Supporting Information

Matoba et al. 10.1073/pnas.1112664108

SI Text

We also sought to explore the possibilities of further improvements to cloning efficiency in mice. Our recent cloning experiments using *Xist*-knockout donors revealed the presence of discrete groups of X-linked genes that showed *Xist*-independent down-regulation in cloned embryos (1). These were the *Xlr* and *Magea* family genes located at positions showing enhanced dimethylation of histone H3 at lysine 9 (H3K9me2), a repressive histone modification needed for the formation of constitutive heterochromatin in somatic cells (2). These genes were inactivated in cloned embryos and could not be rescued by *Xist* knockout (1). Therefore, in the last series of experiments, we examined whether RNAi of the genes coding enzymes responsible for the establishment of H3K9me2 could ameliorate the repressive state of the *Xlr* and *Magea* genes in cloned embryos. Gene expression analysis by DNA microarray using single cloned blastocysts revealed that, although the genes for *G9a* (*Ehmt2*) and *Glp* (*Ehmt1*), the primary H3K9 methyltransferases (3), were successfully down-regulated by specific siRNA injection, the *Xlr* and *Magea* family genes remained fully repressed (Fig. S3). Embryo transfer experiments also revealed that the same siRNA injection had no significant effect on the development of clones (Table S3). We next injected mRNA for *Jhdm2a*, an H3K9-specific demethylase (4), to promote demethylation of H3K9me2, but this resulted in a remarkable decrease in the developmental ability of cloned embryos within 72 h. These findings indicate that, unlike the X chromosome inactivation induced by ectopic *Xist* RNA, the somatic cell-specific gene silencing by H3K9me2 might not be reversed easily by siRNA or mRNA injections, possibly because of the presence of mechanisms that maintain the repressive chromatin state redundantly.

1. Inoue K, et al. (2010) Impeding *Xist* expression from the active X chromosome improves mouse somatic cell nuclear transfer. *Science* 330:496–499.
2. Wen B, Wu H, Shinkai Y, Irizarry RA, Feinberg AP (2009) Large histone H3 lysine 9 dimethylated chromatin blocks distinguish differentiated from embryonic stem cells. *Nat Genet* 41:246–250.
3. Shinkai Y, Tachibana M (2011) H3K9 methyltransferase G9a and the related molecule GLP. *Genes Dev* 25:781–788.
4. Yamane K, et al. (2006) JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell* 125:483–495.

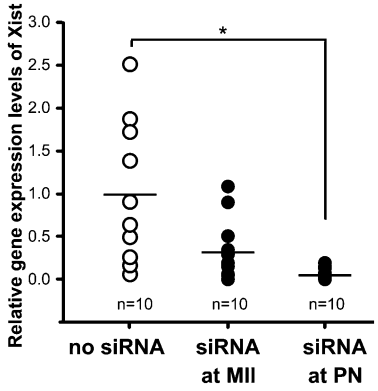

Fig. S1. Examination of the effect of a specific siRNA on *Xist* RNA level in parthenogenetically activated embryos following siRNA injection at different timings. An siRNA construct (*Methods*) was injected into oocytes before or after parthenogenetic activation, and the expression levels of *Xist* RNA were determined by quantitative RT-PCR at 96 h after activation. The horizontal lines in each group indicate mean values. *Xist* RNA level was effectively decreased when injected at 6 h postactivation (pronuclear stage; PN) but not before activation (metaphase II; MII) (*$P < 0.05$ by Student's *t* test).

