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HSPC117 deficiency in cloned embryos causes placental abnormality and fetal death

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ABSTRACT

Somatic cell nuclear transfer (SCNT) has been successfully used in many species to produce live cloned offspring, albeit with low efficiency. The low frequency of successful development has usually been ascribed to incomplete or inappropriate reprogramming of the transferred nuclear genome. Elucidating the genetic differences between normal fertilized and cloned embryos is key to understand the low efficiency of SCNT. Here, we show that expression of *HSPC117*, which encodes a hypothetical protein of unknown function, was absent or very low in cloned mouse blastocysts. To investigate the role of *HSPC117* in embryo development, we knocked-down this gene in normal fertilized embryos using RNA interference. We assessed the post-implantation survival of *HSPC117* knock-down embryos at 3 stages: E9 (prior to placenta formation); E12 (after the placenta was fully functional) and E19 (post-natal). Our results show that, although siRNA-treated *in vivo* fertilized/produced (IVP) embryos could develop to the blastocyst stage and implanted without any difference from control embryos, the knock-down embryos showed substantial fetal death, accompanied by placental blood clotting, at E12. Furthermore, comparison of *HSPC117* expression in placentas of nuclear transfer (NT), intracytoplasmic sperm injection (ICSI) and IVP embryos confirmed that *HSPC117* deficiency correlates well with failures in embryo development: all NT embryos with a fetus, as well as IVP and ICSI embryos, had normal placental *HSPC117* expression while those NT embryos showing reduced or no expression of *HSPC117* failed to form a fetus. In conclusion, we show that *HSPC117* is an important gene for post-implantation development of embryos, and that *HSPC117* deficiency leads to fetal abnormalities after implantation, especially following placental formation. We suggest that defects in *HSPC117* expression may be an important contributing factor to loss of cloned NT embryos *in vivo*.

1. Introduction

Cloning by adult somatic cell nuclear transfer has been used widely to produce viable offspring in a variety of mammalian species. Mammalian embryos derived by nuclear transfer develop apparently normally to the blastocyst stage similar to that of embryos produced by *in vitro* fertilization (IVF) [1]. However, unlike embryos derived from fertilization, most cloned embryos die during post-implantation development, and as a result, the birth rate of cloned animals is very low, regardless of the species or the type of nuclear donor cells. In cloned mice, the birth rate is less than 5% [2] and those that survive to term are frequently defective [3]. To date, nuclear transfer has not resulted in a live primate birth in spite of substantial efforts in multiple laboratories.

Embryos derived by nuclear transfer can develop into "good looking" blastocysts, which are morphologically similar to normal fertilized embryos , but only a few percent of those blastocysts give rise to live offspring. The basis for these failures is unknown, but a popular hypothesis is that inefficient reprogramming of the donor nucleus results in inappropriate expression of genes required for embryonic development. For example, abnormal expression of the pluripotency marker gene *Oct4* (also known as *Pou5f1*) has been reported in cloned embryos [4], although normal expression has also been reported after SCNT [5]. In addition, the trophectoderm transcription factor *Cdx2* in cloned mouse morula expressed very differently at the protein level compared to time- and stage-matched fertilized counterparts [6]. Thus, identification and characterization of genes expressed differentially in cloned and

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fertilized embryos may be a way to understand the basis for the developmental failure of cloned embryos.

In a previous study we used cDNA representational difference analysis (cDNA-RDA) to compare gene expression in normal fertilized and cloned monkey embryos. We identified several genes showing reduced expression in cloned monkey blastocysts. Here, we focus on *HSPC117*, also known as *C22orf28* (Gene ID: 51493) in human and *D10Wsu52e* (Gene ID: 28088) in mouse, which showed strongly reduced expression in cloned rhesus monkey blastocysts. The function of *HSPC117* is not known. It was reported that *HSPC117* is highly expressed in hemopoietic stem cell (HSC) lines [7], and studies have demonstrated the presence of HSPC117 in RNA-transporting granules [8] and the ability of HSPC117 to bind to AU-rich elements within mRNA [9], possible implicating HSPC117 in post-transcriptional regulation. The function of *HSPC117* in embryo development has not been examined. In the present study, we characterize *HSPC117* expression in cloned mouse blastocysts and in *in vivo* produced (IVP) blastocysts. To determine the function of *HSPC117* in embryo development and how it affects embryonic survival rates, we generated *HSPC117* RNAi knock-down embryos and investigated the effects of *HSPC117* deficiency on the pre- and post-implantation stages of mouse embryonic development.

