

**Stem Cell Reports, Volume 14**

**Supplemental Information**

**Compensation of Disabled Organogeneses in Genetically Modified Pig  
Fetuses by Blastocyst Complementation**

**Hitomi Matsunari, Masahito Watanabe, Koki Hasegawa, Ayuko Uchikura, Kazuaki Nakano, Kazuhiro Umeyama, Hideki Masaki, Sanae Hamanaka, Tomoyuki Yamaguchi, Masaki Nagaya, Ryuichi Nishinakamura, Hiromitsu Nakauchi, and Hiroshi Nagashima**

## Supplemental Information

### Compensation of Disabled Organogeneses in Genetically Modified Pig Fetuses by Blastocyst Complementation

Hitomi Matsunari, Masahito Watanabe, Koki Hasegawa, Ayuko Uchikura, Kazuaki Nakano, Kazuhiro Umeyama, Hideki Masaki, Sanae Hamanaka, Tomoyuki Yamaguchi, Masaki Nagaya, Ryuichi Nishinakamura, Hiromitsu Nakauchi, Hiroshi Nagashima

#### Inventory of supplemental information:

Figure S1. Mutation of the *PDX1* gene in cloned pig produced by somatic cell cloning, Related to Figure 1.

Figure S2. Mutation of the *KDR* gene in cloned pig produced by somatic cell cloning, Related to Figure 2 and 3.

Figure S3. Mutation of the *SALL1* gene in pig cloned by somatic cell nuclear transfer, Related to Figure 4.

Figure S4. Morphological and histological features of the *SALL1*-KO cloned fetuses and its compensation by blastocyst complementation, Related to Figure 4.

Figure S5. Mutation of the *HHEX* gene in cloned pig produced by somatic cell cloning, Related to Figure 5.

Table S1. Production performance of chimeric individuals by blastocyst complementation for the organogenesis-disabled traits in pigs, Related to Figure 1-5.

Table S2. Primer sequences for mutation analysis and detection of chimeras, Related to Figure 1-5.



**B**

**Wild-type** TCCCCGTACGAGGTGCCCCCGCTCGCCGACGACCCGGCGGTGGCGCACCTCCA

**Clone #45**  
 (61 bp del) TCCCCGTACGAGGTGCCCCCGCTCGCCGACGAC\*\*\*\*\*  
 \*\*\*\*\*CCGGGCCCTTCC  
 CGGATGGAGCCGAGCCAGGAGCCCTGGAGGAGCCCGGCCGCTCCAGCCGCCT

ggagtcaacgtcctgcagctgacagactccagcgaggtcccgg\*\*\*\*\*  
 \*\*\*\*\*

(1,224 bp del + 6 bp del) \*\*\*\*\* (1224 bp del) \*\*\*\*\*  
 \*\*\*\*\*  
 \*\*\*\*\*CGCACCTCCA  
 CCACCACCTCCCGGCTCAGCTGGCACTCCCGC\*\*\*\*\*CCGGGCCCTTCC  
 CGGATGGAGCCGAGCCAGGAGCCCTGGAGGAGCCCGGCCGCTCCAGCCGCCT

**Clone #114**  
 (4 bp del) TCCCCGTACGAGGTGCCCCCGC\*\*\*CGACGACCCGGCGGTGGCGCACCTCCA

TCCCCGTACGAGGTGCCCCCGCTCGCCGATGGTTAACGAATCCGACTAGGAAC  
**CATGAGGTTGCAGGTTTCGATCCCTGGCCTTGCTCCGTGGGTAAAGGATCTGGC**  
 (195 bp ins) **GTTGCCGTGAGCTGTGGTGTAGGTTGCAGATGCAGCTCAGATCCCACGTTGCT**  
**CGGTGTAGGCAGGTGGCTACAGCTCCCATTAGACCCCTAGCTGGAAACCTCC**  
**ATATGCCCCCGACGACCCGGCGGTGGCGCACCTCCA**