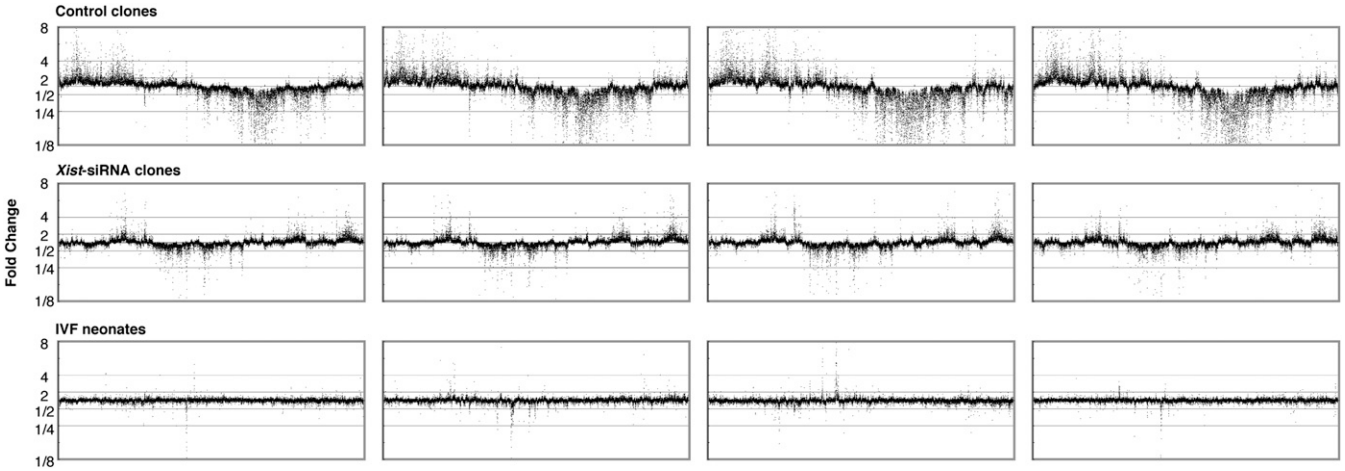

Fig. S2. Gene expression profiles from the liver of Sertoli cell-derived cloned mice and in vitro fertilization (IVF)-derived mice at birth. Each panel represents data from one cloned or IVF individual.

