other transcription factors, such as Sox2, to activate or repress gene expression in mouse early embryos and ES cells (Boyer et al., 2005). A number of genes that were expressed specifically in the mouse early embryos and embryonic stem cells have been reported recently (Table 1; Bortvin et al., 2003; Mitsui et al., 2003). Therefore, these genes might be in association with Oct4 or Nanog for early development of the mouse embryos as well as for maintenance of the pluripotent capacity of the ES cells.

RNAi has been an efficient method to suppress specific gene expression in many organisms. The RNAi method has been also applied to mouse early embryos for specific suppression of gene expression (Wianny and Zernicka-Goetz, 2000). Such RNAi method could substitute generation of knockout mouse for genetic analysis of the mouse early embryogenesis. Furthermore, RNAi could suppress expression of both maternal and zygotic genes simultaneously in the early embryos.

In order to have a clue on molecular mechanisms that reside in mouse early development, we suppressed expression of the embryo-specific genes with RNAi and

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Table 1. List of candidate genes expected to be expressed during mouse early development

Gene	Unigene ID	Description and Reference
Dppa1	Mm.18119	Signal peptide, single transmembrane domain, similarity to *Kim-1* (Ichimura et al., 1998; Bortvin et al., 2003)
Dppa2	Mm.27857	Putative DNA-binding motif SAP, weak similarity to *Dppa3* and *Dppa4* (Aravind and Koonin, 2000; Bortvin et al., 2003)
Dppa3 /Stella	Mm.27982	Putative DNA-binding motif SAP, weak similarity to *Dppa2* and *Dppa4* (Aravind and Koonin, 2000; Bortvin et al., 2003)
Dppa4	Mm.35597	Putative DNA-binding motif SAP, weak similarity to *Dppa2* and *Dppa3* (Aravind and Koonin, 2000; Bortvin et al., 2003)
Dppa5	Mm.139314	Similarity to KH RNA-binding motif (Siomi et al., 1994; Bortvin et al., 2003)
Pramel4	Mm.21737	Similar to human tumor antigen PRAME (van Baren et al., 1998; Bortvin et al., 2003)
Pramel5	Mm.26222	Similar to human tumor antigen PRAME (van Baren et al., 1998; Bortvin et al., 2003)
Pramel6	Mm.21815	Similar to human tumor antigen PRAME (van Baren et al., 1998; Bortvin et al., 2003)
Pramel7	Mm.6018	Similar to human tumor antigen PRAME (van Baren et al., 1998; Bortvin et al., 2003)
Ndp52l1	Mm.27779	Coiled coil protein, similar to nuclear domain10 protein 52 (Korioth et al., 1995; Bortvin et al., 2003)
Ecat1	Mm.157658	ES cell associated transcript (Mitsui et al., 2003)
Ecat3 /Fbx15	Mm.28369	F-box protein, ES cell associated transcript (Mitsui et al., 2003)
Ecat4 /Nanog	Mm.6047	Homeobox protein, ES cell associated transcript (Mitsui et al., 2003)
Ecat5 /ERas	Mm.249524	Ras protein family, ES cell associated transcript (Mitsui et al., 2003)
Ecat6	Mm.158190	ES cell associated transcript (Mitsui et al., 2003)
Ecat7	Mm.13433	ES cell associated transcript (Mitsui et al., 2003)
Ecat8	Mm.47904	ES cell associated transcript (Mitsui et al., 2003)
Ecat9	Mm.4213	ES cell associated transcript (Mitsui et al., 2003)

observed the embryo development. The results showed that Oct4 and Nanog are critical at the morula stage.

