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Identification and characterization of an oocyte factor required for development of porcine nuclear transfer embryos

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Abstract

Nuclear reprogramming of differentiated cells can be induced by oocyte factors. Despite numerous attempts, these factors and mechanisms responsible for successful reprogramming remain elusive. Here, we identify one such factor, necessary for the development of nuclear transfer embryos, using porcine oocyte extracts in which some reprogramming events are recapitulated. After incubating somatic nuclei in oocyte extracts from the metaphase II stage, the oocyte proteins that were specifically and abundantly incorporated into the nuclei were identified by mass spectrometry. Among 25 identified proteins, we especially focused on a multifunctional protein, DJ-1. DJ-1 is present at a high concentration in oocytes from the germinal vesicle stage until embryos at the 4-cell stage. Inhibition of *DJ-1* function compromises the development of nuclear transfer embryos but not that of fertilized embryos. Microarray analysis of nuclear transfer embryos in which *DJ-1* function is inhibited shows perturbed expression of *P53* pathway components. In addition, embryonic arrest of nuclear transfer embryos injected with anti-*DJ-1* antibody is rescued by *P53* inhibition. We conclude that *DJ-1* is an oocyte factor that is required for development of nuclear transfer embryos. This study presents a means for identifying natural reprogramming factors in mammalian oocytes and a unique insight into the mechanisms underlying reprogramming by nuclear transfer.

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Introduction

Embryonic cells differentiate into specific types of cells as development progresses. Once differentiated, the reversion of a differentiated cell state to an original undifferentiated state is strictly inhibited in normal development. However, it has been experimentally shown that differentiated nuclei can be returned to an undifferentiated embryonic state after nuclear transfer (NT) to enucleated eggs or oocytes (1, 2). Such experiments provide an opportunity to reprogram somatic cells as a means to prepare undifferentiated cells, which may be differentiated into any kinds of cells for cell-replacement therapy. Recently, nuclear reprogramming technology has been expanded by the production of induced pluripotent stem (iPS) cells (3). iPS cells can be obtained by overexpressing specific sets of transcription factors such as *Oct4*, *Sox2*, *Klf4*, and *c-myc* in cultured cells. The processes leading to establishment of iPS cell lines are being carefully examined and we are begining to understand how somatic cells acquire pluripotency by this method (4-6). The mechanisms leading to pluripotency may be different between iPS cells and NT embryos because somatic nuclei transferred into unfertilized metaphase II (MII) oocytes must undergo early embryonic development before the inner cell mass (ICM) can give rise to pluripotent embryonic stem (ES) cells. In addition, the molecules and mechanisms that induce somatic cell reprogramming are expected to be different between iPS cells and NT embryos (7, 8). A recent study has shown that nuclear transfer-mediated reprogramming resets differentiated cell states more efficiently than the iPS method (9). However, almost nothing is known about factors and mechanisms involved in the reprogramming of somatic cells by NT to mammalian oocytes.

Some proteins have been identified as reprogramming factors in *Xenopus* eggs and oocytes. These include ISWI (10), FRGY2a/b (11), BRG1 (12), Nucleoplasmin (13), and Histone B4 (14). While the roles of these factors on nuclear remodeling and transcriptional reprogramming have been shown, it has not been demonstrated that these factors are crucial for nuclear reprogramming in NT embryos

during embryonic development.

As shown above, reprogramming factors have been mainly identified in *Xenopus*. This is because *Xenopus* eggs and oocytes are extremely large and easily accessible, as compared to mammalian oocytes. These properties allowed development of cell-free systems using *Xenopus* egg or oocyte extracts. In the extracts, at least some reprogramming events are induced on large numbers of cells (10^5 cells in 100 μl of extracts) (10, 15), allowing the study of reprogramming by biochemical means. In contrast to *Xenopus*, a system to study the molecular mechanisms underlying reprogramming has been lacking in mammalian oocytes. Recently, our group has developed a cell-free system from porcine oocytes (16). We have shown that a porcine oocyte extract from the metaphase II (MII) oocytes is able to displace transcription factors from donor nuclei and deacetylate the histone tails of somatic nuclei. These reprogramming events related to the erasure of gene-expression memories are also observed in somatic nuclei transferred to MII oocytes, suggesting that a part of reprogramming events can be reproduced in the oocyte extracts. Besides the character of the MII extracts, somatic nuclei can be fully reprogrammed to totipotency when transplanted in MII oocytes. Reprogramming factors related to totipotency are also present in oocytes at the germinal vesicle stage (GV) (17), and GV oocyte extracts induce reprogramming events, which are different from reprogramming in MII extracts, such as activation of pluripotent genes (16). However, the ability of these GV factors to reprogram transferred nuclei toward totipotency has never been directly shown because cloned offspring have never been obtained by direct injection of nuclei to GV oocytes. Taken together, MII oocyte extracts are ideal to explore reprogramming factors important for development of NT embryos as these factors might be accumulated and active.

In this study, we use porcine oocyte extracts to identify a unique oocyte factor involved in nuclear reprogramming toward totipotency. We focused on protein exchange in extract-treated nuclei because the exchange of nuclear proteins between a donor nucleus and an oocyte cytoplasm is a

prominent phenomenon during reprogramming (18-20). Additionally, some proteins, such as ISWI and nucleoplasmin, that are incorporated from *Xenopus* egg extracts into donor nuclei have been shown to have a role in reprogramming (10, 13). After incubation of somatic nuclei in oocyte extracts, proteins that become associated with somatic nuclei from the extracts were identified by mass spectrometry. The function of the identified proteins was then examined in NT embryos and cultured cells. These experiments revealed that *PARK7* (*DJ-1*) is required for successful reprogramming in NT embryos.

Results

Identification of Oocyte Proteins That Associate with Somatic Nuclei. The strategy to identify a reprogramming factor in oocytes is diagramed in Fig. 1*A*. Briefly, porcine fibroblast cell nuclei were incubated in extracts from oocytes at the MII stage or the GV stage or extracts from fibroblast cells (own cell extracts). After the extract treatment, nuclei were collected and proteins were separated by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) (Fig. 1*B* and Fig. S1). As shown in Fig. S1, nuclear proteins were greatly changed after oocyte-extract treatment, in agreement with a previous *in vivo* study in which nuclei were transferred into eggs (18). In particular, we focused on the proteins that were specifically and abundantly accumulated in nuclei after treatment with MII oocyte extracts. More than 60 unique protein spots were detected in MII-extract treated nuclei and these spots were excised and subjected to mass spectrometry. MALDI-TOF/TOF analysis and database searches revealed identity of 31 unique protein spots, and 25 proteins were finally identified (Fig. S2 and Table S1). Proteins were classified according to their function (Fig. S3*A*). Proteins with chaperone function were most commonly found, reflecting a marked change of nuclear proteins during reprogramming. Proteins related to DNA replication initiation were also found. This proteomic result agrees with a previous report (21) showing that reprogramming of somatic type replication forks to embryonic ones starts from the metaphase stage

in oocyte extracts. Some examples of unique protein spots are shown in Fig. S3*B*.

DJ-1 Is Incorporated in Oocyte Extracts Treated Nuclei. Two unique protein spots (20-25 kDa and p*I* 5-7) were detected in the MII-extract treated nuclei (Fig. 1*B*, red box; and 1*C*, arrows). MALDI-TOF/TOF analysis and database searches revealed that these two spots were both matched to *PARK7*, also known as *DJ-1*. This result was repeated in three independent experiments using different oocyte extracts. Two spots of different p*I* were identified as DJ-1, because oxidized DJ-1 has a lower p*I* (22). Incorporation of DJ-1 protein into fibroblast cell nuclei after treatment with MII oocyte extracts was also observed by western blot (Fig. 1*D*) and immunofluorescence analysis (Fig. 1*E*). DJ-1 was also detected in nuclei treated with GV oocyte extracts (Fig. 1*D*), but the total amounts of incorporated DJ-1 proteins were higher in those with MII oocyte extracts than GV oocyte extracts, agreeing with the 2D PAGE result (Fig. 1*C*). Considering that GV and MII oocyte extracts had similarly large amounts of DJ-1 (Fig. 1F) and both extracts have differential reprogramming abilities (16), greater incorporation of DJ-1 into cells treated with MII extracts may result from an active incorporation mechanism, implying that DJ-1 might have a specific role in MII oocytes extracts.