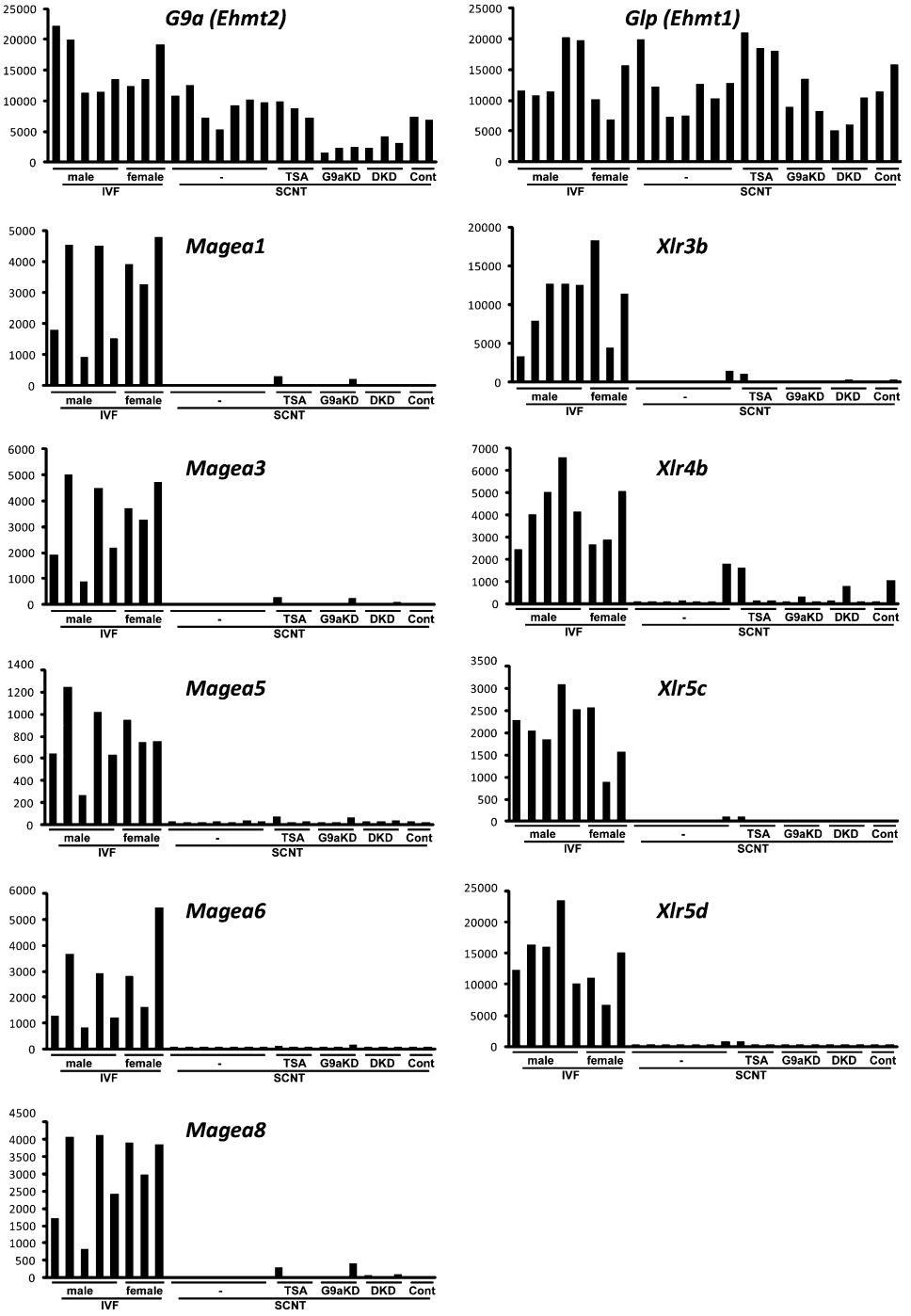

Fig. S3. Expression levels of *Xlr* and *Magea* genes in IVF-generated, untreated cumulus cell-derived cloned (–), trichostatin A (TSA)-treated cumulus cell-derived cloned, and siRNA-injected cumulus cell-derived cloned blastocysts. For the siRNA-injected groups, siRNA against *G9a* alone (G9aKD), a combination of two siRNA constructs against *G9a* and *Glp* (DKD), or negative-control siRNA (Cont) was injected. As expected, *G9a* was repressed in both G9aKD and DKD embryos ($n = 3$), and *Glp* was repressed in DKD embryos ($n = 3$). However, the expression levels of the *Xlr* and *Magea* genes examined remained basal. SCNT, somatic cell nuclear transfer.

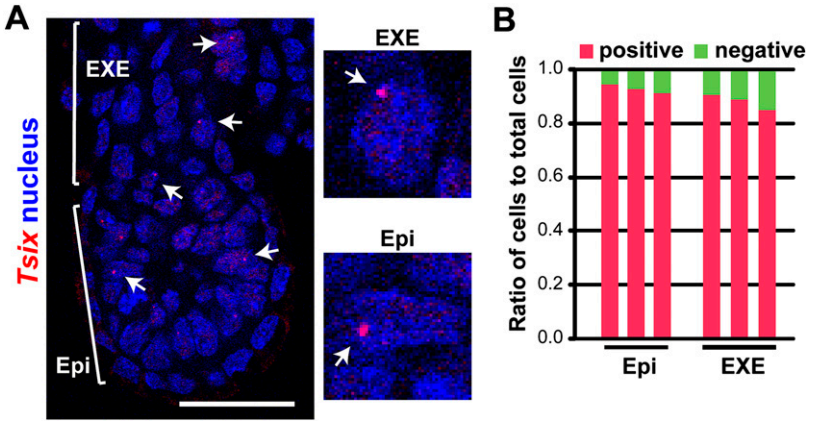

Fig. S4. Expression pattern of *Tsix* in *Xist*-siRNA–injected cloned embryos at embryonic day (E)5.5. (*A*) RNA-fluorescence in situ hybridization (FISH) analysis of *Tsix* (red). Most cells showed a positive signal for *Tsix* as a single spot (arrows) in both extraembryonic ectoderm (EXE) and epiblast (Epi) regions. (Scale bar, 50 μm.) (*Right*) High-magnification images of representative *Tsix*-positive cells in EXE or Epi. (*B*) Ratios of *Tsix*-positive cells in the Epi or Exe region analyzed by RNA FISH. Each column represents a single embryo. *Tsix*-positive cells occupied more than 80% of the cells in both regions.

Table S1. In vitro and in vivo development of siRNA-injected embryos

Embryo*	siRNA	TSA (nM)	No. cultured	No. two-cell embryos (% of cultured)	No. blastocysts (% of cultured)	No. blastocysts (% of cleaved)	Embryos transferred at two-cell stage (ET)	Implanted (% of ET) At E5.5	Implanted (% of ET) At term	Embryos recovered at E5.5 (% of ET) Total	Embryos recovered at E5.5 (% of ET) Normal	Term pups (% of ET)	
Sertoli clone													
	Control	0	116	84 (72)	44 (38)	(52)							
	Xist	0	103	80 (78)	53 (52)	(66)							
	Control	0	166	120 (72)			102	59 (58)		20 (20)	1 (1)		
	Xist	0	79	57 (72)			57	32 (56)		12 (21)	9 (16)		
	Control	0	107	87 (81)			87		34 (39)			1 (1)	
	Xist	0	125	89 (71)			89		47 (53)			11 (12)	
	Control	5	172	138 (80)			138		32 (23)			1 (1)	
	Xist	5	193	146 (76)			146		84 (58)			20 (14)	
	Control	50	84	68 (81)			68		29 (43)			4 (6)	
	Xist	50	85	69 (81)			69		43 (62)			14 (20)	
IVF													Male:Female
	Control	0	61	58 (95)			43		31 (72)			25 (58)	13:12
	Xist	0	105	100 (95)			66		43 (65)			29 (44)	14:15

Results from statistical analyses are indicated in the main text or corresponding figures.
*Each result reflects the sum of different experiments performed by two or three operators.