MATERIALS AND METHODS

Recovery and culture of the mouse embryos

Embryos were obtained from Fvb female mice of 6-10 week old. Superovulation was induced by intraperitoneal injections of pregnant mare serum gonadotropin (PMSG, Sigma, St. Louis, MO) followed by human chorionic gonadotropin (hCG, Sigma) 46-48 h later. The females were immediately caged with Fvb males and checked for vaginal plugs in the following morning. One-cell zygotes were collected from oviducts by tearing off ampulla region with fine forceps in the CZB medium containing hyaluronidase (1 mg/ml, Sigma), washed with fresh CZB media, and then cultured in the CZB medium (Chatot et al., 1989) containing 0.5% BSA (Sigma) under paraffin oil in sterile culture dishes in an atmosphere of 5% CO_2 at 37°C. In the late afternoon of day 2 when embryos reached 4-cell stage, they were washed several times and transferred to the CZB medium lacking EDTA but supplemented with glucose (1 mg/ml).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent. Fifteen embryos were pooled in a 1.5 ml tube containing 30 µl of TRIzol on ice and 0.2 volume of chloroform-isoamyl alcohol (49 : 1) was added. After pipetting mildly, the mixture was incubated on ice for 15 min. Total RNA was precipitated by addition of the same volume of isopropanol followed by centrifugation at 12,000 rpm for 20 min at 4°C. The pellet was washed twice with 75% ethanol and dissolved in diethyl pyrocarbonate (DEPC)-treated water after drying completely. The isolated RNA was denatured in the presence of 100 pmol random hexanucleotide in a final volume of 8 µl at 65°C for 5 min. After incubation on ice for 5 min, 12 µl of master mix [200 U RNaseH-MMLV reverse transcriptase, 4 µl dNTP mix (2.5 mM each), 1 µl RNasin (26 U/Al), and 4 µl of 5 × RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol)] were added and further incubated at 37°C for 1 h. The RT reaction was terminated by raising the temperature to 70°C for 15 min. PCR amplification was carried out with 2 µl of RT reaction mixture in 40 µl of PCR reaction solution containing 4 µl of 10 × PCR buffer

(Takara), 4 μl dNTP mix (2.5 mM each), and 1 U Ex-Taq polymerase (Takara). In each reaction, 10 pmol of PCR primers was added. Samples were subjected to amplification on a Gene Amp PCR System 2400 (Perkin Elmer). PCR amplification conditions for each candidate genes as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 second, annealing at 57°C for 30 second, and polymerization at 72°C for 30 second. The final cycle had an extended incubation at 72°C for 7 min, followed by a decrease to 4°C. Six microliters of PCR products was electrophoresed on a 3% agarose gel. Gels were stained with ethidium bromide and photographed under UV illumination (BioRad).

The sequences of primers used for PCR are as follows: *Dppa1*, 5'-ctccttctcccagcagtttg-3' and 5'-atcagcaatgcagcagacac-3'; *Dppa2*, 5'-gcctggagactttcaacgag-3' and 5'-tttccaggacaccactctcc-3'; *Dppa3*, 5'-gatgaagaggacgctttgga-3' and 5'-tcccgttcaaactcatttcc-3'; *Dppa4*, 5'-cctgcaaaggctaaagcaac-3' and 5'-gcaggtatctgctcctctgg-3'; *Dppa5*, 5'-cagtcgctggtgctgaaata-3' and 5'-tccatttagcccgaatcttg-3'; *Pramel4*, 5'-ggccaagcagagaaaagatg-3' and 5'-tttgagctgatggatgcttg-3'; *Pramel5*, 5'-gaagccttgaccatctctgc-3' and 5'-tgtcgtcggcagtacttgag-3'; *Pramel6*, 5'-cctgccctaaccctagaagg-3' and 5'-gactgccaaagttggagagc-3'; *Pramel7*, 5'-caggatcaggctttcaggag-3' and 5'-cagcttcaaacgctctttcc-3'; *Ndp52l1*, 5'-cccatacctaccttgctgga-3' and 5'-gccacctgcttcctctgtag-3'; *Ecat1*, 5'-cagttggctcctgtgaaggt-3' and 5'-tcccttggaggctgaactat-3'; *Ecat3*, 5'-acattgcctcccgacactac-3' and 5'-tcaaaccaccctaggtctgc-3'; *Ecat4*, 5'-aagtacctcagcctccagca-3' and 5'-cgtaaggctgcagaaagtcc-3'; *Ecat5*, 5'-aggcagctacccgagtacaa-3' and 5'-aaaggcttcctccacacctt-3'; *Ecat6*, 5'-ggtgacctcctgctgtctgt-3' and 5'-ctccagtcgggtcttcacat-3'; *Ecat7*, 5'-tgatgctgacagtcctctgg-3' and 5'-ccatggcattgatcctctct-3'; *Ecat8*, 5'-gacagtgcagccatccagta-3' and 5'-ttttggttggccagtacaca-3'; *Ecat9*, 5'-tgctctgtctgtggttcagg-3' and 5'-tcattgagaaggggcactct-3'.