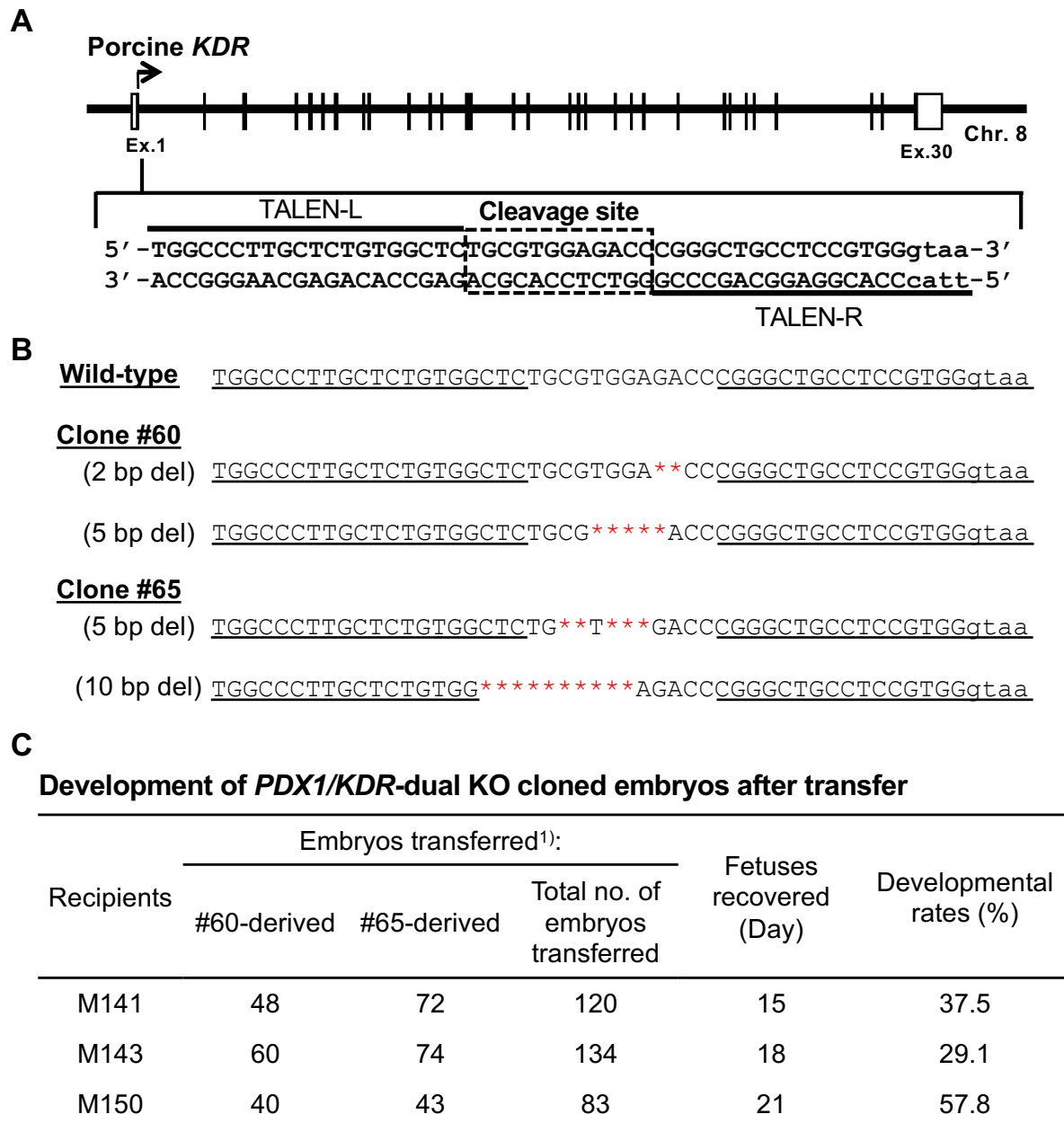
**C**

**Development of the cloned embryos with *PDX1*-KO trait**

Recipient No.	Cloned embryos transferred:			Fetuses obtained:		
	Total	#45-derived <sup>1)</sup>	#114-derived <sup>1)</sup>	Total	#45-derived	#114-derived
M70	101	51	50	4 (4.0%)	3	1

1) Nuclear donor cell with 61 bp / 1,230 bp deletion mutations (#45) and 4 bp deletion / 195 bp insertion (#114) were used to generate the cloned embryos.