Expression Pattern of DJ-1. In addition to the unique incorporation pattern of oocyte DJ-1 proteins to somatic nuclei, *DJ-1* has been reported to interact with a known reprogramming factor, *c-myc* (23, 24). Large amounts of DJ-1 are stored in oocytes (Fig. 1F), while its function in early embryonic development has never been examined. For these interesting characteristics of *DJ-1*, we asked whether *DJ-1* plays a role in nuclear transfer embryos. We first investigated its expression pattern. DJ-1 was abundantly expressed in all porcine organs examined (Fig. 2*A*). *DJ-1* expression was examined in porcine oocytes and NT embryos. These transcripts and proteins were highly expressed throughout oocyte maturation and persisted until the 4-cell stage in NT embryos (Fig. 2*B*). Expression was

downregulated until the blastocyst stage (Fig. 2*B*). Generally, large amounts of DJ-1 proteins were detected throughout oocyte cytoplasm excluding lipid droplets (Fig. 2*C*). In oocytes at the GV stage, DJ-1 was present within the germinal vesicles as well as in cytoplasm (Fig. 2*Ci*, a germinal vesicle in the enlarged yellow box). It was dispersed in the MII oocyte cytoplasm (Fig. 2*Cii*) and on condensed MII chromosomes (Fig. 2*D*). Incorporation of DJ-1 into transferred donor nuclei was observed from 1 h after nuclear transfer although the signal is weaker than that in cytoplasm (Fig. 2 *Ciii* and *E*). Three h after nuclear transfer, nuclear DJ-1 is comparable to cytoplasmic DJ-1 (Fig. 2*Civ*) and it remained in nuclei 12 h after transfer (Fig. 2*Cv* and *F)*, suggesting that DJ-1 has been already incorporated into nuclei before entering the first S phase (25). Together, maternally accumulated DJ-1 was incorporated into transferred somatic nuclei in NT embryos.

Inhibition of DJ-1 Function in NT Embryos. To test the role of *DJ-1* on nuclear reprogramming, its function was inhibited in porcine NT embryos by several methods. Because DJ-1 is present in transferred nuclei 1 hour after NT, its function should be inhibited before introduction of donor nuclei into oocyte. However, neither siRNA injection nor antisense oligonucleotide injection during oocyte maturation caused significant degradation of maternal DJ-1 proteins although maternal mRNA was decreased (Fig. S4 *A* and *B*), indicating that it is difficult to reduce DJ-1 proteins during oocyte maturation.

We then tried anti-DJ-1 antibody injection into MII oocytes before nuclear transfer, parthenogenetic activation (PA), and intracytoplasmic sperm injection (ICSI). The polyclonal antibody used here is raised against a peptide mapping near the N terminus of *DJ-1* of human origin whose sequence matches perfectly to that of pig and is highly specific for recognizing the porcine DJ-1 among whole oocyte proteins (Fig. S4*C*). The antibody was injected into cytoplasm of MII oocytes more than 1 h before fusion with donor cells in order to allow binding to maternal DJ-1. The proportion of embryos

that cleaved normally was less for NT embryos injected with anti-DJ-1 antibody (αDJ1-NT embryos) than control NT embryos injected with IgG (IgG-NT embryos), at 24, 27, and 30 hour after NT (Fig. S5), but the final proportion of normally cleaved embryos, judged 48 h after NT, was not different (Table 1). This result suggests that the first cleavage in αDJ1-NT embryos is delayed. Interestingly, the proportion of αDJ1-NT embryos that developed to the blastocyst stage was significantly lower than that of control IgG-NT and non-injected NT embryos ($P < 0.004$: 1.67 ± 1.67%, 13.40 ± 2.39% and 14.22 ± 2.92%, respectively) (Table 1). Similarly, significantly fewer embryos developed to the morula stage in αDJ1-NT embryos compared to controls ($P < 0.002$) (Table 1). Many of αDJ1-NT embryos arrested at the 2-cell or 4-cell stage. In contrast to NT embryos, anti-DJ-1 antibody injection to ICSI and PA embryos did not affect development to the morula and blastocyst stages (Table 1).

Finally, *DJ-1* was inhibited by injecting mRNA of *DJ-1* dominant negative form [DJ1(L166P)] (26). Similar to the result of antibody injection, lower proportion of NT embryos injected with DJ1(L166P) mRNA developed to the morula and blastocyst stages, as compared with *EGFP* mRNA-injected or non-injected NT embryos, although proportion of cleaved embryos did not differ (Table 1).

P53 Activation Is Involved in Developmental Arrest after Inhibition of DJ-1 Function. In order to uncover the basis for developmental arrest in *DJ-1*-antibody-injected NT embryos, global changes in gene expression were examined by Affymetrix porcine microarrays. NT embryos were collected 28 h after nuclear transfer when many of the embryos had just reached the 2-cell stage. We chose this stage because major zygotic genome activation occurs at the 4-cell stage, which might mask altered expression of direct target genes, and because *DJ-1* seems to be active before the 2- or 4-cell stage.

Global transcripts were compared among αDJ1-NT, IgG-NT, non-injected NT embryos, and donor cells. Comparing αDJ1-NT embryos and donor cells, 6411 probes (26.78%) were underexpressed

and 4256 probes (17.78%) were overexpressed among total 23,937 probes (≥2.0-fold), indicating a significant reprogramming in a large number of genes. To find genes affected specifically by *DJ-1* inhibition, we examined differentially-expressed probes comparing αDJ1-NT and IgG-NT embryos and 401 downregulated probes (1.68%) and 349 upregulated probes (1.46%) were found. These genes were involved in RNA metabolism, oxidative stress, signaling pathways, and cell metabolism. *DJ-1* has been reported to be engaged in these cellular processes (27-31). In particular, previous reports have identified a set of DJ-1 related genes using cultured cells, and the expression of these genes is largely disturbed in αDJ1-NT embryos (Ras oncogenes [*RABL3*: 2.3-fold down, *RAP2C*: 2.1-fold down, *RAB26*: 12.5-fold up] and serine/cysteine protease inhibitor [*SERPINB1*: 3.3-fold up]) (27, 32), suggesting that antibody injection specifically inhibited *DJ-1* function in NT embryos.

Microarray analysis also showed that the expression of genes involved in *P53* pathway were largely disturbed in αDJ1-NT embryos compared to IgG-NT as summarized in Fig. S6 (genes whose expression was upregulated or downregulated were marked with asterisk). Moreover, it is reported that *DJ-1* inhibition is correlated with *P53* activation (33, 34). We therefore decided to examine *P53* expression after *DJ-1* inhibition. The correlation between *P53* and *DJ-1* expression was first examined in cultured cells. *DJ-1* expression in porcine fibroblasts was efficiently and significantly inhibited at both transcriptional and translational levels by siRNA against pig *DJ-1* (Fig. 3 *A* and *B*). Lipid droplets reminiscent of cellular stress appeared in the knockdown cells, but not control cells (Fig. S7, arrows). Upregulation of *MDM2*, a main regulator of *P53*, was observed in *DJ-1* knockdown cells (Fig. 3*A*). Concomitantly, P53 and phospholylated P53 at the Serine 20 [P53(pS20)], which is a stabilized form of *P53* (35), were slightly upregulated in *DJ-1* knockdown cells (Fig. 3*B*). These results suggest that *DJ-1* inhibition induces activation of *P53* in cultured cells.