2. Materials and methods

2.1. Production of NT embryos and IVP embryos in mouse

Mouse SCNT was performed as previously described [10]. Briefly, B6D2F1 (C57BL/6 × DBAF1) F1 female mice (8–10 weeks old) were superovulated and cumulus–oocyte complexes (COCs) were collected 13–14 h after hCG injection. Cumulus cells were washed 2–3 times in drops of CZB-Hepes and transferred directly to a micromanipulation chamber. SCNT embryos were reconstituted by injection of donor nucleus nuclei (cumulus cell) into enucleated oocytes and then activated in strontium-containing CZB medium. IVP embryos (B6D2 F2) were flushed at specific developmental stages: pronucleus stage (1-cell, 20 h post-hCG) and blastocyst stage (96–98 h post-hCG) in CZB-Hepes.

2.2. Quantitative RT-PCR analysis of gene expression on blastocysts or tissues

RNA extraction and reverse transcription (RT) of each blastocyst was done using the SuperScript™ III Cells Direct cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA). For fetals or placentals, total RNA was prepared using RNAeasy Mini kit (Qiagen, Hilden, Germany). First-stand cDNA was synthesized from 1 μg of total RNA with oligo dT primer (Promega, Madison, USA). Each experimental group included at least four samples and was replicated at least three times. Real-time PCR was carried out with the ABI PRISM7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). SYBR Green (Sigma, St. Louis, MO) was used for quantitative PCR as a double-stranded DNA-specific fluorescent dye. The relative quantification of gene expression between multiple samples was achieved by normalization against the endogenous housekeeping gene *GAPDH* using the ΔC_T method of quantification. Fold changes were calculated as $2^{-S\Delta C_T - C\Delta C_T}$. The sequences of primers for qPCR were: *HSPC117* 5′-GCAGTTGGCTTG TGCTCG-3′ (forward primer) and 5′-AAGGTCATAGAGGAGCGGT-3′ (reverse primer); *Oct4* (*Pou5f1*) 5′-GGAAGCCGACAACAATGAG-3′ (forward primer) and 5′-TCGGGCACTTCAGAAACAT-3′ (reverse primer); *Cdx2* 5′-TGCCACACTTGGGCTCTC-3′ (forward primer) and 5′-GCTGTGGAGGCTGTTGTTG-3′ (reverse primer); *GAPDH* 5′-CAG CAACTCCCACTCTTCCAC-3′ (forward primer) and 5′-TGGTCCAGG GTTTCTTACTC-3′ (reverse primer).

2.3. HSPC117 siRNA microinjection

IVP 1-cell mouse embryos (B6D2 F2) were obtained and denuded of cumulus cells. Three pairs of Stealth™ RNAi against *HSPC117* were synthesized by Invitrogen, Inc. (Carlsbad, CA, USA). Approximately, 20 pl of individual siRNA (200 μm) was microinjected into the cytoplasm of embryos using an Eppendorf FemtoJet express Microinjector under a Leica DIC microscope. Control embryos were microinjected with the same amount of Neg Control Stealth™ RNAi (Invitrogen, Stealth™ RNAi NEG CTL, CN 12935300). Embryos were cultured to the blastocyst stage at 37 °C in a 5% CO_2 atmosphere. Relative expression of *HSPC117* in blastocysts was detected by RT-qPCR. Then, the siRNA with the highest knock-down efficiency of about 90% was selected for subsequent microinjection. Lastly, the *HSPC117* siRNA and control siRNA injected 1-cell embryos were transferred into pseudo pregnant ICR females to compare the development ability.

2.4. Embryos transfer and dissection

Mouse embryos were transferred at the late 1-cell stage into pseudopregnant ICR females or cultured in CZB medium to the blastocyst stage. Recipients were euthanized at different stages and the embryos were dissected into PB1 medium containing 10% fetal calf serum. The recovered embryos or placentas were examined and stored at −80 °C for RT-PCR.