**Figure S1. Mutation of the *PDX1* gene in cloned pig produced by somatic cell cloning**  
 A: Cleavage site of *PDX1* gene in the nuclear donor cells used for producing cloned pigs. Recognition sites of *PDX1*-targeted TALENs are indicated by lines. B: The uppercase sequences represent the coding region of porcine *PDX1* gene. The deletion and insertion mutations are indicated by asterisks and bold letters, respectively. The *PDX1* allele of cell clone #45 with 1224 bp deletion has another 6 bp deletion at the outer side of TALEN binding sites. C: Production efficiency of the *PDX1*-KO cloned fetuses.



1) Embryos were generated by somatic cell cloning from two lines (#60, #65) of the *PDX1/KDR*-dual KO fetal fibroblasts.

**Figure S2. Mutation of the *KDR* gene in cloned pig produced by somatic cell cloning**

A: Cleavage site of *KDR* gene in the nuclear donor cells for producing cloned pigs. Recognition sites of *KDR*-targeted TALENs are indicated by lines. B: The uppercase sequences represent the coding region of porcine *KDR* gene. The deletion mutations are indicated by asterisks. C: Production efficiency of *PDX1/KDR*-dual KO cloned fetuses.



**B Wild-type** CAGTCCCTCTCCCCATCATCCCGATGAACAAATGAATGAC

**Clone #81**

(4 bp del) CAGTCCCTCTCCCCATCATC\*\*\*\*TGAACAAATGAATGAC

(521 bp del) CAGTCCCTCTCC\*\*\*\*\*  
 \*\*\*\*\*  
 \*\*\*\*\* (521 bp del) \*\*\*\*\*  
 \*\*\*\*\*  
 \*\*\*\*\*ACTTGCCAGCATCTTCTAGTCCTTCTCAAGG

**Clone #91**

(5 bp del + 183 bp del) CAGTCCCTCTCCCC**AAAT**\*\*\*\*TGAACAAAT**TTTCAAT**  
**TTGT**\*\*\*\*\*  
 \*\*\*\*\* (183 bp del) \*\*\*\*\*  
 \*\*\*\*\*  
 \*\*\*\*\*GCAGCAGCAGCTCCTGCACAGGTACCTCAGCGATCA