We examined whether *P53* is similarly activated in *DJ-1* deficient NT embryos. RT-PCR analysis showed *MDM2* transcripts were only detected in porcine αDJ1-NT embryos (Fig. 3*C*), agreeing

with the result from *DJ-1* knockdown in cultured cells. Faint bands of P53 and P53(pS20) proteins were detected in αDJ1-NT embryos although their expression did not show a clear difference between αDJ1-NT and control IgG-NT embryos (Fig. 3*D*, lanes 1 and 2). We further tested the correlation between *P53* activation and the early embryonic arrest, P53 protein expression was compared between normal and arrested PA embryos at the 2-cell stage. Mitomycin C treatment (10 μM) with the 2-cell embryos caused developmental arrest of embryos and upregulated P53 proteins (Fig. 3*D*, lanes 3 and 4). Furthermore, when P53 expression was compared between the 2-cell embryos (day 2) and the arrested 2-cell embryos (day 5), large amounts of P53 and P53(pS20) were accumulated in the arrested embryos (Fig. 3*D*, lanes 3 and 5). These results suggest that *P53* activation is correlated with early embryonic arrest. Finally, we asked if *P53* inhibition on αDJ1-NT embryos can rescue the early embryonic arrest. *P53* was inhibited by a specific inhibitor, Pifithrin-α, at a concentration of 1 μM (36, 37). Remarkably, Pifithrin-α treatment successfully rescued the embryonic arrest of αDJ1-NT (Table 1). Pifithrin-α did not improve development of normal nuclear transfer embryos to the blastocyst stage (38), suggesting that a rescued phenotype observed by this inhibitor treatment should be specific to αDJ1-NT. These results indicate that embryonic arrest of αDJ1-NT can, at least to some extent, be attributed to *P53* activation. Considering that *DJ-1* inhibition did not change amounts of *P53* transcripts (Fig. 3 *A* and *C*) and weakly changed total P53 protein amounts (Fig. 3 *B* and *D*), regulation of *P53* activation by *DJ-1* may be carried out at the post-translational level. This idea agrees with previous reports in which *DJ-1* represses *P53* transcriptional activity through direct binding to P53 proteins rather than repressing *P53* transcription (39, 40).

Discussion

Factors necessary for the successful reprogramming of somatic cells by mammalian oocytes have been

explored since the first successful production of the cloned sheep (2). Although more than a decade has passed, they remain largely unknown, partly because many biochemical analyses have not been applied to oocytes and embryos whose quantities are limited. Here, we used functional oocyte extracts to overcome this issue and combined them with proteome analysis. We have identified an oocyte factor, *DJ-1*, necessary for the successful development of porcine NT embryos.

DJ-1 was initially identified as an oncogene that transformed mouse NIH3T3 cells weakly on its own and more strongly in combination with Ras (24). Further studies have revealed that *DJ-1* has multiple functions (Fig. S6). *DJ-1* modulates the PTEN/Akt survival pathway (30, 41), suppresses Ask1-mediated apoptosis (42), has RNA binding activity (28) and chaperone activity (43), and protects cells from oxidative stress (44). *DJ-1* is a dimeric protein and has some structural similarities with heat shock proteins (45). Deletion and a point mutation (L166P) of *DJ-1* that disrupts normal dimer formation are responsible for the onset of familiar Parkinson's disease (46). In germ cells, high amounts of expression in testis and sperm have been reported and disturbed testicular *DJ-1* expression is related to male infertility (47). We find that large amounts of *DJ-1* are accumulated in oocytes during oogenesis (Fig. 2 *B* and *C*). Nevertheless, nothing is known about the role of maternally stored *DJ-1* on early embryonic development.

Inhibition of maternal *DJ-1* function indicated that *DJ-1* is indispensable for early embryonic development in NT embryos (Table 1). Furthermore, microarray analysis revealed that the *P53* pathway is disturbed in DJ1-NT embryos compared to control IgG-NT embryos (Fig. S6). *P53* responds to diverse cellular stresses and induces cell cycle arrest, apoptosis, cell senescence, or DNA repair. *P53* expression is mainly regulated by *MDM2*, which binds to and targets p53 for ubiquitin-dependent proteolysis (48, 49). It has been generally considered that *P53* and *MDM2* exist as an autoregulatory feedback loop; *MDM2* transcription is induced by *P53* and in turn inactivates *P53* by ubiquitination. Therefore, activation of *MDM2* is often due to elevated *P53* activity as was observed in this study (Fig.

3*A*). *P53* activation critically affects embryonic viability (50, 51). Its activation also plays a crucial role in reprogramming somatic cells toward iPS cells (52-56). However, it has not been revealed whether *P53* activation is involved in reprogramming of NT embryos. Our study shows that *P53* activation is implicated in embryonic arrest in NT embryos. Interestingly, other identified oocyte proteins that are associated with somatic nuclei (Table S1), such as HSP27 (57), 14-3-3-zeta (58), and HSP90 (59), are reported to repress *P53* activity. Several oocyte factors may synergistically inhibit *P53* activation in NT embryos.

One of the interesting properties that *DJ-1* possesses is that its function is necessary only for NT embryos, but not for ICSI and PA embryos (Table 1). NT embryos undergo higher rates of apoptosis (60) and are less tolerant to *in vitro* culture environment than fertilized embryos (61). Lower levels of gene expression related to cell protection from stress and apoptosis have been reported in NT embryos, compared to fertilized embryos (62). Considering that NT embryos are experimentally produced whereas fertilized embryos are made by the natural process, it is reasonable to think that transferred somatic nuclei undergo higher cellular stress than sperms. This notion is supported by the fact that NT embryos exhibit higher rates of apoptosis and weaker stress-coping functions than fertilized embryos (60-62). Nevertheless, proportion of NT embryos that develop to the blastocyst stage is similar to that of *in vitro* fertilized embryos in many reports. This comparable developmental ability to the blastocyst stage implies that oocytes intrinsically possess robust mechanisms to relieve cellular stresses and these mechanisms might work as a safeguard to ensure preimplantation development of NT embryos. These stress-coping mechanisms are probably relying on many different molecules since we found a lot of anti-stress proteins by mass spectorometry (Table S1). *DJ-1* is one of the key players among these proteins and the lack of *DJ-1* function has a detrimental effect on NT embryos, suggesting that the other redundant factors with stress-coping functions may not be sufficient to complement *DJ-1* function in NT embryos. In contrast, the loss of *DJ-1* in fertilized embryos might not be as harmful as in NT embryos

because fertilized embryos have less cellular stress and/or stronger stress-coping functions than NT embryos as described above.

Thus far, many studies have focused on chromatin remodeling proteins and transcription factors as reprogramming-related factors in oocytes. *DJ-1* rather acts as a positive regulator of cell survival by modulating signaling pathways than by directly modifying chromatin structures. Our results show that such a process is critical for nuclear reprogramming in embryos. This notion is further supported by a recent publication in which inhibition of HDAC6 that is involved in induction of apoptosis responding to cellular stress greatly improves cloning efficiency in mice (63). During reprogramming, original cell states are forced to change. This process must be accompanied by a lot of cellular stresses. *DJ-1* may relieve those stresses and protect NT embryos. Exploring downstream networks of *DJ-1* in embryos might provide further clues to the long-standing question why cloned embryos are able to develop to term.

Materials and Methods

Oocyte-Extract Treatment. Porcine oocyte extracts were prepared as described previously (16). Briefly, 1000-1200 of GV oocytes or 800 of *in vitro* matured MII oocytes were collected. After denudation and zona pellucid removal, oocytes were suspended in 4-5 μl of extraction buffer (50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 2 mM 2-mercaptoethanol, 0.1 mM PMSF, protease inhibitor cocktail, and 50 mM HEPES, pH 7.6) containing an energy-regenerating system (2 mM ATP, 20 mM phosphocreatine, 20 U/ml creatine kinase, and 2 mM GTP). Oocytes were disrupted by ultra-centrifugation and the oocyte components were extracted. These extract solutions were kept on ice before use. Porcine fibroblast cells were treated with 30 μg/ml digitonin (Calbiochem) for permeabilization (64). Cell nuclei (2×10^5) were incubated in the oocyte extracts or fibroblast-cell extracts for 1 h at 37°C. After the incubation, nuclei

were suspended in sucrose buffer (75 mM KCl, 4 mM $MgCl_2$, 250 mM sucrose, 0.5 mM spermidine trihydrochloride, 0.15 mM spermine tetrahydrochloride, 1 mM DTT, 10 mM β-glycerophosphate, protease-inhibitor cocktail, and 10 mM HEPES pH:7.8) and collected by centriguation. Nuclear pellets were dissolved in 150 μl of the lysis buffer (7M urea, 2M thiourea, 4% CHAPS, bromophenolblue, protease inhibitor, and phosphatase inhibitor) and these samples were used for 2D PAGE. For the purpose of protein identification, five different samples were pooled, which equals about 4,000 oocytes.

Statistical Analysis. The differences of developmental abilities of embryos were analyzed by student's t-test. A *P* value of less than 0.05 was considered statistically significant. SI Materials and Methods provides complete information on materials and methods.