Double-stranded RNA (dsRNA) preparation

Double-stranded RNA was prepared by annealing two complementary RNAs transcribed by T3 or T7 RNA polymerase in vitro. The cDNA fragments for dsRNA were initially subcloned into the *pGEM-T* vector and transferred to the *pBluescriptSK(+)* vector. For microinjection, we prepared five kinds of dsRNAs: *GFP* dsRNA (a 443 bp-fragment prepared using primers 5'-cacatgaagcagcacgactt-3' and 5'-acgaactccagcaggaccat-3'); *E-Cadherin* dsRNA (a 330 bp-fragment prepared using primers 5'-ctgctgctcctactgttttct-3' and 5'-gaacaccaacagagagtcgt-3'), *Oct4* dsRNA (a 305 bp-fragment prepared using primers 5'-ccagaagggcaaaagatcaa-3' and 5'-cccaaagctccaggttctct-3'), *Sox2* dsRNA (a 466 bp-fragment prepared using primers 5'-agaaccccaagatgcacaac-3' and 5'-atgtaggtctgcgagctggt-3'), and *Nanog* dsRNA (a 424 bp-fragment prepared using primers 5'-aagtacctcagcctccagca-3' and 5'-cgtaaggctgcagaaagtcc-3').

After RNA was synthesized using the T3 and T7 RNA polymerase (Roche), DNA templates were removed with the DNase I treatment. The RNA products were extracted with phenol/chloroform and precipitated with isopropanol. To anneal sense and antisense RNAs, equimolar quantities of both sense and antisense RNAs were mixed in the annealing buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) to a final concentration of 2 mM each, heated for 1.5 minutes in the boiling water, and incubated at room temperature for several hours. The quality of dsRNA was confirmed by running on an agarose gel. The dsRNA samples were diluted to a final concentration of 2-4 mg/ml and stored at −70°C before use.

Microinjection of dsRNA

Microinjection of dsRNA was carried out as described previously (Sonn et al., 2004). Briefly, microinjections of dsRNAs were performed as follows: the holding and the injection pipettes were fabricated from prewashed borosilicate glass tubes with an outer diameter of 1.0 mm and inner diameter of 0.75 mm (Humagen Fertility Diagnostic, Inc., Charlotteville, VA). The injection pipette was prepared on a micropuller (P-97, Sutter Instrument Co., Navato, CA) by pulling a capillary containing a filament which facilitates its loading of solutions by capillary action. Holding pipettes were made by cutting and fire-polishing a pulled pipette on a microforge (Narishige), followed by S-shaping on a small flame.

Micromanipulation of embryos was performed according to a standard procedure (Nagy et al., 2002). One-cell embryos were placed in the HEPES-buffered CZB medium containing 20 mM HEPES and 5 mM sodium bicarbonate under light mineral oil (all from Sigma, St. Louis, MO). A holding pipette was used to keep the 1-cell embryos stationary during manipulation. An injection pipette loaded with dsRNA solution was inserted into the cytoplasm of each zygote, followed by the microinjection of ~10 pl dsRNA using a constant flow system (Transjector, Eppendorf). After microinjection, embryos were cultured in CZB medium up to 4 days.

Embryo fixation and immunocytochemical staining

Embryos were fixed in 4% formaldehyde in PBS for 10 min at room temperature, and washed with 0.3% PBST (PBS containing 0.3% Triton X-100 and 3% BSA) several times. Immunocytochemical staining was performed by incubating the fixed samples with primary antibodies for 90 minutes, followed by secondary antibodies conjugated with TRITC or FITC for 30 minutes. The monoclonal antibody against Oct4 (Santa Cruz Biotechnology, s-5279) and the monoclonal antibody against Troma1 (Developmental Studies Hybridoma Bank) were diluted to 1 : 100. The secondary antibodies conjugated to TRITC or FITC