2.5. Molecular cloning of HSPC117 and antibody preparation

The *HSPC117* cDNAs were prepared by RT-PCR from mouse placental mRNA and an *HSPC117* prokaryotic expression plasmid was constructed. HSPC117 protein was expressed in *Escherichia coli.* Polyclonal anti-HSPC117 antibody was obtained from a rabbit immunized with purified HSPC117 protein. The specificity of antibodies was tested by Western blot in cells of mouse placenta; fibroblasts and R1 ES cells (gift from Prof. Nagy's laboratory).

2.6. Immunofluorescence studies on embryos using HSPC117 antibody

Immunofluorescence on embryos was performed as previously described with our own anti-HSPC117 rabbit polyclonal antibody [5]. Nuclei were counterstained with Yopro 10 μM (Invitrogen, Carlsbad, CA). The experiments were replicated at least three times.

2.7. Statistical analysis

The numbers of embryos/fetus were expressed as means ± SD. Results were analysed by Newman–Keuls Multiple Comparison Test or unpaired *t*-test, with $P < 0.05$ considered significant.

3. Results

3.1. Decreased HSPC117 expression in mouse NT blastocysts compared with IVP blastocysts

To characterize differences in rhesus monkey IVP embryos and NT embryos, we previously used cDNA-RDA and identified several genes whose expression is substantially reduced in NT embryos. *HSPC117*, one of the identified genes with unknown function, was selected for further study. In this study, we extended this analysis by examining *HSPC117* expression in cloned mouse embryos.

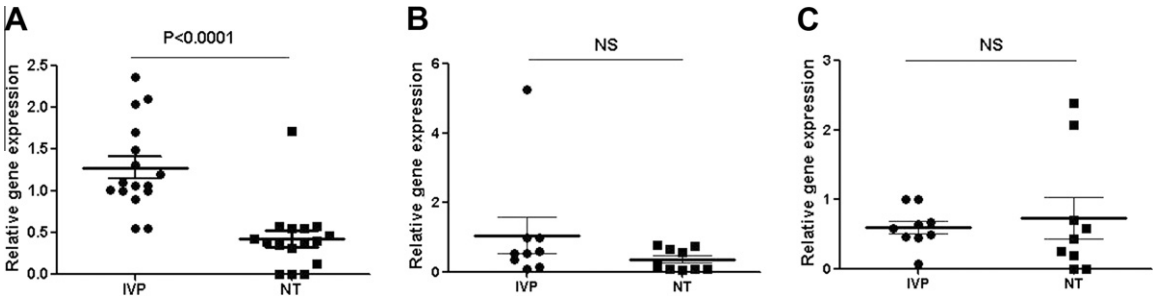

Fig. 1. Relative expression of *HSPC117* (A), *Oct4* (B) and *Cdx2* (C) in mouse IVP and NT blastocysts.

Sixteen good blastocyst stage NT and 16 control IVP mouse blastocysts were collected and RT-qPCR was performed on each embryo. Notably, NT blastocysts with 'excellent morphology' displayed significantly decreased *HSPC117* gene expression (Fig. 1A). However, *Oct4* and *Cdx2* expression did not differ substantially between NT and IVP embryos (Fig. 1B and C).

3.2. Expression of HSPC117 in mouse pre- and post-implantation embryos

To better understand the role of this protein in embryonic development, we investigated the timing of *HSPC117* expression in mouse pre- and post-implantation embryos by RT-PCR or immunofluorescence. Antibody against HSPC117 was prepared and the specificity was confirmed by Western blot (Fig. 2A). Mouse embryos were collected at the blastocyst stage (E3.5), early implantation stage (E6.5) and 'fully functional placenta' stage (E12). At E3.5, HSPC117 protein was widely expressed in the cytoplasm of blastomeres in mouse blastocysts, both in the inner cell mass and trophectoderm (Fig. 2B). At E6.5, embryos were separated into epiblast and extraembryonic ectoderm (EXE) (Fig. 3A) and *HSPC117* was expressed in all epiblast and EXE parts by RT-PCR (Fig. 3B). At E12, by which time the placenta has developed, we detected *HSPC117* expression both in the fetus and placenta (Fig. 3C). The results of *HSPC117* expression and location were consistent with expression in both blastocysts and post-implantation stages of embryos.

