

## Short Communication

# Post-mortem re-cloning of a transgenic red fluorescent protein dog

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Recently, the world's first transgenic dogs were produced by somatic cell nuclear transfer. However, cellular senescence is a major limiting factor for producing more advanced transgenic dogs. To overcome this obstacle, we rejuvenated transgenic cells using a re-cloning technique. Fibroblasts from post-mortem red fluorescent protein (RFP) dog were reconstructed with *in vivo* matured oocytes and transferred into 10 surrogate dogs. One puppy was produced and confirmed as a re-cloned dog. Although the puppy was lost during birth, we successfully established a rejuvenated fibroblast cell line from this animal. The cell line was found to stably express RFP and is ready for additional genetic modification.

**Keywords:** re-cloned dog, RFP dog, serial cloning, somatic cell nuclear transfer, transgenic dog

Somatic cell nuclear transfer (SCNT) is currently the only technique suitable for producing transgenic dogs because the use of technologies appropriate for other species, such as germline-transmissible embryonic stem cells and *in vitro* embryo culture, have not yet been established in dogs. However, SCNT is associated with a number of limitations. To establish transgenic donor cells for SCNT, transfection and selection procedures must be performed [14]. During this process, the donor cells can easily become senescent and the number of transgenic cells that can be used for SCNT are limited [4]. In particular, gene targeting by homologous recombination or multiple transfections that require two or more rounds of selection [3] is extremely hard to perform with primary cultured cells that are necessary for SCNT.

In 1998, Cibelli *et al.* [3] suggested an alternative strategy to overcome these obstacles by using a serial cloning

technique. In their study, they produced cloned fetuses derived from senescent bovine fibroblasts and then successfully isolated non-senescent fibroblasts from the fetuses. It had already been proven that non-senescent cells from cloned animals can be used to produce re-cloned offspring in several species including cattle [7], pigs [2], and cats [1]. Thus, the life-span of the cells can be theoretically elongated infinitely using this serial cloning technique. Furthermore, complex genetic modification could be performed as much as desired if the transgenes were successfully transferred to the re-cloned transgenic animals. Currently, the potential for serial cloning in dogs and the extent of transmission of the transgene from transgenic dogs to re-cloned dogs is unclear. Therefore, the present study was performed to produce re-cloned offspring from our red fluorescent protein (RFP) transgenic dog and to analyze expression of the RFP gene in the re-cloned dog. A re-cloned transgenic cell line for further serial cloning was also established.

In our previous report [6], four female and two male RFP dogs were successfully produced by SCNT. However, one of the male dogs was died due to chronic bronchopneumonia at 11 weeks after birth. To re-clone the deceased RFP dog (R6), we harvested fibroblasts 2 h after the death of the puppy and established a cloned transgenic cell line. The same SCNT and embryo transfer procedures described in our previous reports [6,8-10] were used for re-cloning in the present study. In total, 174 re-cloned embryos reconstructed with fibroblasts derived from R6 were transferred into the oviducts of 10 estrous-synchronized surrogate dogs. Two surrogates became pregnant but one experienced an abortion around 1 month of gestation. On Day 62 of gestation, the pregnant surrogate delivered one male puppy (rcR6). Unfortunately, the puppy was lost during birth.

To validate that rcR6 was a clone of R6, microsatellite and mitochondrial DNA sequencing analyses were performed [11]. As shown in Table 1, rcR6 was genetically identical to the cell donor, R6, while the mitochondrial DNA sequence was identical to the oocyte donor but different from that of

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**Table 1.** Microsatellite analysis of the re-cloned dog

	Marker*							
	PEZ 001	PEZ 005	PEZ 006	PEZ 008	PEZ 012	PEZ 020	FH 2010	FH 2079
R6	114/122	101/105	199/199	223/231	265/277	175/179	228/232	273/273
rcR6	114/122	101/105	199/199	223/231	265/277	175/179	228/232	273/273
Oocyte donor	114/126	97/109	175/183	231/235	269/277	175/183	232/236	269/273
Surrogate	118/118	101/101	175/183	223/243	261/293	175/175	232/236	269/269

\*The isolated genomic DNA samples were used for microsatellite assay with eight specific markers originally derived from dogs. R6: red fluorescent protein transgenic dog, rcR6: re-cloned dog derived from R6.