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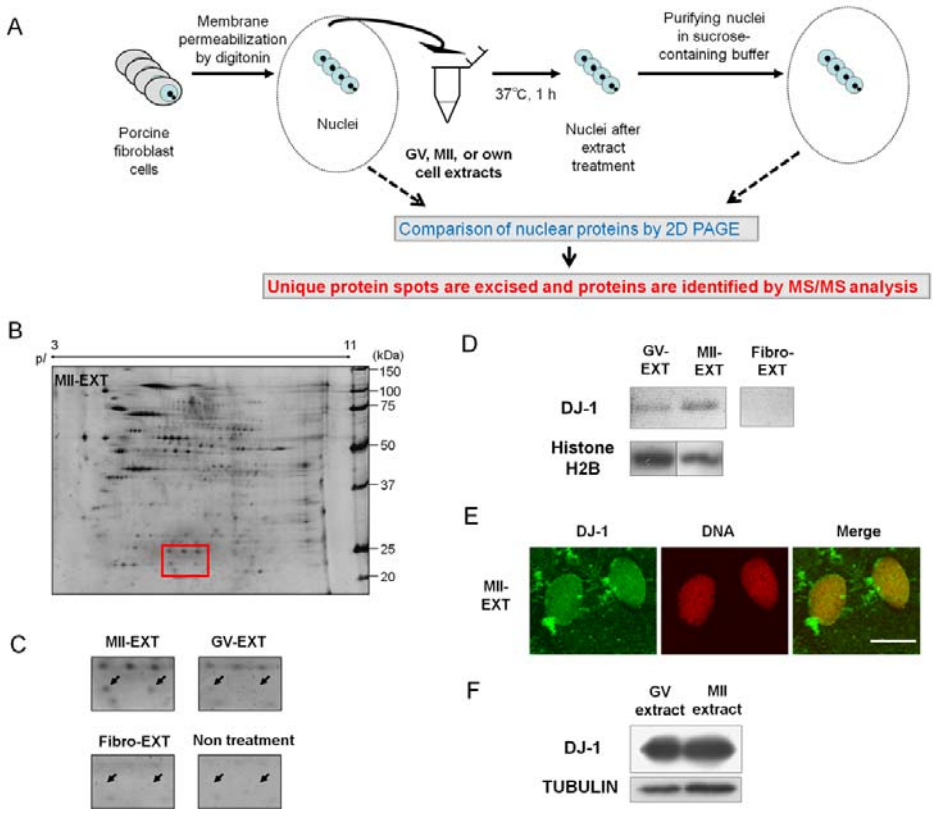

Fig. 1. Identification of protein DJ-1 as a candidate reprogramming factor present in the MII oocytes. (*A*) Schematic diagram of the strategy to identify reprogramming factors in oocytes. (*B*) Total protein of nuclei treated with MII oocyte extracts (MII-EXT) separated by 2D PAGE. The gel marked by the red square box (20-25 kDa) contains protein DJ-1 spots. p*I* means isoelectric point. (*C*) DJ-1 spots (arrows) in different nuclear samples from MII-EXT, GV oocyte-extract-treated cells (GV-EXT), fibroblast extract-treated cells (Fibro-EXT), and permeabilized cells without extract treatment (Non treatment). (*D*) Western blotting on fibroblast nuclei after extract treatment. Incorporation of DJ-1 proteins into nuclei was detected using a DJ-1 specific antibody. Histone H2B was used as a loading control. (*E*) Immunofluorescence analysis of DJ-1 in nuclei after treatment with MII oocyte extracts. DNA was stained with propidium iodide. (Scale bar: 10 μm.). (*F*) Large amounts of DJ-1 are present in both GV and MII extracts, examined by western blotting. TUBULIN was used as a loading control.

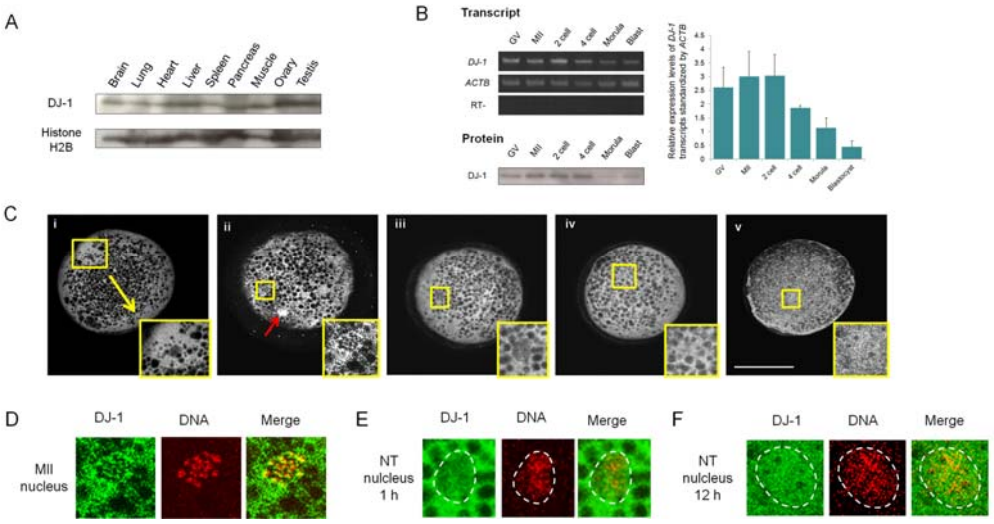

Fig. 2. *DJ-1* expression in porcine tissues, oocytes, and nuclear transfer embryos. (*A*) DJ-1 proteins exist in various types of tissues examined by western blotting. (*B*) Expression of *DJ-1* transcripts and proteins was examined in oocytes (GV and MII stages) and NT embryos during preimplantation development. The graph shows the relative expression levels of *DJ-1* transcripts examined by semiquantitative RT-PCR (normalized by ACTB transcripts). The number of oocytes and embryos used for western blotting are as follows; GV:8, MII:8, 2 cell: 8, 4 cell: 7, Morula: 2, Blast: 3. Error bars, mean ± SEM. (*C*) Localization of DJ-1 protein in oocytes and NT embryos, observed by immunofluorescence analysis. Each panel shows a whole oocyte or NT embryo, with an enlargement of the yellow boxed area shown to the lower right. The enlarged areas contain nuclei of oocytes or NT embryos. White area shows staining with anti-DJ1 antibody. i: GV oocyte, ii: MII oocyte, iii: NT embryo 1 h after nuclear transfer, iv: NT embryo 3 h after transfer, v: NT embryo 12 h after transfer. A red arrow in ii represents a first polar body in a MII oocyte. (Scale bar: 100 μm.). (*D*) Distribution of DJ-1 in a MII nucleus at high magnification. Yellow color on merged photos (Merge) represents nuclear localization of DJ-1. (*E*) DJ-1 distribution in transferred fibroblast nuclei 1 h after nuclear transfer into a MII oocyte. The border between nucleus and oocyte cytoplasm was marked with dotted white lines. (*F*) DJ-1 in transferred fibroblast nuclei 12 h after nuclear transfer.