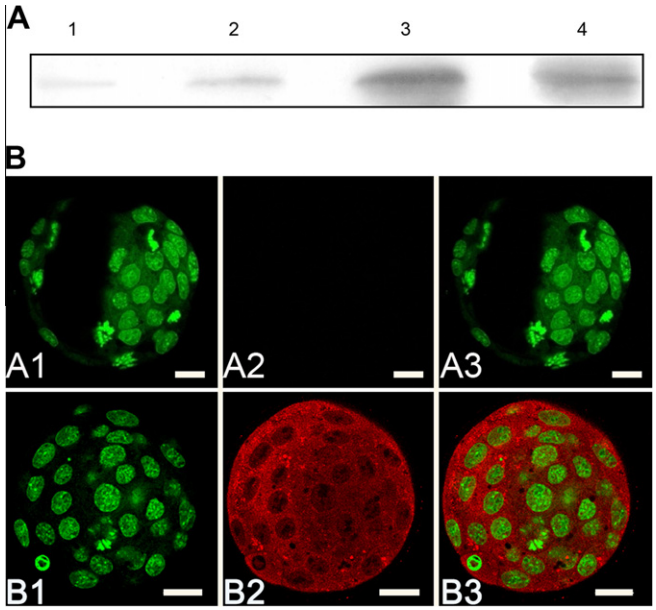

Fig. 2. *HSPC117* protein expression in mouse blastocysts. (A) HSPC117 expression by Western blot. 1. mouse placentas; 2. fibroblasts; 3. R1 ES cells; 4. mouse HSPC117 protein. (B) *HSPC117* protein expression in mouse blastocyst by immunofluorescent using isotype control (A1–A3) or anti-HSPC117 antibody (B1–B3). Green indicates DNA and red indicates *HSPC117* protein. Scale bar: 20 μm.

3.3. Effects of microinjection of HSPC117-specific Stealth™ RNAi on embryo development

3.3.1. Pre-implantation development of embryos with knock-down HSPC117 expression

Development to the blastocyst stage was not affected by injection with Stealth™ RNAi. Blastocysts with reduced *HSPC117* expression had 'excellent morphology' similar to blastocysts injected with negative control Stealth™ RNAi and IVP blastocysts. Both inner cell mass and trophectoderm of these embryos appeared normal and the embryos could hatch during *in vitro* culture.

3.3.2. Post-implantation development of embryos with knock-down HSPC117 expression

We assessed post-implantation survival of *HSPC117* knock-down embryos in 3 stages: E9 (before placenta formation); E12 (after the placenta was fully functional) and E19 (new born) to cover the whole period of development (Table 1). Strikingly, lack of *HSPC117* expression did not cause any apparent abnormality in implantation. There was no difference between control embryos and *HSPC117* RNAi embryos in the frequency of implantation sites (77–78% vs. 80%, respectively). At the early post-implantation stage (E9), most of the implantation sites still contained a conceptus (69% in control group and 73% in *HSPC117* RNAi group). The others were composed of only decidual tissues with blood (Fig. 4A: a1, b1). Fetuses recovered from conceptuses were alive, with a beating heart and they had turned. We found one developmentally-retarded E8.5 fetus in the *HSPC117* RNAi group. There was no significant difference between the control and *HSPC117* RNAi groups at E9 (Fig. 4A: a2, b2).

In contrast to our results with E9 embryos, a large number of deaths had occurred by E12, by which time the embryos had a fully functional placenta. Compared with the control group, most uteruses in the *HSPC117* RNAi group were filled with degenerated decidual tissues and blood clots (Fig. 4A: a3, b3). Only 50% of the implantation sites in the RNAi group had a conceptus vs. 65% in the control group ($P < 0.05$). Thirty-three percent of fetuses and 41% of placentas derived from these RNAi group conceptuses were abnormal. Fetuses had blood spots on their bodies, especially on the heads. Placentas also had blood clots and some were filled with blood (Fig. 4A: b4). Both the fetuses and placentas in the *HSPC117* RNAi group had significant differences from the control group ($P < 0.01$). *HSPC117* knock-down dramatically affected the percentage of live births: at E19, only 13% of *HSPC117* RNAi transferred embryos were born alive, compared to 65% of controls.