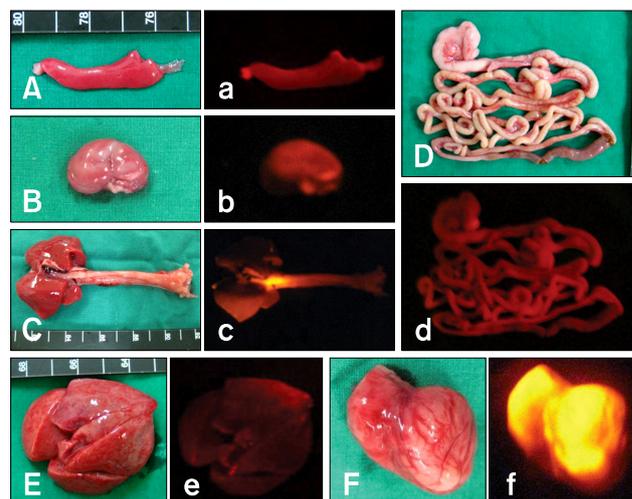
**Table 2.** Sequence alignments within 661 bases of the hypervariable mitochondrial DNA region

Sample	Nucleotide position																				
	15435	15483	15508	15526	15595	15611	15612	15627	15632	15439	15643	15650	15652	15710	15800	15814	15815	15912	15955	16003	16083
Reference*	G	C	C	C	C	T	T	A	C	T	A	T	G	C	T	C	T	C	C	A	A
R6	G	C	C	C	C	T	T	A	C	T	A	T	G	C	T	T	T	C	C	A	A
rcR6	G	T	C	C	C	T	T	G	C	A	A	T	G	C	T	T	T	T	C	A	A
Oocyte donor	G	T	C	C	C	T	T	G	C	A	A	T	G	C	T	T	T	T	C	A	A
Surrogate	A	C	C	T	T	T	C	A	T	G	G	T	A	C	C	T	C	T	T	G	G

\*The nucleotide positions are numbered according to those of GenBank accession no U96639 v.2; 661 bases (from 5431 to 16091) were examined.

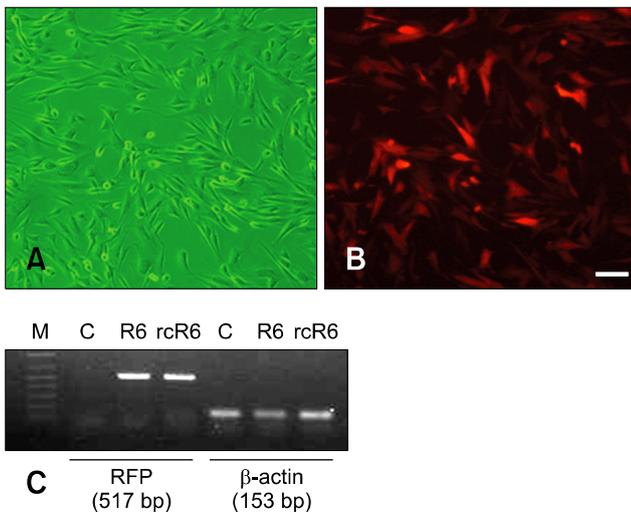
the R6 and surrogate dog (Table 2). These data revealed that rcR6 was a clone of R6. The expression of RFP in rcR6 was also evaluated. Similar to the RFP expression in R6 [6], rcR6 also expressed RFP in all of the examined organs (Fig. 1). Therefore, we concluded that the phenotypes of transgenic dogs can be inherited by the re-cloned offspring. To establish a re-cloned transgenic cell line, fibroblasts were harvested from rcR6 then cultured *in vitro*. The re-cloned fibroblasts grew robustly, were morphologically normal (Fig. 2A), and stably expressed RFP (Figs. 2B and C).

In the present study, we failed to obtain a viable re-cloned dog although the puppy almost developed to full-term and died during the delivery process. Additionally, the overall re-cloning efficiency in this report was inferior to that of a previous report on cloning R6 [6]. Thus, the canine re-cloning procedure developed in the present study still requires some improvement. Several previous reports have shown that cloning efficiency is decreased by re-cloning [5,13] especially if adult cells isolated from cloned animals are used as donor cells for SCNT [14] as was the case in the present study. However, this phenomenon can be overcome in mice by using histone deacetylase inhibitors such as trichostatin A to epigenetically reprogram donor



**Fig. 1.** Expression of red fluorescent protein (RFP) in the organs of the re-cloned dog. (A and a) spleen, (B and b) kidney, (C and c) trachea and lung, (D and d) stomach and intestine, (E and e) liver, (F and f) heart. (A~F) Visible light images. (a~f) Fluorescence images.

cells, or by using stem cells derived from cloned animals as donor cells for SCNT [12]. We also found similar



**Fig. 2.** Transgenic cell line established from the re-cloned dog. (A) Visible light image. (B) Fluorescence image. Scale bar = 100  $\mu$ m. (C) PCR analysis of the RFP gene. M: marker, C: wild-type, R6: RFP transgenic dog, rcR6: re-cloned dog derived from R6.

tendencies for both of these strategies during canine re-cloning (data not shown) and further studies are being conducted to confirm our observation.

In conclusion, we demonstrated the possibility of producing a re-cloned transgenic dog. The RFP transgene was inherited by the offspring and was highly expressed after re-cloning. A re-cloned transgenic cell line suitable for further serial cloning was also successfully established. This technology will be useful for producing dogs with multiple modified genes or gene targeting in canines.

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