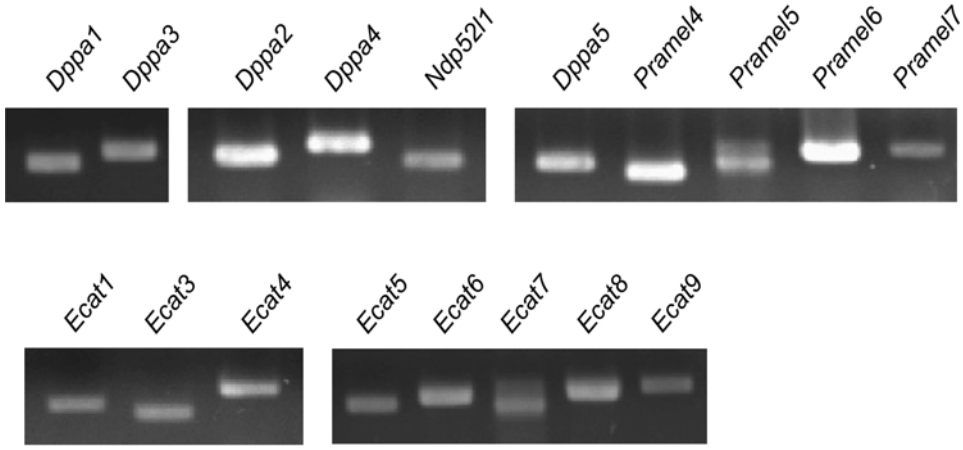

Fig. 1. RT-PCR analysis of the expression of the candidate genes in mouse blastocysts. Total RNA was extracted from cultured mouse blastocysts and reverse transcribed. PCR was carried out with primers specific to the candidate genes. Expression of a candidate gene was determined by ethidium bromide staining of the PCR product at an expected molecular size.

(Jackson ImmunoResearch Laboratories) were diluted 1 : 200. The slides were observed under a confocal microscope.

RESULTS

In order to identify genes whose products are critical for early development, we chose candidate genes that were known to be expressed specifically in the mouse early embryos and embryonic stem cells (Table 1; Bortvin et al., 2003; Mitsui et al., 2003). Expression of the candidate genes in the mouse early embryos was examined with the RT-PCR method (Fig. 1). Total RNA was prepared from the mouse blastocysts and reverse transcribed for PCR amplification. We observed specific PCR bands that correspond to the candidate genes, indicating that all candidate genes were expressed in mouse early embryos (Bortvin et al., 2003).

We initially microinjected the *Oct4*-specific dsRNA and observed the target gene expression immunohistochemically. As reported previously (Kim et al., 2002; Shin et al., 2005), the Oct4 signal was detected at nuclei of the ICM cells (Fig. 2). However, we were not able to observe Oct4 signals in the embryos microinjected with the *Oct4*-specific dsRNA (Fig. 2). The Troma1 antibody stained cytoplasm of the trophectoderm cells of the both embryos. These results indicated that the dsRNA injection was able to suppress the target gene expression efficiently.

In order to identify genes critical for mouse early embryogenesis, we microinjected dsRNAs specific to the candidate genes listed in Table 1 and observed in vitro development of the embryos morphologically. However, we did not detect any developmental defect in the candidate

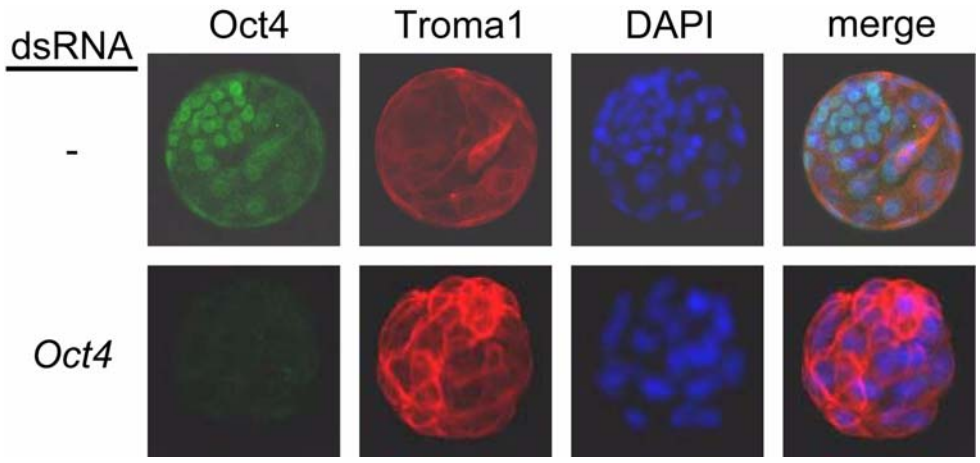

Fig. 2. Suppression of the *Oct4* expression in mouse early embryos. Double-stranded RNA specific to *Oct4* was microinjected into the mouse fertilized eggs and cultured for 4 days. The embryos were immunostained with the Oct4 antibody. Antibody specific to Troma1 was co-immunostained for detection of the trophectoderm cells. DNA was stained with DAPI.