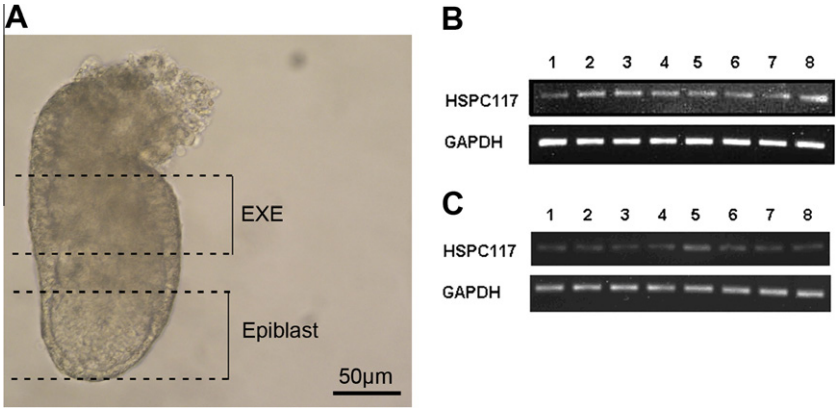

Fig. 3. *HSPC117* expression in mouse embryo epiblasts/fetuses and EXE/placentas by RT-PCR. (A) Mouse E6.5 embryo. The embryo was separated along the dotted lines to yield the pure epiblast and EXE. Scale bar: 50 μm. (B) *HSPC117* expression in E6.5 embryo epiblasts and EXE by RT-PCR. Four E6.5 embryos were divided two parts: epiblasts (1, 3, 5, 7) and EXE (2, 4, 6, 8). (C) HSPC117 expression in E12 embryo fetuses and placentas by RT-PCR. Four E12 embryos were used: fetuses (1, 3, 5, 7) and placentas (2, 4, 6, 8).

Table 1
In vivo development of mouse embryo at E9, E12 and E19 after *HSPC117* knock-down.

Treatment group	No. of transferred 1-cell embryos (*n*)	No. of implantations (% of transferred)	No. of conceptuses (% of transferred)	Morphology of conceptuses (F + P)	
				No. of abnormal fetuses (% of conceptuses)	No. of abnormal placentas (% of conceptuses)
RNAi control					
E9	61	47(77 ± 12)	42(69 ± 10)	0(0)	NA
E12	80	61(78 ± 13)	50(65 ± 18)[a]	0(0)[a]	0(0)[a]
E19	83	NA	53(65 ± 12)[d]	NA	NA
RNAi HSPC117					
E9	78	61(80 ± 12)	55(73 ± 18)	1(2 ± 4)	NA
E12	78	62(80 ± 14)	39(50 ± 10)[b]	12(33 ± 25)[c]	16(41 ± 20)[c]
E19	139	NA	19(13 ± 15)[e]	NA	NA

a vs b, $P < 0.05$; a vs. c, $P < 0.01$; d vs. e, $P < 0.01$.