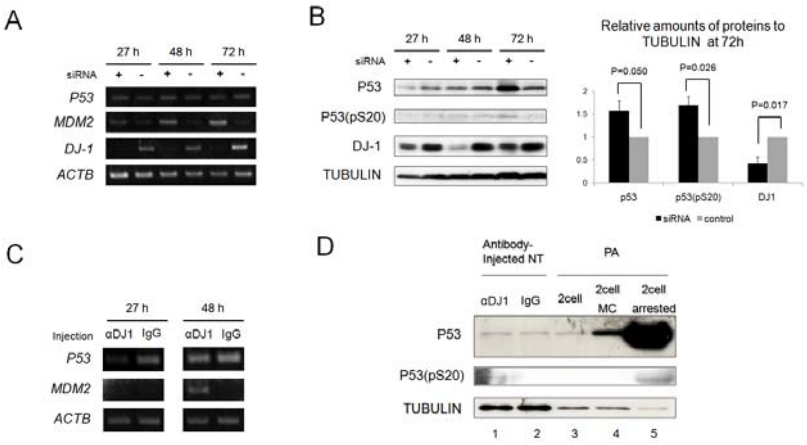

Fig. 3. Activation of *P53* after inhibition of *DJ-1*. (*A*) RT-PCR analysis of cultured porcine fibroblast cells after knockdown of *DJ-1* by specific siRNA. *DJ-1* expression was specifically inhibited by siRNA and, at the same time, activation of *MDM2* was observed. Duration (h) after siRNA transfection was indicated. (*B*) Activation of P53 after knockdown of DJ-1 in porcine fibroblasts was examined by western blotting analysis (left panel). The right graph represents the relative band intensities of P53, P53(pS20), and DJ-1 when standardized with TUBULIN. P53 and Phosphorylated P53 at serine 20 [P53(pS20)] was significantly upregulated, while DJ-1 was successfully downregulated by knockdown. Error bars, mean ± SEM (*t*-test, $n = 3$). (*C*) Transcripts in NT embryos injected with anti-DJ-1 antibody (αDJ1) or IgG (IgG) were examined by RT-PCR analysis. *MDM2* was only detected in embryos injected with DJ-1 antibody. (*D*) Activation of P53 was examined in NT and PA embryos by western blotting analysis. Anti-DJ-1 antibody was injected into NT embryos (αDJ1, lane 1) and IgG was injected as a control (IgG, lane 2). NT embryos were collected 72 h after nuclear transfer. For western blots of PA embryos, three types of 2-cell stage PA embryos were collected; non-treated embryos were collected 48 h after parthenogenetic activation (2cell, lane 3), Mitomycin C was added to the culture medium from 24 h to 48 h after activation and sampling was performed at 48 h (2cell MC, lane 4), and embryos arrested at the 2 cell stage were collected at 120 h (2cell arrested, lane 5).

Table 1. In Vitro Development of Anti-DJ-1 Antibody-injected Embryos to the Blastocyst Stage*

	Number of trials	Total no. of embryos	1st cleavage (%)	Morulae (%)	Blastocysts (%)	Embryos arrested at the 2- and 4-cell stages (%)
NT embryos						
a. anti-DJ1-injected embryos	6	103	70 (68.04±5.04%)	3 (2.50±2.50%)[b,d,e,f,g]	2 (1.67±1.67%)[d,f,g]	59 (57.61±4.27%)[b,d,f]
b. anti-DJ1-injected (+Pifithrin-α)	3	35	26 (75.37±6.94%)	8 (21.10±5.09%)[a]	6 (12.98±9.40%)	11 (34.78±7.97%)[a]
c. anti-DJ1-injected (+DMSO)	3	33	26 (75.56±8.89%)	3 (7.22±3.89%)[d,f,g]	2 (4.44±4.44%)	20 (61.11±5.56%)[d,f]
d. IgG-injected embryos	6	111	73 (64.76±4.52%)	26 (25.41±4.81%)[a,c]	15 (13.40±2.39%)[a]	36 (30.31±5.34%)[a,c]
e. DJ1(L166P) mRNA-injected embryos	4	74	53 (71.07±5.23%)	10 (13.04±1.96%)[a,f,g]	5 (6.25±2.39%)	35 (46.43±7.92%)
f. EGFP mRNA-injected embryos	4	83	57 (68.30±5.48%)	19 (23.16±1.90%)[a,c,e]	11 (13.08±3.47%)[a]	30 (35.65±4.81%)[a,c]
g. Control (Non-injected embryos)	4	134	93 (68.94±2.43%)	28 (21.07±1.05%)[a,c,e]	19 (14.22±2.92%)[a]	n.d. †
ICSI embryos						
anti-DJ1-injected embryos	5	63	21 (33.06±0.85%)	18 (28.55±1.82%)	18 (28.55±1.82%)	3 (4.51±2.09%)
IgG-injected embryos	5	60	21 (34.58±1.33%)	17 (27.86±1.56%)	17 (27.86±1.56%)	4 (6.72±1.88%)
Control (Non-injected embryos)	5	68	25 (36.15±2.36%)	21 (30.82±0.65%)	21 (30.82±0.65%)	4 (5.33±2.26%)
PA embryos						
anti-DJ1-injected embryos	3	63	49 (76.18±3.21%)	n.d.	33 (51.99±5.03%)	n.d.
IgG-injected embryos	3	72	58 (79.39±2.56%)	n.d.	39 (51.49±6.79%)	n.d.

*Three experimental groups are set as NT (nuclear transfer) embryos, ICSI (intracytoplasmic sperm injection) embryos, and PA (parthenogenetically activated) embryos. DJ-1-injected embryos represent the embryos injected with antibody against DJ-1. Similarly, IgG-, DJ1(L166P) mRNA-, and EGFP mRNA-injected embryos are shown.

[a~g]Each sample in NT embryos is expressed as a-g, and samples that show significant differences within the same column in NT embryos are indicated as superscripts ($P<0.05$). For example, sample f shows significant differences from a, c, and e in development to the morula stage. ICSI and PA embryos do not show any significant differences. Percentages are expressed as mean±SEM

†n.d. represents not determined

Classification: Biological Sciences, Cell Biology

Identification and characterization of an oocyte factor required for development of porcine nuclear transfer embryos

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Data deposition footnote: GenBank accession no: GSE18104

Supporting Information (SI)

SI Materials and Methods

Proteomic Analysis. Total protein concentrations were determined by the modified Bradford-HCl assay using γ-globulin as a standard and aligned among all samples. Separation of proteins by first-dimensional IEF electrophoresis was performed using IPG gels (Immobiline DryStrip, pH 3–11 NL, GE Healthcare). The strips were transferred onto SDS-PAGE gels. After the 2D PAGE, gels were stained with Sypro Ruby (Invitrogen). Differentially expressed spots were picked up manually and subjected to in-gel trypsin digestion. MS spectrometric analysis of the tryptic digests was performed using a 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). To identify proteins, sets of MS and MS/MS spectra were subjected to MS/MS Ion search (Matrix Science) against Swiss Prot or NCBI mammalian protein database.

Embryo Manipulation. Porcine nuclear transfer was performed as described previously (1). Porcine fibroblast cells from an adult male Meishan pig were cultured in DMEM with 10% FBS and used for donor cells within passage 8. Five pl of antibody solution or 10 pl of 500 ng/μl poly(A)-tailed RNA was injected into enucleated oocytes. After injection of antibody, embryos were kept for at least 1 h before fusion with donor cells, which allows the antibody to bind endogenous DJ-1. Total number of cleaved embryos was judged 48 h after nuclear transfer. Porcine ICSI followed the previous protocol (2) and the antibody was injected at least 1 h before sperm injection. Antibodies used are as follows: DJ-1 (sc-27004, Santa Cruz), rabbit IgG (sc-2027, Santa Cruz), and goat IgG (sc-2028, Santa Cruz).

Gene Expression Analyses. Incorporation of DJ-1 protein into extract-treated nuclei and distribution of DJ-1 protein in oocytes and embryos were examined by immunofluorescence analysis. The following antibody was used: DJ-1 (sc-27004). Anti-goat secondary antibody conjugated with Alexa488

(Invitrogen) was used. Nuclei were stained with propidium iodide. The amounts of proteins expressed in cells, oocytes, and embryos were measured by western blotting. The following primary antibodies were used: DJ-1 (sc-27004), P53 (628201, Biolegend), P53(S20) (DR1023, CALBIOCHEM or 55427, ANASPEC), Histone H2B (07-371, Millipore), β-Actin (clone AC15, Sigma), and Tubulin (T9026, Sigma). Anti-Tubulin, anti-Histone H2B, and anti-β-Actin antibodies were used for standardization. The blots were visualized using Immobilon Western HRP Substrate (Millipore). The amounts of transcripts expressed were measured by RT-PCR analysis. Total RNA from cells or from oocytes and embryos was extracted by a phenol-chloroform method or by Picopure RNA extraction kit (Arcturus), respectively. Purified RNA was reverse transcribed by ReverTra Ace (TOYOBO) and PCR reaction was performed by ExTaq (Takara) or KOD plus. Primers used are listed on Table S2. In both analyses, the band intensities were determined by a model 4.0 ATTO densitograph (ATTO).

Microarray Analysis. Nuclear transfer embryos (23-26 embryos per each sample) were collected 28 h after nuclear transfer. Total RNA was purified by the use of Picopure RNA extraction kit. Two rounds of amplification for isolated RNA were performed for microarray analysis (3). The final yield of biotinylated cRNA was hybridized to the Affymetrix GeneChip Porcine Genome Array. The output data were analyzed with GeneSpring software (Agilent).