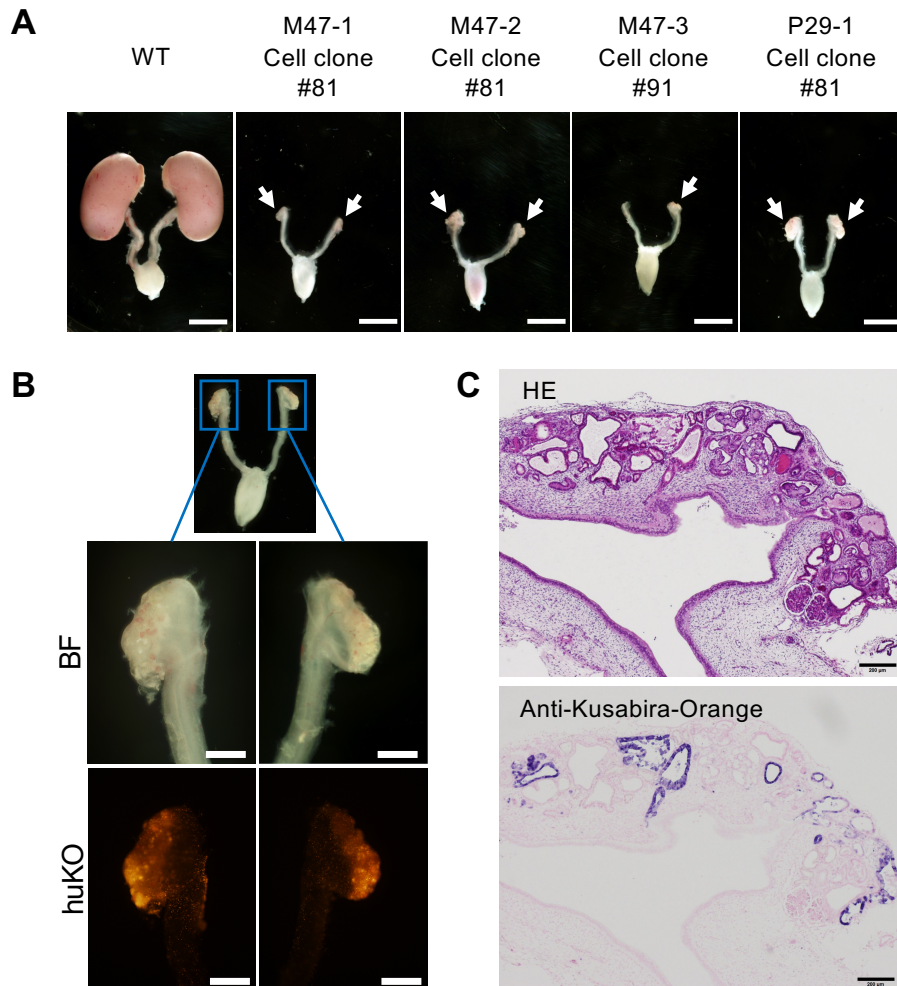
**C. Development of the cloned embryos with SALL1-KO trait**

Recipient No.	Cloned embryos transferred:			Anephrogenic fetuses obtained:			
	Total	#81-derived <sup>1)</sup>	#91-derived <sup>2)</sup>	Total	#81-derived	#91-derived	Age
M35	109	0	109	1 (0.9%) <sup>3)</sup>	0	1	day-66
M36	108	0	108	0	0	0	miscarried <sup>4)</sup>
M47	161	81	80	3 (1.9%) <sup>5)</sup>	3	0	day-43
P29	161	81	80	1 (0.6%) <sup>5)</sup>	1	0	day-45

- 1) Nuclear donor cells with 4 bp / 521 bp deletion mutations (#81) were used to generate the cloned embryos.
- 2) Nuclear donor cells with homozygotic 188 bp deletion mutation (#91) were used to generate the cloned embryos.
- 3) Degenerating fetus
- 4) day-44 of gestation
- 5) Another five (M47) and two (P29) fetuses were obtained, but nephrogenesis in these fetuses could not be analyzed due to severe retardation of the development.