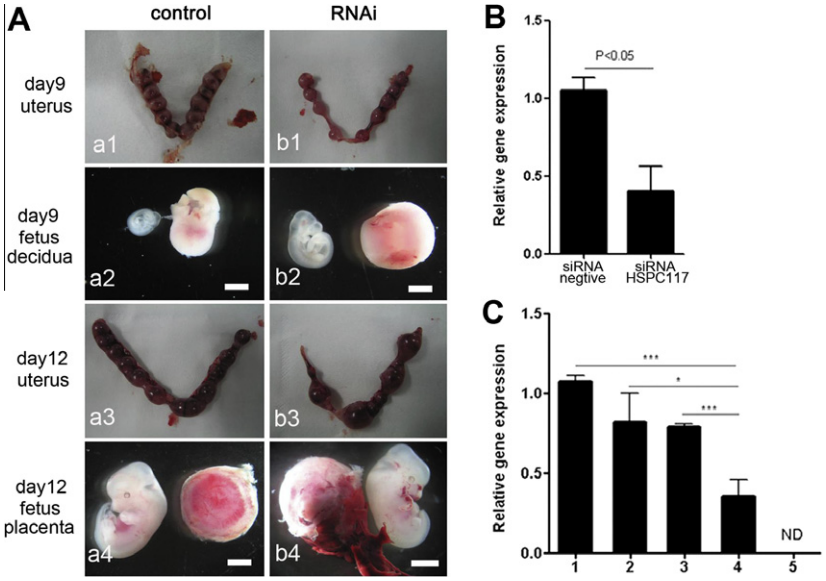

Fig. 4. *In vivo* development of mouse embryos after *HSPC117* knock-down and *HSPC117* expression in the placentas. (A) Uteruses of control and RNAi groups at E9 (a1, b1) and at E12 (a3, b3); deciduas of control and RNAi groups at E9 (a2, b2); placentas and fetuses of control and RNAi groups at E12 (a4, b4). a4 displayed normal control placenta and fetus at E12 and b4 displayed abnormal placenta and fetus at E12 after RNAi treatment. Scale bar: 1 mm. (B) Relative *HSPC117* expression in the placentas after RNAi treatment. (C) *HSPC117* expression in the placentas derived from IVP embryos (1), ICSI (2), NT with live fetus (3), NT without fetus (4) and degenerated (5). ($^{*}P < 0.05$, $^{***}P < 0.0001$, ND means no detection).

From these *in vivo* observations, we found that most *HSPC117* knock-down embryos started to die after E9. The majority of conceptuses degenerated by E12 and the remaining ones exhibited abnormal growth of fetuses and placentas. These *HSPC117* knock-down embryos continued to die throughout the remainder of gestation until birth. Notably, the average amount of *HSPC117* expression in RNAi placentas was significantly reduced compared with control placentas ($P < 0.05$) (Fig. 4B).

3.4. Abnormal HSPC117 expression in placentas of cloned animals

To test the hypothesis that *HSPC117* might be abnormally expressed in the placenta of cloned animals, we examined *HSPC117* mRNA expression in mouse placentas (E19) by RT-qPCR (Fig. 4C). We analysed placentas derived from IVP, ICSI and NT embryos, and from degenerated NT-derived conceptuses. Each experimental group included at least three placentas and data from at least three independent experiments were shown. The cloned (NT) group comprised 5 pairs of placentas from 5 different surrogate mothers, each pair including a placenta with a fetus and one without a fetus from the same surrogate mother.

The *HSPC117* transcripts were decreased in NT-derived placentas compared with its transcripts in IVP-derived placentas and ICSI-derived placentas. Even in the cloned placentas, those with or without fetus showed a substantial difference in *HSPC117* expression. In those NT-derived placentas without a fetus, *HSCP117* transcript levels were much lower than in the NT-derived placentas with a fetus. Degenerated conceptuses (both placenta and fetus) had no *HSPC117* expression at all.

4. Discussion

Successful nuclear transfer depends on nuclear reprogramming of an adult somatic nucleus to an embryonic state [1]. In the development of naturally fertilized embryos, epigenetic marks are established over long periods of time during gametogenesis and fertilization [11]. In nuclear transfer, however, an adult somatic pattern of epigenetic modification that is normally very stable must be reversed within a short period of time before zygotic genome activation [12]. Therefore, incomplete nuclear reprogramming may be responsible for the failure of development in NT embryos.

There are two stages of embryo development: pre-implantation and post-implantation. Most cloned embryos die *in utero*, and the few that develop to term show a high incidence of abnormalities [13]. However, cloned embryos develop to the blastocyst stage with a high success rate and embryonic stem cells from blastocysts cloned by NT (ntESCs) can be derived from such embryos with high efficiency [14]. It is possible that these cloned blastocysts are reprogrammed incompletely and show abnormalities at the level of gene expression. Surprisingly, however, it has been observed in several studies that global gene expression is similar in cloned embryos and fertilized controls [15]. Moreover, the global gene expression pattern in cloned bovine blastocysts closely resembled that of naturally fertilized embryos, with <1% of genes showing differential expression [16]. It is reasonable to hypothesize that expression differences in a small number of genes, present in this 1% subset of differentially regulated genes, may be responsible for developmental failures in NT embryos. Identifying the relevant genes may lead to improvements in cloning efficiency, and help us to understand the basic parameters of embryonic development.

