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Supplemental Information

Successful Serial Recloning in the Mouse over Multiple Generations

Sayaka Wakayama, Takashi Kohda, Haruko Obokata, Mikiko Tokoro, Chong Li, Yukari Terashita, Eiji Mizutani, Van Thuan Nguyen, Satoshi Kishigami, Fumitoshi Ishino, and Teruhiko Wakayama

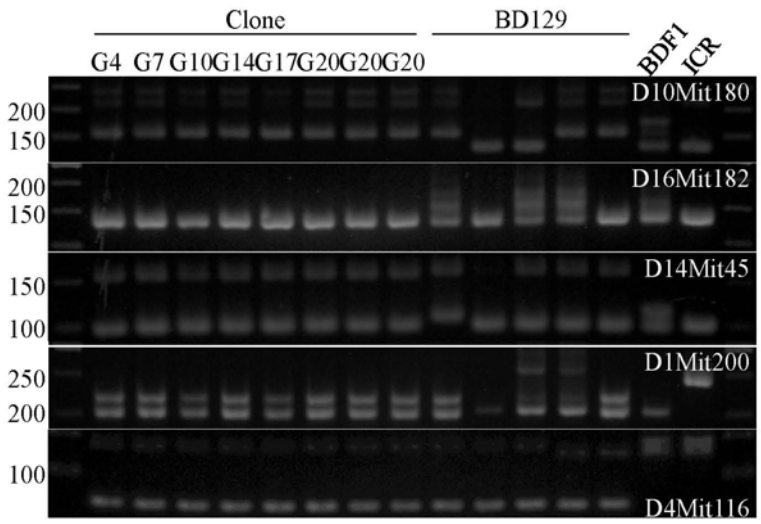

Figure S1. Genotyping of Recloned Mice, Related to Figure 1

The mice used in this study were produced by three-way crosses between C56/BL6, DBA/2 and 129/Sv strains, so each individual was genetically unique. These differences could be detected by genotyping, using polymorphic DNA markers for the mouse strains used. In total, eight recloned mice were selected from each of the G4, G7, G10, G14, G17 and G20 generations. When five DNA markers (D10Mit180, D16Mit182, D4Mit45, D1Mit200 and D4Mit116) were used, the possibility that all the mice had the same banding pattern was theoretically $1/10^{12}$. Furthermore, all cloned mice were female. These data indicate that all the clones were derived from the same original nuclear donor mouse.

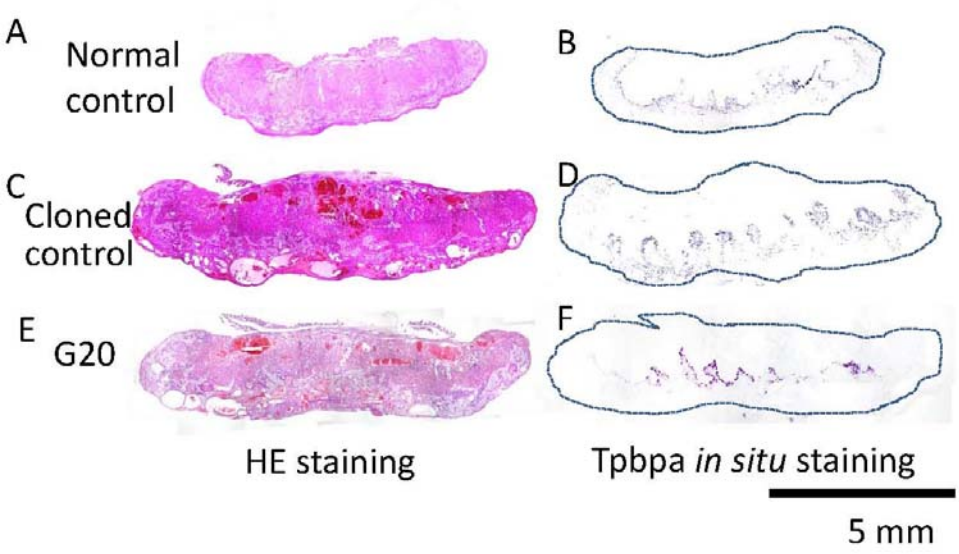

Figure S2. Histology of Recloned Mouse Placenta, Related to Figure 1

(A, B) Sections of control placentas derived from embryos fertilized using intracytoplasmic sperm injection (Normal control). (C, D) Sections of cloned control mouse placentas. (E, F) Sections of G20 cloned mouse placentas. (A, C, E) Hematoxylin and eosin (HE) staining. (B, D, F) In situ hybridization analysis of the gene for trophoblast-specific protein α, *Tpbpa*. The expansion of the spongiotrophoblast layer, which is a cloned mouse-specific abnormality, was slightly reduced compared with that of the CC mouse (n=5, respectively).