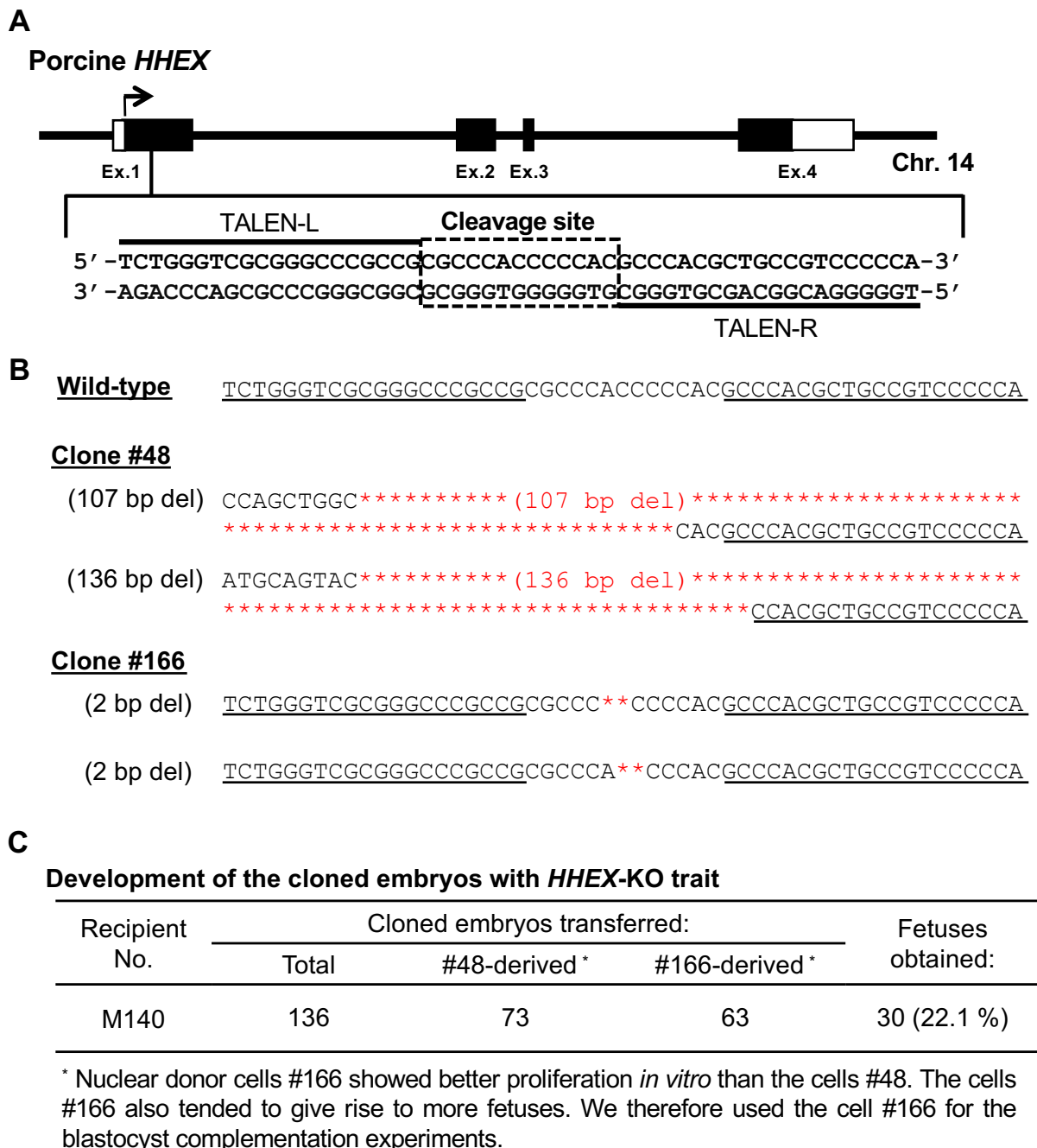
**Figure S3. Mutation of the SALL1 gene in pig cloned by somatic cell nuclear transfer**

A: Cleavage site of the SALL1 gene in the nuclear donor cells for producing cloned pigs. Recognition sites of SALL1-targeted ZFNs are indicated by lines. B: The uppercase sequences represent the coding region of porcine SALL1 gene. The deletion mutations are indicated by asterisks. The 13 bp (2 bp + 11 bp) substitution mutations occurred in clone #91 are indicated in bold letters. C: Production efficiency of the SALL1-KO cloned fetuses.



**Figure S4. Morphological and histological features of the *SALL1*-KO cloned fetuses and its compensation by blastocyst complementation**

A: Typical features of the aneprogenic phenotype in the *SALL1*<sup>-/-</sup> fetuses. Note that features of the vestigial kidney with the same mutation (#81) vary among the fetuses. Scale bars, 5 mm. B: A case of incomplete compensation of the *SALL1*<sup>-/-</sup> aneprogenic trait after blastocyst complementation with exogenous cells expressing huKO. Note that distribution of huKO expressing cells are limited in both ureter and hypoplastic kidney tissue, indicating insufficient level of chimerism of this fetus. Scale bars, 1 mm. C: An HE section of a hypoplastic kidney (upper) and immunostained section showing limited distribution of the huKO positive cells. Scale bars, 200  $\mu$ m.