In previous studies, we used cDNA-RDA to compare gene expression profiles in normal fertilized and cloned monkey embryos. *HSPC117* was one of the differentially regulated genes that interested us. *HSPC117* expression was absent or very low in cloned monkey blastocysts in comparison with normal fertilized monkey blastocysts. This indicated that *HSPC117* may be an important gene for embryo development, and raised the possibility that its deficient expression could cause abnormalities or even death in cloned monkey embryos. In the present study, we detected *HSPC117* expression in cloned mouse blastocysts and in *in vivo* produced blastocysts. Our results show strikingly that, although all the embryos showed excellent morphology, *HSPC117* expression in NT blastocysts was significantly decreased or even missing compared with control IVP blastocysts. In the same embryos, expression of the pluripotency marker gene *Oct4*, and *Cdx2*, a required gene for embryo implantation [17], did not show significantly different expression between IVP and NT blastocysts. Consistent with our results, other groups also reported normal *Oct4* [5] and *Cdx2* [18] expression after somatic cell nuclear transfer. These results confirm a previous study which showed that the pattern of expression of *Oct4* is not a reliable indicator of developmental competence *in vivo* [19]. More importantly, *HSPC117* showed significant differential expression in mouse IVP and NT blastocysts, suggesting that failures in *HSPC117* expression might contribute to developmental failure of cloned embryos.

Our results showed that the blastocyst formation rate and the implantation potential of embryos microinjected with Stealth™ RNAi was not significantly different from the control group, suggesting that decreased expression of *HSPC117* did not affect development of embryos up to the blastocyst stage. In fact, in the early post-implantation stage, *HSPC117* knock-down embryos developed normally and were still alive at E9. In the late post-implantation stage, by which time the placentas of embryos became fully functional, we observed a large number of deaths in *HSPC117* knock-down embryos. At E12, one-third of conceptuses degenerated quickly, while 33% of fetuses and 41% of placentas derived from the remaining conceptuses were abnormal. In addition, the abnormal conceptuses continued to die over the next several days and only a few embryos developed to term.

There is a good correlation between our observations with *HSPC117* knock-down embryonic development and development of NT embryos. It has been shown that NT embryos are very similar to fertilized embryos in terms of their ability to differentiate *in vitro* and *in vivo*, and NT embryos can easily be reprogrammed into blastocysts [14]. Most cloned embryos also implant after transfer into recipient females. Despite this, only a few percent of those blastocysts give rise to live offsprings [20]. Most cloned embryos die during post-implantation development, which may be attributable to problems with placental development and function, which correlates well with the deaths of *HSPC117* knock-down embryos between E9 and E12 in the present study, and with the observed failure of proper placental development in knock-down embryos.

In cloned embryos, defects involving the placenta have been noted in several species [3]. Dysregulated gene expression has been identified in the placentas or placentomes of cloned bovine fetuses [21]. Cloned mouse embryos die before a functional placenta can fully develop and placentas of cloned mice are enlarged [5]. Since, *HSPC117* knock-down embryos died at high rates during the period of placental development, we monitored expression of *HSPC117* in RNAi-treated placentas, and found expression was only one-third of its expression in control placentas. Moreover, all NT embryos with a fetus, as well as IVP and ICSI embryos, had normal placental HSPC117 expression while those NT embryos showing reduced or no expression of HSPC117 failed to form a fetus. These results imply that *HSPC117* is essential for placental development and thus plays a very important role post-implantation. Placentas from *HSPC117* knock-down embryos were clearly defective, showing blood clots and some almost entirely filled with blood. However, the placentas were normal in shape and were not overweight (data not shown) as is frequently observed in placentas of

NT embryos in mice. We would predict that additional dysregulated genes contribute to these other abnormalities of placental function in NT embryos.

In summary, *HSPC117*, a gene that was found to be expressed differentially in normally-produced and nuclear transfer embryos, and was shown to be essential for development and function of placentas in post-implantation stages. We hypothesize that deficiency of *HSPC117* in pre-implantation stages and throughout gestation in cloned embryos may be responsible for dysfunctional placental development and contribute to the high incidence of fetal death of cloned embryos.

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