Table S1. Success Rate of Recloned Mice from BDF1 or Donor's Oocytes, Related to Figure 1

Table S1. Success rate of recloned mice from BDF1 or Donor's oocytes

Generation	Oocyte strain	No. of oocyte used	No. of oocyte survived	No. of oocyte PN formed	Embryo development		No. of recipient female	No. of transferred embryos	No. of cloned mice	Percent**		
					Fragment or 1-cell	2-cell				Average	Maximum	Minimum
G1*	BDF1	521	469	410	67	392	18	392	29	7.4	18.0	2.1
G2		87	78	67	6	65	3	60	3	5.0	-	-
G3		135	126	70	47	67	4	66	3	4.5	-	-
G4		253	210	197	14	193	6	193	13	6.7	7.4	5.3
G5		381	303	245	55	238	12	238	17	7.1	9.7	8.5
G6		330	276	266	22	252	7	202	14	6.9	7.1	6.3
G7		606	469	367	100	338	16	344	17	4.9[a]	3.7	6.5
G8		251	192	158	34	152	6	152	14	9.2	10.4	8.7
G9		247	213	175	48	115	9	154	14	9.1	9.8	8.7
G10		523	409	362	66	333	15	333	27	8.1[a]	20.8	4.3
G11		762	604	434	141	422	20	422	20	4.7[a]	10.8	1.3
G12		684	589	471	95	462	19	462	38	8.2	16.7	4.9
G13		648	510	410	83	392	18	382	38	9.9	14.5	7.5
G14		210	185	133	48	129	6	129	15	11.6	11.9	4.0
G15		160	147	118	16	115	4	115	14	12.2	19.0	3.1
G16		128	112	100	14	80	5	96	14	14.6[b]	-	-
G17		243	214	155	45	140	3	118	12	10.2	10.6	9.9
G18		276	257	219	43	187	9	187	18	9.6	21.7	2.8
G19		186	169	139	32	113	6	113	12	10.6	-	-
G20		438	370	343	33	321	16	321	34	10.6	15.6	5.9
G21		1219	1045	882	124	819	39	791	56	7.1[a]	14.9	1.1
G22		376	333	281	94	228	11	207	21	10.1	15.9	7.5
G23		399	358	330	38	305	15	305	26	8.5	11.0	5.5
G24		919	833	680	97	498	19	430	49	11.4[b]	14.0	5.3
G25		393	335	237	75	200	11	200	27	13.5[b]	25.0	4.8
Sub total		9942	8413	6902	1385	6225	281	6081	545 (527)***	8.7		
G4	Donor's oocyte	31	29	25	3	26	1	26	4	15.38	-	-
G5		85	69	67	3	66	3	66	7	10.61	12.1	9.1
G6		94	69	69	2	67	3	67	6	8.96	15.0	0
G7		123	72	62	9	60	3	60	5	8.33	16.7	0
G8		48	36	32	5	31	2	31	3	9.68	-	-
G9		45	32	30	11	21	1	21	0	0		
G10		143	113	103	8	101	4	101	10	9.90	33.3	0
G11		64	28	25	6	22	1	22	0	0		
G12		77	66	65	0	65	3	65	6	9.23	15.0	6.7
G14		30	18	16	2	16	1	16	0	0		
G15		38	26	24	1	24	1	24	2	8.33	-	-
G20		57	26	24	4	21	1	21	2	9.52	-	-
Sub total		835	584	542	588	520	24	520	45	8.65		
Total		10777	8997	7444	1973	6745	305	6601	590	8.9		

*G1 cloned mice were generated from four donor mice. The donor mouse that showed the highest success rate in producing G1 clones was then selected as the original donor and used to initiate the sequential mouse cloning experiment.

** In some experiments, there were not enough recipient female or some recipients died after 2-cell embryos transfer. Therefore, to clarify the effect of recloning for full term development, we calculate the percentage against transferred embryos into recipient female, rather than total 2-cell embryos.

*** The total 527 cloned mice were derived from one original donor mouse.

[a] Significant differences between generations ($p<0.05$).

[b] Significant differences between this generation and G1 ($p<0.05$).

This table presents the exact number of nuclear transfer experiments, as shown in Figure 1B.

Table S2. Fertility of G20 Recloned Mice, Related to Figure 1

Table S2 Fertility of G20 recloned mice

ID	Day of birth	Day of 1st delivery	Period (day)	No. pups			Day of 2nd delivery	Period (day)	No. pups		
				Total	♂	♀			Total	♂	♀
1	4/5/2011	6/6/2011	61	8	5	3	7/4/2011	28	13	8	5
2	4/5/2011	6/12/2011	67	9	3	6	7/24/2011	45	10	4	6
3	4/5/2011	6/6/2011	61	7	3	4	8/8/2011	62	10	5	5
4	5/5/2011	7/7/2011	62	10	3	6	8/6/2011	29	12	9	3

This table presents the one of normality experiments "fertility" in G20 recloned mice

Supplemental Experimental Procedures

Mice

BD129F1 (BDF1 × 129/Sv) mice, aged 3 months, were used as the original donor animals and cumulus cells were used as nuclear donors. The mice were produced by three-way crosses between C56/BL6, DBA/2 and 129/Sv strains. Although all polymorphic DNA markers for the 129/Sv strain are inherited by all offspring, markers for the C57BL/6 and DBA/2 strains are inherited randomly. Therefore, each individual was genetically unique and these differences could be detected easily by genotyping. If several DNA markers are used and all show an identical pattern between mice, they must be clones from the original mouse. See Figure S1 legend. BDF1 (C57BL/6 × DBA/2) mice, aged 8–10 weeks, were used to produce oocytes. The surrogate pseudopregnant females used as embryo transfer recipients were ICR strain mice mated with vasectomized males of the same strain. The BDF1 and ICR mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). The BD129F1 strain was bred in our mouse facility. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the RIKEN Center for Developmental Biology.