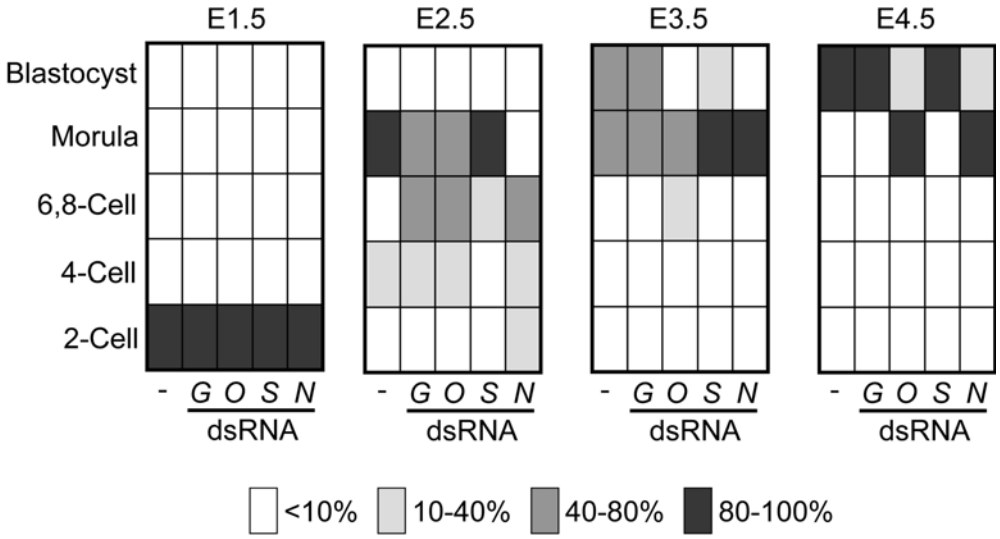

Fig. 3. Development of the cultured embryos microinjected with dsRNA specific to *Oct4* (*O*), *Sox2* (*S*) and *Nanog* (*N*). Fertilized eggs were microinjected with the indicated dsRNAs and cultured up to 4 days. As a control, *GFP* dsRNA (*G*) was microinjected. Development of the embryos was determined daily. Proportion of the eggs at indicated developmental stages were represented with gray tones. The experiments were repeated three times.

gene-suppressed embryos except *Oct4* and *Ecat4/Nanog* (data not shown). These results suggest that the most of candidate genes may not be critical for mouse early embryogenesis at least in a morphological aspect. However, we do not rule out the possibility that the target genes may not be suppressed sufficiently to produce visible phenotypes.

Since we observed developmental arrests in the *Oct4* or *Nanog* dsRNA-injected embryos, we decided to determine phenotypes of these embryos carefully. After microinjection of the dsRNAs into the fertilized eggs, morphology of the embryos was observed daily (Fig. 3). At E1.5, most of embryos were at the 2-cell stage. At E2.5, most of the control embryos reached to morula while development of the *Nanog*-suppressed embryos progressed somewhat slowly. At E.3.5, about a half of the un-injected and non-specific *GFP* dsRNA-injected embryos reached to blastocysts while *Oct4* or *Nanog* dsRNA-injected embryos reached to morula. Since Sox2 was also known to be an important transcription factor that interacts with Oct4 and *Nanog*, its expression was suppressed with dsRNA injection. A significant fraction of the *Sox2* dsRNA-injected embryos also reached to blastocyst. At E4.5, most of the *Oct4* or *Nanog* dsRNA-injected embryos were arrested at morula stage while *GFP* or *Sox2* dsRNA-injected embryos reached to the blastocyst stage. These results revealed importance of Oct4 and Nanog in the mouse early embryogenesis.

Morphology of the *Oct4*- or *Nanog*-suppressed embryos was observed (Fig. 4). When mouse embryos were cultured for 4 days, most of them reached to blastocysts. Development of the most of *E-Cadherin*-suppressed embryos was blocked at morula as reported previously (Wianny and Zernicka-Goetz, 2000; Sonn et al., 2004). Development of the most of the *Oct4*- or *Nanog*-suppressed embryos was also blocked at morula, suggesting that the Oct4 and Nanog activities are required for development to blastocyst.