Plasmid Construction, RNA Synthesis, and Transfection. Site-directed mutagenesis was induced by different primer sets containing a point mutation (Table S2) to generate constructed plasmids containing pig DJ1(L166P) cDNA. The plasmids were used as templates for *in vitro* transcription. RNA synthesis and poly(A) tailing were performed with MEGAscript T7 kit (Ambion). Antisense oligonucleotid and siRNA against porcine DJ-1 were designed and the sequences are listed on Table S2. *DJ-1* siRNA was transfected to fibroblast cells using Lipofectamine 2000 (Invitrogen) according to the vendor's

instructions.

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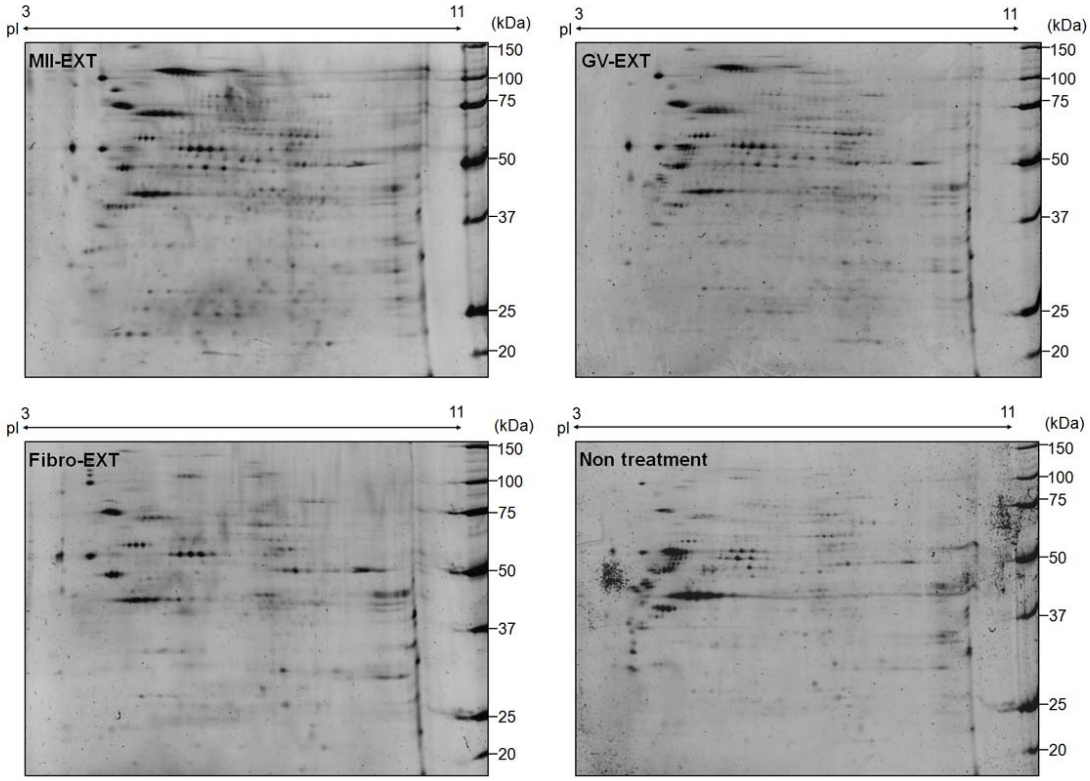

Fig. S1. Fibroblast cell nuclei were treated with oocyte extracts and nuclear proteins were separated by 2D PAGE. MII-EXT: MII oocyte-extract-treated cells, GV-EXT: GV oocyte-extract-treated cells, Fibro-EXT: Fibroblast-extract-treated cells, Non treatment: Cells before extract treatment. p*I* means isoelectric point.

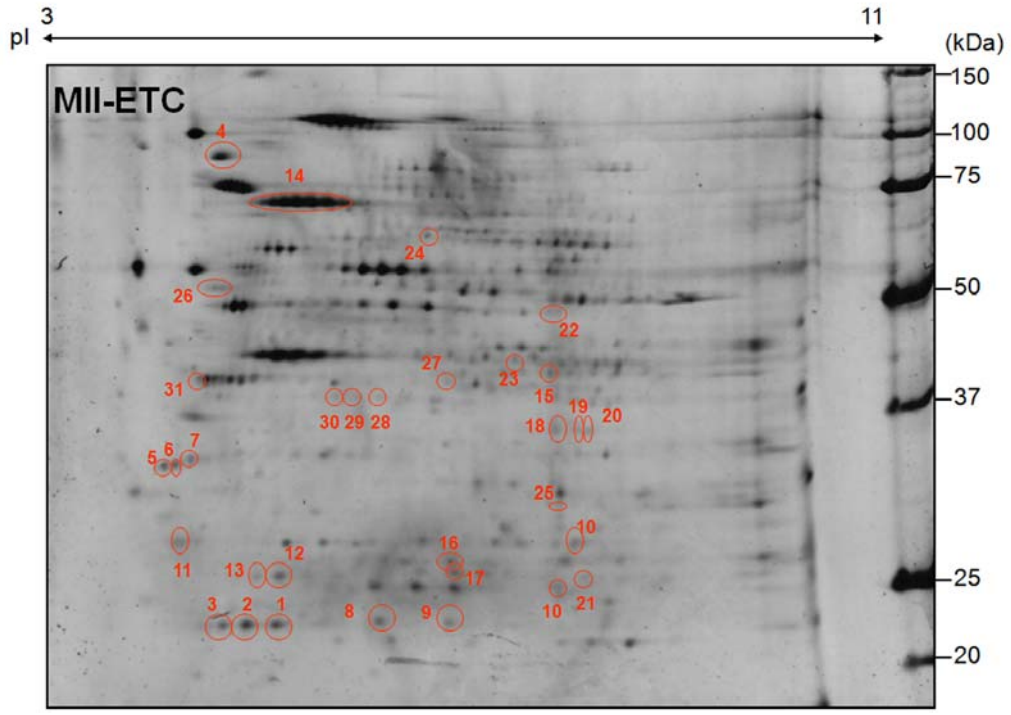

Fig. S2. Protein spots identified by MS/MS analysis. Protein spots marked by red circles were specifically and abundantly expressed in MII oocyte-extract-treated cells (MII-EXT). The number on the 2D PAGE gel corresponds to the Spot No in Table S1. MII-EXT: MII oocyte-extract-treated cells. p*I* means isoelectric point.