Nuclear Transfer and Production of Cloned Mice

Nuclear transfer and reconstructed oocyte activation were performed as described (Kishigami et al., 2006). Briefly, enucleated BDF1 oocytes were injected individually with a cumulus cell nucleus recovered from a recloned mouse at 3 months of age. After nuclear transfer, the reconstructed oocytes were activated using 10 mM $SrCl_2$ in Ca-free CZB or EGTA-KSOM medium in the presence of 5 μg/mL cytochalasin B supplemented with trichostatin A (TSA) (50 nM) for 6 h. Our previous study demonstrated that those two media did not affect the success rate of mouse cloning (Kishigami and Wakayama, 2007). Oocytes were then cultured in KSOM for a further 3 h. After three washes in KSOM, cloned embryos were cultured in the same medium for development. When any cloned embryos developed to the 2-cell stage, they were transferred into

pseudopregnant ICR female mice at 0.5 days post copulation (dpc) and the live offspring were collected by caesarean section at 19.5 dpc. The body and placental weights were measured immediately and the placentas were fixed for histology.

After commencing this long-term study, we reported several new nuclear transfer methods, such as different types of histone deacetylation inhibitor (HDACi) treatments instead of TSA (Ono et al., 2010; Van Thuan et al., 2009) or latrunculine A treatment instead of cytochalasin B (Terashita et al., 2012). However, to clarify the effect of recloning, we used the above method as much as possible. For example, donor recloned mice were always used at 3 months of age, and cumulus cells were always used as donor cells. TSA was used at the same concentration (50 nM) and for the same duration (10 h) throughout this experiment. To minimize variations in technical skill, S.W. and T.W. collaborated for all recloning experiments. Several persons also joined this study when their skill level was judged adequate. On the other hand, the oocyte activation medium was changed during this period. At first we used Ca-free CZB medium for this, but the medium was replaced with EGTA-KSOM, because we confirmed that this difference did not affect the cloning success rate, and it simplified the oocyte activation protocol (Kishigami and Wakayama, 2007).

Generation of Natural Control Mice by Intracytoplasmic Sperm Injection

To generate genetically identical normal control (NC) mice, oocytes were collected from BDF1 females and spermatozoa were collected from 129/Sv males. Therefore, the offspring were BD129F1, the same as the original donor strain. To match the conditions of cloned embryos, NC mice were generated by ICSI, which gave a similar in vitro manipulation stress and 1 day of culture in a CO_2 incubator. ICSI was performed as described (Kimura and Yanagimachi, 1995).

Statistical Analysis

Outcomes were evaluated using χ^2 tests and $p < 0.05$ was regarded as statistically significant. Coefficient of regression was also used between generation and body or placenta weight.

Genotyping Recloned Mice

The microsatellite markers D1Mit26, D3Mit18 and D3Mit21 were amplified using primer pair sequences obtained from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/). DNA was extracted from the spleens and tail tips of the cloned and control mice. Thirty cycles of polymerase chain reaction amplification were performed and the products were separated on 3% agarose gel before visualization.

Histology

Placentas were fixed with 4% paraformaldehyde immediately after caesarian section. Hematoxylin and eosin staining or in situ hybridization were performed as described (Lescisin et al., 1988; Tanaka et al., 2001). The plasmid clones for making the RNA probes were generously provided by Dr Janet Rossant (*Tpbpa*) and Dr Satoshi Tanaka.

Telomere Length

DNA was collected from the tail tips of G14–G21 mice, all on the same day. Genomic DNA (3 μg) was digested with RsaI and HinfI and separated electrophoretically on 0.8% agarose gels using a CHEF DR-II pulsed-field apparatus (Bio-Rad Laboratories, Hercules, CA). Southern blotting was performed with the Telo TAGGG Telomere Length Assay (Roche Applied Science, IN), according to the manufacturer's manual.

DNA Microarrays

Brain and liver tissues were collected from G20, cloned control (CC) and normal control (NC) mice at the time of caesarean section, frozen immediately in liquid nitrogen and stored at –80 °C until analysis. The analysis was performed using an Agilent G4122F DNA microarray as described (Kohda et al., 2012). To extract transcripts that commonly exhibit more than twofold upregulation in the cloned pups, the fold change in each signal in the individuals within one group (G20 or CC) was calculated and compared with the mean signal in the control and the mean difference was then assessed with Tukey's test ($p < 0.05$) using R. Hierarchical

clustering was done using cluster and visualized using TreeView software (http://en.bio-soft.net/tree/TreeView.html).

Supplemental References

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