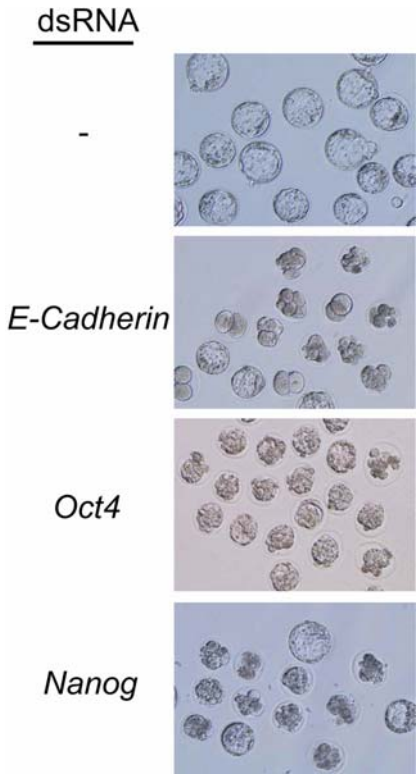

Fig. 4. Morphology of the mouse embryos microinjected with dsRNAs specific to *E-Cadherin*, *Oct4* and *Nanog*. Fertilized mouse eggs were microinjected with indicated dsRNAs and cultured in vitro. Four days later, morphology of the embryos was observed with stereo-microscope.

DISCUSSION

In the present study, we identified genes whose products are required for successful development of mouse early embryos. Our results showed that Oct4 and Nanog are critical at the morula stage.

It was known that the *Oct4* knockout embryos developed to blastocyst without ICM and concluded that Oct4 is critical for ICM but not for trophectoderm formation (Nichols et al., 1998). Similar phenotypes were observed in the *Oct4* knockdown embryos with siRNA injection (Kim et al., 2002; Shin et al., 2005). In the present study, we observed that development of the *Oct4*-suppressed embryos was arrested at morula stage. These results suggest that the Oct4 activity is required not only for ICM formation as reported but also for development to blastocyst in an earlier stage. In fact, *Oct4* expression in earlier stages of the mouse pre-implantation embryos has been observed. The *Oct4* mRNA was detected at as early as zygote stage, and it became abundant after 8-cell stage (Shin et al., 2005). In the blastocyst stage, *Oct4* expression was limited to the ICM cells (Nichols et al., 1998). It is not clear why developmental arrest of the *Oct4*-suppressed embryos in earlier stages than blastocyst has not been reported in the previous papers (Nichols et al., 1998; Kim et al., 2002; Shin et al., 2005). One possibility may be that the maternal *Oct4* mRNA might play a role in the *Oct4* knockout embryos reaching to the blastocyst stage. Suppression efficiency of the *Oct4* mRNA might be also a critical factor in overcoming the morula arrest of the *Oct4*-suppressed embryos.

Nanog is a transcription factor with a homeobox domain (Mitsui et al., 2003). The *Nanog* knockout embryos developed to blastocyst and were indistinguishable from wild type embryos. However, ICM of *Nanog* knockout blastocysts failed to proliferate in vitro, indicating that Nanog is essential for maintenance of pluripotency of ICM (Mitsui et al., 2003; Chambers et al., 2003). In the present study, we observed that development of the *Nanog*-suppressed embryos blocked at morula stage. These results suggest that Nanog is also required for embryogenesis earlier than the blastocyst stage. Again, it is not clear why the *Nanog* knockout embryos proceeded to blastocysts without developmental arrest. One possible explanation may be that the maternal *Nanog* mRNA worked to overcome early development of the *Nanog* knockout mice.

Except Oct4 and Nanog, we did not observe any visible defect in development of the embryos in which the target gene expression was suppressed. In fact, knockout studies of some of the candidate genes also revealed no specific phenotypes in early development (Takahashi et al., 2003; Amano et al., 2006). However, we do not rule out the possibility that the residual target gene products might play key roles in early embryogenesis of the target gene-suppressed embryos. It is also possible that some of target gene products might be critical for development of the post-implantation embryos. Since our works proposed importance of Oct4 and Nanog in the morula stage of the mouse embryo, we currently investigate to identify downstream genes of Oct4 and Nanog at this stage.

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