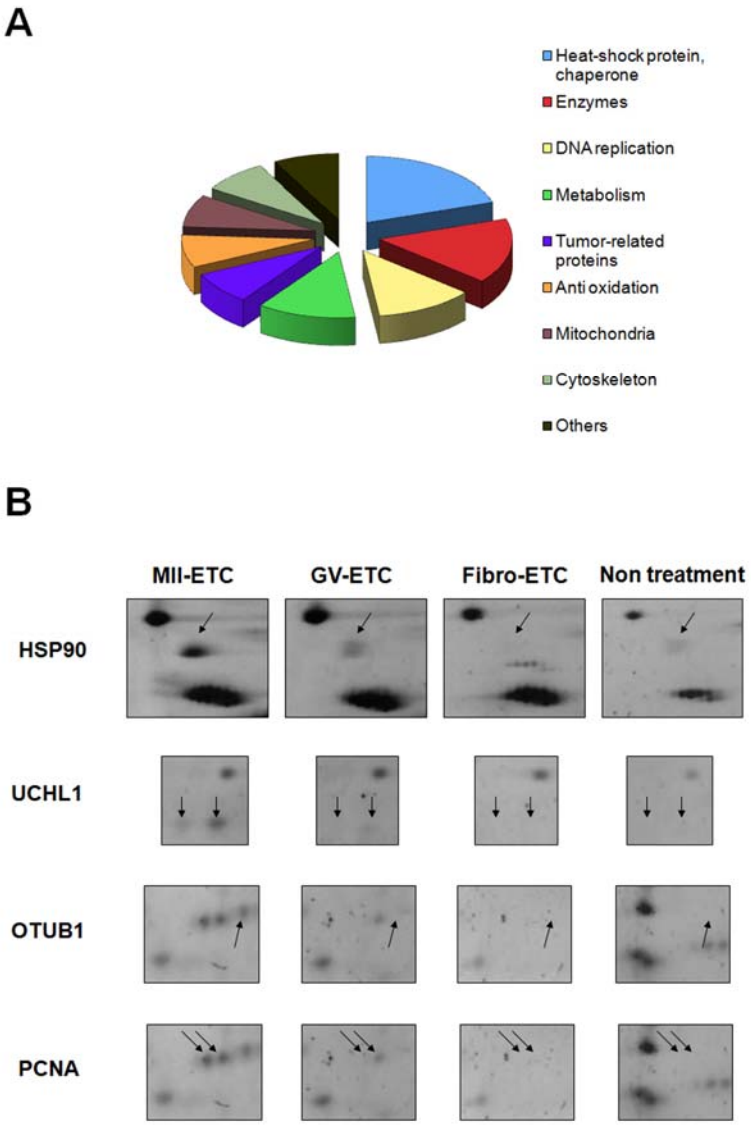

Fig. S3. Oocyte proteins that are specifically and abundantly accumulated in somatic nuclei after treatment with MII oocyte extracts. (*A*) Identified proteins are classified according to their cellular function. (*B*) Some examples of proteins that are highly expressed in MII oocyte-extract-treated cells (MII-EXT). Protein spots are indicated by arrows. GV-EXT: GV oocyte-extract-treated cells, Fibro-EXT: Fibroblast-extract-treated cells, Non treatment: Cells before extract treatment.

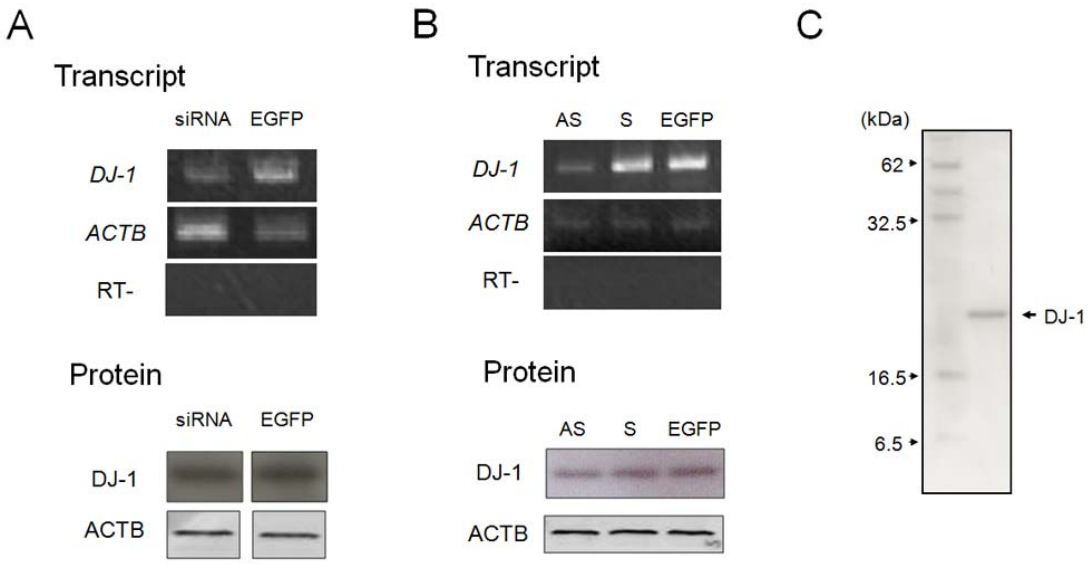

Fig. S4. Inhibition of *DJ-1* expression and specificity of anti-DJ-1 antibody. (*A*) siRNA against *DJ-1* was injected into GV oocytes and then *in vitro* matured to the MII stage. Although *DJ-1* transcripts were somewhat decreased, no significant degradation of DJ-1 proteins was observed during oocyte maturation. As a control, EGFP mRNA was injected at the same time. β-Actin (ACTB) was used as a loading control. (*B*) Oocytes injected with antisense oligonucleotide against pig *DJ-1* were *in vitro* matured to the MII oocytes and specific inhibition of *DJ-1* expression was examined. Although transcripts were efficiently downregulated by the antisense oligonucleotide, no apparent degradation of DJ-1 proteins was detected (AS). As a control, sense oligonucleotide (S) and EGFP mRNA were injected. β-Actin was used as a loading control. (*C*) Goat polyclonal antibody against human DJ-1 specifically recognized porcine DJ-1 proteins in MII oocytes, observed by western blotting.

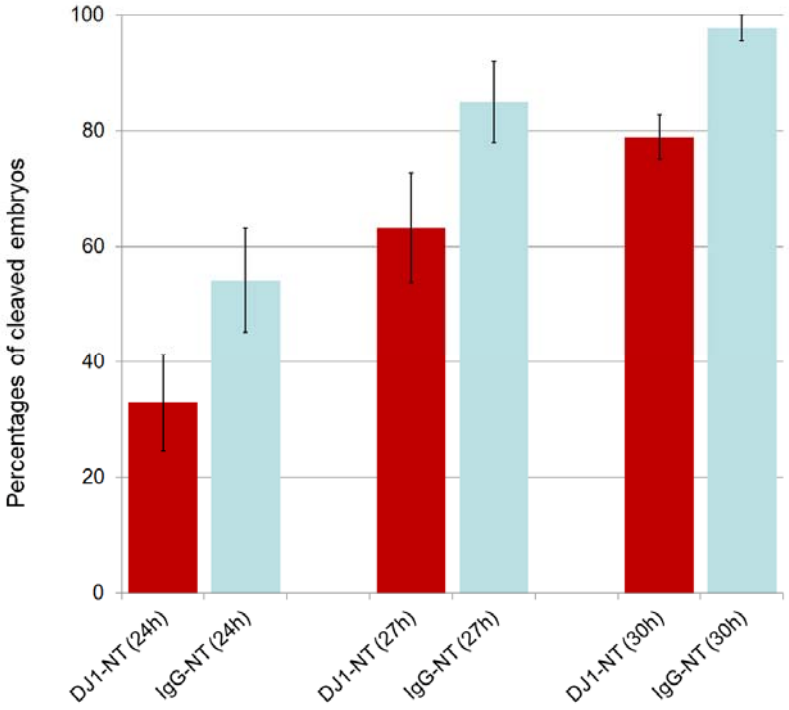

Fig. S5. Timing of first cleavage was compared between NT embryos injected with DJ-1 antibody (DJ1-NT) and those with IgG (IgG-NT). The y axis represents ratios of cleaved embryos at different times (24 h, 27 h, and 30 h) to total cleaved embryos at 48 h.