**Figure S5. Mutation of the *HHEX* gene in cloned pig produced by somatic cell cloning**

A: Cleavage site of the *HHEX* gene in the nuclear donor cells for producing cloned pigs. Recognition sites of *HHEX*-targeted TALENs are indicated by lines. B: The uppercase sequences represent the coding region of porcine *HHEX* gene. The deletion mutations are indicated by asterisks. Both lines of the mutant cells (#48, #166) were used as nuclear donors for somatic cell cloning. C: Production efficiency of *HHEX*-KO cloned fetuses. The *HHEX*<sup>-/-</sup> fetuses exhibited a hepatogenic phenotype with varying degree of anterior abnormalities. No significant phenotypic difference was seen in the mutant fetuses derived from the two cell lines.

**Table S1. Production performance of chimeric individuals by blastocyst complementation for the organogenesis-disabled traits in pigs**

Type of host embryos	Recipient pigs	Embryos transferred	Type of fetuses obtained (%)			Age of fetuses examined (day)
			Chimera	Host embryo-derived	Donor embryo-derived	
<i>PDX1</i> -KO <sup>1</sup>	M101	66	0 (0)	0 (0)	0 (0) <sup>3</sup>	— <sup>4</sup>
	M102	65	3 (4.6) <sup>5</sup>	4 (6.2)	0 (0) <sup>3</sup>	107
	M109	97 <sup>2</sup>	1 (1.0)	2 (2.2)	0 (0) <sup>3</sup>	114
	M110	97 <sup>2</sup>	0 (0)	1 (1.0)	0 (0) <sup>3</sup>	114
<i>PDX1/KDR</i> -dual KO <sup>6</sup>	M203	38	9 (33.3) <sup>7</sup>	13 (48.1)	5 (18.5) <sup>8</sup>	21
<i>SALL1</i> -KO <sup>9,10</sup>	M82	82	1 (1.2) <sup>11</sup>	0 (0) <sup>9,10</sup>	1 (1.2)	44
	M84	81	0 (0)	1 (1.2) <sup>9,10</sup>	0 (0)	47
	M105	64	0 (0)	0 (0) <sup>10</sup>	0 (0)	— <sup>4</sup>
	M120	64	1 (1.6) <sup>11</sup>	1 (1.6) <sup>10</sup>	2 (3.1)	40
	M223	48	0 (0)	3 (6.3) <sup>10</sup>	2 (4.2) <sup>13</sup>	41
	M224	49	1 (2.0) <sup>12</sup>	5 (10.2) <sup>10</sup>	1 (2.0) <sup>13</sup>	43
<i>HHEX</i> -KO <sup>14</sup>	M207	37	4 (26.7) <sup>15</sup>	9 (60.0)	2 (13.3) <sup>16</sup>	23
	1703	50	2 (33.3) <sup>15</sup>	0 (0)	4 (66.7) <sup>16</sup>	102
	1717	45	1 (100) <sup>15</sup>	0 (0)	0 (0) <sup>16</sup>	101

<sup>1</sup> Host embryos were prepared by somatic cell cloning from two lines (#45, #114; Fig. S1) of *PDX1*-KO fetal fibroblasts.

<sup>2</sup> Nuclear donor cells #45 were used to prepare the host embryos.

<sup>3</sup> Morula blastomeres collected from the cloned embryos expressing huKO or Plum were used for complementation of the host *PDX1*-KO embryos.

<sup>4</sup> The recipient was not impregnated.

<sup>5</sup> Two #45-derived and one #114-derived.

<sup>6</sup> Host embryos were prepared by somatic cell cloning of *PDX1/KDR*-dual KO fetal fibroblasts (#65; Fig. S2).

<sup>7</sup> Chimerism was determined by analysis of genomic DNA of the fetal tissue samples. Samples indicating both the sequence of the mutation site and huKO signal were determined as chimeric.

<sup>8</sup> Morula blastomeres collected from the *in-vivo*-derived embryos expressing huKO were used for complementation of the host *PDX1/KDR*-KO embryos.

<sup>9</sup> Nuclear donor cells with 4 bp / 521 bp deletion mutations (#81, Fig. S3) were used to generate the cloned host embryos.