Table S2. List of genes with enhancement of their expression levels by *Xist*-siRNA injection in cloned embryos

Gene symbol	Description	Normalized value of expression level*			Fold change (*Xist* siRNA vs. control siRNA)	Transcript ID RefSeq/Ensembl
		IVF male	Control siRNA	*Xist* siRNA		
Fras1	Fraser syndrome 1 homolog	3.133	−5.609	−0.489	34.758	NM_175473
Zhx1	Zinc fingers and homeoboxes 1	−0.003	−2.619	1.194	14.052	NM_001042438
Car2	Carbonic anhydrase 2	2.470	−3.679	−0.014	12.685	NM_009801
Slc4a4	BI100969 602886295F1 NCI_CGAP_Kid14 cDNA clone	1.985	−3.606	−0.333	9.668	BI100969
Ccdc120	Coiled-coil domain containing 120	1.432	−3.249	−0.314	7.648	NM_207202
Pdk3	Pyruvate dehydrogenase kinase, isoenzyme 3	2.154	−2.844	0.076	7.565	NM_145630
Raver2	Ribonucleoprotein, PTB-binding 2	0.790	−2.120	0.628	6.718	NM_183024
Bach2	BTB and CNC homology 2	2.035	−2.329	0.132	5.505	NM_001109661
Tet3	Tet oncogene family member 3	0.873	−2.178	0.029	4.616	NM_183138
Ly6g6d†	Lymphocyte antigen 6 complex, locus G6D	0.343	−1.529	0.204	4.259	NM_033478
Sestd1†	SEC14 and spectrin domain 1	1.071	−1.620	0.416	4.100	NM_175465
Tyw3	tRNA-yW-synthesizing protein 3 homolog	1.121	−1.777	0.190	3.909	NM_172474
Igdcc3	RIKEN full-length cDNA 2810401C09	0.329	−1.499	0.358	3.621	AK012957
Tmem80	Transmembrane protein 80	0.890	−1.699	−0.001	3.246	NM_027797
Pabpn1	Poly(A)-binding protein, nuclear 1 gene	0.961	−1.626	−0.031	3.020	ENSMUST00000133397
Mtvr2	Mammary tumor virus receptor 2	1.003	−1.341	0.242	2.997	NM_181452
B4galt4	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4	1.207	−1.254	0.171	2.685	NM_019804
Ahnak	AHNAK nucleoprotein (desmoyokin)	1.085	−1.135	0.184	2.495	NM_009643
Ikbkb	Inhibitor of kappaB kinase beta	0.623	−1.244	−0.069	2.258	NM_010546
Tia1	Cytotoxic granule-associated RNA-binding protein 1	0.687	−0.852	0.216	2.096	NM_011585
Pacs2	Phosphofurin acidic cluster sorting protein 2	0.623	−1.042	−0.134	1.877	NM_001081170
Ccni	Cyclin I	0.351	−0.676	0.057	1.663	NM_017367
Gatad1	GATA zinc finger domain containing 1	0.370	−0.549	0.083	1.549	NM_026033
Nid1	Nidogen 1	0.055	−0.364	0.208	1.487	NM_010917
Rbbp9	Retinoblastoma-binding protein 9	0.401	−0.394	0.083	1.392	NM_015754
Trabd	TraB domain containing	0.434	−0.296	0.095	1.311	NM_026485
Ift52	Intraflagellar transport 52 homolog (*Chlamydomonas*)	0.268	−0.289	0.070	1.283	NM_172150

*One-way ANOVA with Tukey's post hoc test was applied to the microarray data from IVF (n = 4), *Xist*-siRNA–treated (n = 7), and control siRNA-treated embryos (n = 5). The list consists of genes with a significant ($P < 0.05$) level of down-regulation in control siRNA embryos compared with IVF-generated and *Xist*-siRNA–treated embryos but without a significant difference between the two latter groups.
†Mean value of two probes.

Table S3. In vitro and in vivo development of cloned embryos injected with siRNAs against histone methyltransferases responsible for H3K9 dimethylation

siRNA (μM)	No. cultured	No. cleaved (% of cultured)	No. four-cell embryos		Embryos transferred at four-cell stage(ET)	Implanted (% of ET)	Term pups (% of ET)
			(% of cultured)	(% of cleaved)			
Control (10)	77	59 (76)	30 (38)	(49)	30	0 (0)	0 (0)
G9a (5)	77	46 (60)	31 (40)	(67)	31	9 (45)	1 (3)
Glp and G9a (5, 5)	184	128 (70)	92 (50)	(72)	80	29 (36)	1 (1)