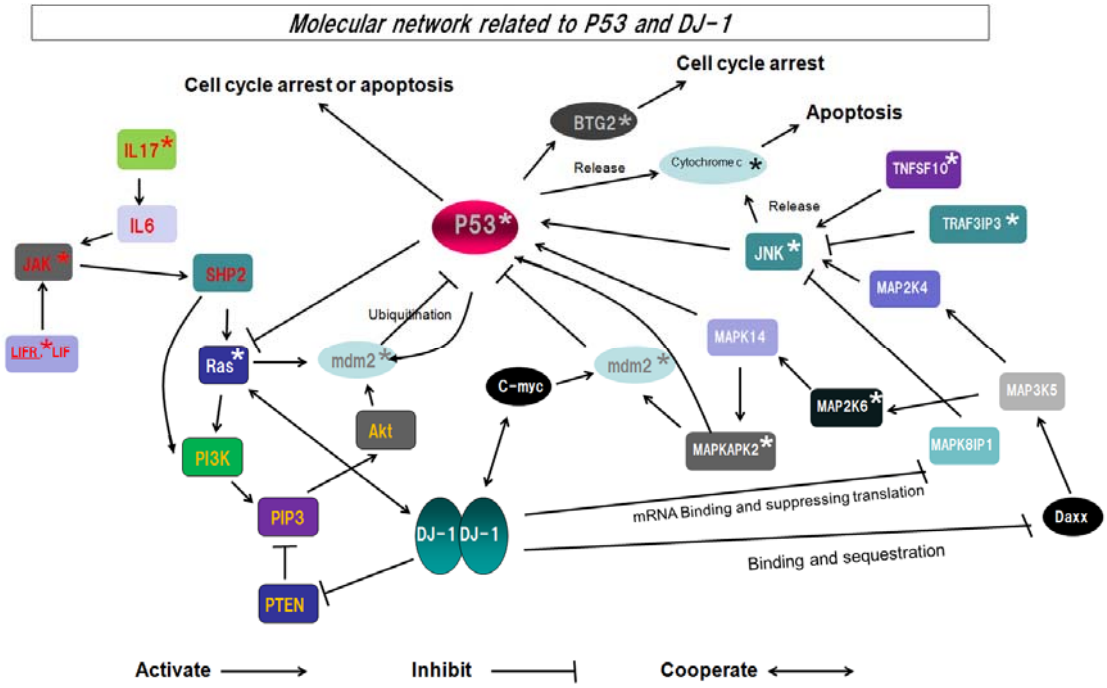

Fig. S6. Molecular networks related to *P53* and *DJ-1* are depicted. Signaling pathways and relationship among these genes are shown. These pathways are based on published reports. Genes whose expression was disrupted after inhibition of DJ-1 function in nuclear transfer embryos in this report are marked with asterisk. The abnormal expression was detected by microarray analysis (more than 2-fold differences), RT-PCR, western blotting analysis, or inhibitor treatment for P53. Large sets of genes and molecular pathways are perturbed after DJ-1 inhibition in NT embryos including some critical genes involved in cell arrest or apoptosis.

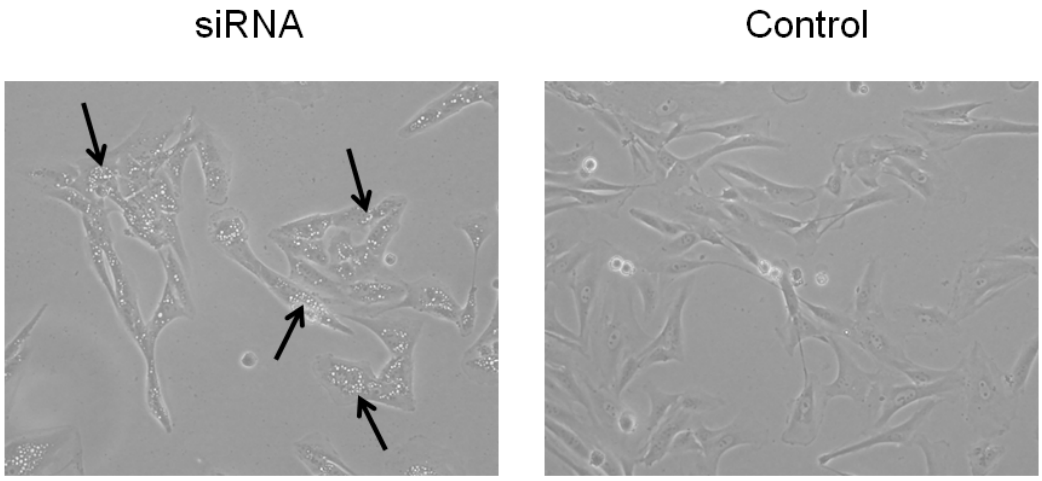

Fig. S7. Fibroblast cells after treatment with specific siRNA against DJ-1. Many lipid droplets (some of them were marked with arrows) were accumulated 24 h after transfection with siRNA against pig DJ-1 (siRNA). As a control, cells were treated with lipofectamine 2000 without siRNA.

Table S1. Oocyte Proteins That Are Incorporated into Somatic Nuclei during Incubation in MII Oocyte Extracts

Spot No.*	Protein Name	Accession ID	Mascot PS [a]	Classification
1-3	Peroxiredoxin-2 - Sus scrofa (Pig)	PRDX2_PIG	420,366,344	Anti oxidation
4	Heat shock protein HSP 90-alpha - Bos taurus (Bovine)	HSA90A_BOVIN	376	Chaperone
5, 6	Proliferating cell nuclear antigen - Bos taurus (Bovine)	PCNA_BOVIN	214,278	DNA replication
7	Ubiquitin thioesterase OTUB1 - Homo sapiens (Human)	OTUB1_HUMAN	102	Ubiquitin enzyme
8, 9	Protein DJ-1 - Bos taurus (Bovine)	PARK_BOVIN	33,144	Chaperon, Oncogene
10	Heat shock protein beta-1 - Bos taurus (Bovine)	HSPB1_BOVIN	72	Chaperone
11	14-3-3 protein zeta/delta - Bos taurus (Bovine)	1433Z_BOVIN	94	Oncogene
12, 13	Ubiquitin carboxyl-terminal hydrolase isozyme L1 - Sus scrofa (Pig)	UCHL1_PIG	224,76	Ubiquitin enzyme
14	Heat shock cognate 71 kDa protein - Equus caballus (Horse)	HSP7C_HORSE	117	Chaperone
	Protein-arginine deiminase type-6 - Homo sapiens (Human)	PADI6_HUMAN	63	Arginine deiminase
15	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor - Sus scrofa (Pig)	ACADM_PIG	107	Mitochondria
16	Peroxiredoxin-4 - Bos taurus (Bovine)	PRDX4_BOVIN	47	Anti oxidation
17	Thioredoxin-dependent peroxide reductase, mitochondrial precursor - Bos taurus (Bovine)	PRDX3_BOVIN	121	Mitochondria
18	Polymerase delta-interacting protein 2 - Homo sapiens (Human)	PDIP2_HUMAN	87	DNA replication
19	Alcohol dehydrogenase [NADP+] - Sus scrofa (Pig)	AK1A1_PIG	112	Metabolism
20	DNA replication complex GINS protein PSF1 - Bos taurus (Bovine)	PSF1_BOVIN	67	DNA replication
21	Uncharacterized protein C6orf118 - Homo sapiens (Human)		75	Others
22	Alpha-enolase - Homo sapiens (Human)	ENOA_HUMAN	74	Glycolytic enzyme
23	Actin, aortic smooth muscle - Bos taurus (Bovine)	ACTA_BOVIN	37	Cytoskeleton

24	Lamin-A/C - Sus scrofa (Pig)	LMNA_PIG	230	Nuclear membrane
25	PREDICTED: similar to Nitrilase family, member 2 [Pan troglodytes]	gi|114588194	105	Putativel tumor suppressor
26	Vimentin - Homo sapiens (Human)	VIME_HUMAN	229	Cytoskeleton
27	DnaJ homolog subfamily B member 11 precursor - Canis familiaris (Dog)	DJB11_CANFA	213	Chaperone
28-30	Galactokinase - Bos taurus (Bovine)	GALK1_BOVIN	102,130,177	Metabolism
31	Spermine synthase - Bos taurus (Bovine)	SPSY_BOVIN	300	Metabolism

*See Figure S2.

[a] Mascot protein score (Mascot PS) was calculated by MASCOT MS/MS Ion search.

Table S2. Primer, siRNA, and oligonucleotide sequences

Gene	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
A. Gene expression analysis (pig genes)		
ACTB	GCCCATCTACGAGGGGTACG	CACGTCGCACTTCATGATCG
DJ-1	CGAGGTTCAGTTAAGTCTTCAGA	CGGGAGGGTTCCGGGCAGCCCGCTCG
MDM2	AATGAATCCTCCCCTTCCAC	TTGGGATAGGAAGGCTTGTG
P53	CCTCACCATCATCACACTGG	GGCTTCTTCTTTTGCACTGG
B. Cloning of pig DJ-1		
Pig DJ-1 cloning 1	CGGAATTCCGAGGTTCAGTTAAGTCTTC AGA	CGGGATCCCGGGAGGGTTCCGGGCAGCCC GCTCG
Pig DJ-1 cloning 2	CGGGGTACCCCGAGGTTCAGTTAAGTCT TCAGA	CCCATCGATGGGAGGGTTCCGGGCAGCCCG CTCG
Pig DJ-1 L166P	CTTCGAGTTTGCTCCGGCCATTGTTGAGG	CTCAACAATGGCCGGAGCAAACTCGAA
C. siRNA against pig DJ-1		
sense	GAAGCAAAGUUACGACGCAUU	
antisense	UUCUUCGUUUCAAUGCUGCGU	
D. Oligonucleotide against pig DJ-1		
DJ-1 sense	CAGAAAATACAACATAAGAATGGCT	
DJ-1 antisense	AGCCATTCTTATGTTGTATTTTCTG	