<sup>10</sup> Nuclear donor cells with homozygous 188 bp deletion mutation (#91, Fig. S3) were used to generate the cloned host embryos.

<sup>11</sup> Retained the anephrogenic phenotype. Limited contribution of the exogenous huKO expressing cells were observed in the hypoplastic kidney tissue.

<sup>12</sup> Restored kidneys were developed from exogenous huKO expressing cells.

<sup>13</sup> Fetuses derived from the donor embryos. *In-vivo*-derived or *in vitro* fertilization (IVF) derived embryos expressing huKO transgene were used as donor embryos.

<sup>14</sup> Host embryos were prepared by somatic cell cloning of *HHEX*-KO fetal fibroblasts (#166; Fig. S5).

<sup>15</sup> Chimerism was determined by analysis of genomic DNA of the fetal tissue samples. Samples indicating both the mutant and WT sequences of the *HHEX*-KO site were determined as chimeric.

<sup>16</sup> Morula blastomeres collected from the *in-vivo*-derived embryos expressing huKO were used for complementation of the host *HHEX*-KO embryos.



**Table S2. Primer sequences for mutation analysis and detection of chimeras**

Mutation analysis			
Target gene	1st PCR primers	Nested PCR primers	Sequencing primer
<i>PDX1</i>	5'-ACTGCCAACTGCACATGCAG	5'-TGCGGAGCTGTCAAAGCAGCAGG	5'-CACGCAGCTCTACAAGGACC
	5'-AGCATCCCAGAGCCCTACTCG	5'-TGCTTTAGTCCGACTCTGC	
<i>KDR</i>	5'-CAGTGTTCAGCATCTCG	5'-TCAGAACCCCTGATGGACTAC	5'-TCGGACACTTGGGATAACTTC
	5'-TCAGGAGACCACCAACTCGC	5'-ATCTTGGGCCAGGCATTGAGC	
<i>HHEX</i>	5'-AGGCAGCTGCTTGCCTCAGC	5'-GACTTCTTTCGAGGGCGTCAC	5'-TCCCGAGGGCGGGGAGCTCAG
	5'-CGACGCCTGCAAGGTGCAGG	5'-AGTCACAGTGCGGTCACTCTG	
<i>SALL1</i>	5'-ATTAGGCACCAATGTCGGCAG	5'-TGATAGGAAGTCCTCCAACAG	5'-TCGAAGTCACAGGTGGCTCC
	5'-TGCAGAGCTAAGAGCTGCTCC	5'-TCTCGATGATGACGTTGCTG	
Detection of chimeras			
Target gene	1st PCR primers	Nested PCR primers	
<i>PDX1</i>	5'-TCTGCAGAGACGTCCTGATTC	5'-TCAATCCGTACACAGACGACG	
	5'-AGTGCAGGTGTGACGAGAAGTG	5'-AGTGAATTAGCATCGCAGACG	
<i>KDR*</i>	5'-CTTGCTCTGTGGCTCTGT	5'-TGCTCTGTGGCTCTGTGA	
	5'-ATCTTGGGCCAGGCATTGAGC	5'-CTATGATTCCGAGTTAGGTCC	
<i>SALL1</i>	5'-ATTAGGCACCAATGTCGGCAG	5'-TGATAGGAAGTCCTCCAACAG	
	5'-TGCAGAGCTAAGAGCTGCTCC	5'-TCTCGATGATGACGTTGCTG	
<i>huKO</i>	5'-TGACCCTGAGGGTGACAATG	5'-ACGTGACCATGTTCTGAAGCTGG	
	5'-TCTGTTCCCTAACCTTGATCTGAAC	5'-AGCTAGCTTGCCAAACCTACAGG	
<i>Plum</i>	5'-CTACAAGACCGACATCAAGCTG	5'-ACGAGGACTACACCATCGTGG	
	5'-ACAGCTATGACTGGGAGTAGTCAGG	5'-TGTTTCATGGCAGCCAGCATATGG	

\* Primers specific to *KDR*-KO mutant allele in clone 65 (5 bp deletion) were used.