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Monoallelic gene targeting in hypoblast stem cells reveals X chromosome inactivation

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ABSTRACT

We recently isolated hypoblast stem cells (HypoSC), which are related to embryonic stem (ES) cells. ES cells efficiently perform homologous recombination (HR) and lack X chromosome inactivation (Xi), but it is unknown whether the same applies to HypoSC. Using the X-linked hypoxanthine phosphoribosyl transferase (HPRT) gene, we find that HypoSC perform HR with similar frequency as ES cells. Monoallelic targeting in female HypoSC eliminated HPRT gene expression, implying epigenetic inactivation of the other allele. Although density-induced differentiation complicated selection, the targeted clones maintained their original properties. These results will facilitate targeted genetic manipulation of HypoSC and the study of Xi.

1. Introduction

Gene targeting through homologous recombination (HR) is the main method to introduce precisely planned changes into genomes [1]. In mammals, it has been used especially for the genetic manipulation of embryonic stem (ES) cells. In these cells, HR is reasonably frequent compared to random integration of transgenes, but HR tends to be less efficient in more differentiated cell types [1–3]. The higher background of randomly integrated transgenes, together with the lower proliferative capacity of somatic cells, make identification and isolation of targeted somatic cells more difficult, and therefore facilitative strategies, especially the introduction of double strand breaks, are used [1]. Thus, in unassisted form, HR has rarely been used for gene targeting in mammalian cells other than ES cells. However, highly proliferative cells other than ES cells may also show a reasonable frequency of HR. Rat spermatogonial stem cells (SSCs) showed a similar frequency as rat ES cells (comp. [4,5]). Rat mesenchymal stem cells, which are more representative of a somatic stem cell and might not have unlimited proliferative capacity, were also efficiently modified by unassisted HR [6]. Overall, however, reports of unassisted HR in non-ES cell types are rare.

We previously isolated a new stem cell type named XENP cells (extraembryonic endoderm precursor cells) and most recently, with a different protocol, stem cells named HypoSC (hypoblast stem cells) that so far have been indistinguishable from the XENP cells [7,8]. The XENP/HypoSC appear to represent the committed precursor of the yolk sac endoderm in the inner cell mass of preimplantation embryos, and as such they are the developmental siblings of the epiblast precursor, which in turn is represented by ES cells in vitro. Thus, XENP/HypoSC and ES cells can be seen as sister cell types (discussed in [7]). Like ES cells, XENP/HypoSC show lineage plasticity, enabling them to differentiate into somatic cell types in vitro [8]. Further, XENP/HypoSC exhibit a phenotype similar if not identical to that of Multipotent Adult Precursor Cells (MAPC) [8]. Thus, although XENP/HypoSC cannot transmit their DNA to the next generation, their precise genetic manipulation is attractive.

We therefore asked whether unassisted HR is feasible in XENP/HypoSC and if so, whether the frequency of HR is similar to the one previously described in ES cells. We also realized that when targeting an X-linked gene, we may simultaneously clarify whether XENP/HypoSC exhibit X chromosome inactivation (Xi). XENP/HypoSC may be expected to show Xi since their presumed in vivo equivalent, the extraembryonic endoderm precursor [7], exhibits Xi [9]. On the other hand, in view of their close developmental relationship with ES cells, which do not show Xi [10], and in view of the lineage plasticity and in vitro pluripotency of XENP/HypoSC resulting from this developmental context, it is equally conceivable that cultured XENP/HypoSC do not exhibit Xi.

Thus, we decided to examine HR in XENP/HypoSC with the X-linked HPRT gene, which has previously been targeted by unassisted HR in rat ES cells [4], allowing direct comparison if using

Abbreviations: HypoSC, hypoblast stem cells; HPRT, hypoxanthine phosphoribosyl transferase; XENP cells, extraembryonic endoderm precursor cells; Xi, X chromosome inactivation.

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the same construct. The HPRT gene has also previously been targeted in mouse [11,12] and human (e.g. [13]) ES cells, as well as in murine hematopoietic progenitor [14] and human fibrosarcoma [15] cells.

2. Materials and methods

2.1. Cell lines and cell culture

For this study, we used lines MX13 (strain F344, male), MX18 (strain WKY, female), and MX14 (BNxF344 hybrid, female). The cell lines were derived and cultured under "MAPC conditions" as previously described [8].

2.2. Gene targeting

The PGK/neo gene targeting construct HPRT 2.2 [4] was generously provided by Dr. T. Burdon (Roslin Institute, U. of Edinburgh, UK). The cells to be targeted were electroporated in a Gene Pulser™ (Bio-Rad, Hercules, CA, USA) set at 2.5 kV, 25 μFD, 200 Ω, 0.7 ms. 3–7 million cells were harvested by trypsinization and electroporated in 0.9 ml of Hepes-buffered saline with 10 μg of DNA (linearized with XhoI) per 1 million cells. Selections were performed with G418 followed by 6-thioguanine (6TG). The electroporated cells were plated into 10-cm plates ($5–6 \times 10^5$ cells per plate), and after 36 h, G418 was added at 150 μg/ml. 4–5 days later, the G418 was replaced by 6TG (5 μM), and after another 3 days, colonies were picked as follows: the medium was replaced with Mg/Ca-free PBS, replaced with the same again, and colonies were aspirated with a mouth-controlled glass capillary in one continuous act of suction, taking care to avoid escapees. The cells were then seeded into 4-well plates and expanded under continued 6TG selection. In total, the selection procedure took 8.5–10 days. After expansion, the identities of all targeted clones were confirmed using a panel of sex- and strain-specific primer pairs (Table S1, Fig. S1A).

2.3. Genomic PCR

Cells were lysed in general DNA lysis buffer and 2 μl of the lysate directly analyzed by PCR using DiaStar™ Taq polymerase (SolGent Co., Daejeon, South Korea) for cDNA templates and DiaStar™ EF-Taq polymerase (SolGent) for gDNA templates, at an annealing temperature of 60 °C, with the following primer pairs: HPRT 5′F-GGTAGTAACAAGTGGTGGAC and HPRT 5′R-CCACTTTCG CTGATGACAC for 5′ screening (wild type product size 3.5 kb, K/O product size 4.6 kb); HPRT 3′F-AGCACGTACTCGGATGGAAG and HPRT 3′R-CAAATGCAGGGAACGACACC for 3′ screening (wild type no product, K/O product size 5.6 kb).

2.4. RT-PCR

RNA was isolated by the RNAGEM™ (ZyGem Co., Hamilton, New Zealand) procedure. RT-PCR and real-time quantitative RT-PCR (qRT-PCR) were performed according to standard procedures [16]. Delta Ct values were calculated by subtracting the Ct value for PGK1 (phosphoglycerate kinase 1) from the Ct value of the gene of interest. See Table S1 for a list of primers.

2.5. Assessment of growth properties

To obtain growth curves, cells were plated at 1500 cells/dish of 12-well dishes and counted with a hemocytometer at the indicated time points. For the plating experiments, cells were washed one additional time with PBS to remove residual hormones, seeded at 500 cells/well of 24- or 4-well dishes, supplemented with the hormones as needed, and the resulting colonies were fixed and stained with Crystal Violet after 1 week.

3. Results and discussion

3.1. HypoSC efficiently perform unassisted homologous recombination at the HPRT locus

Using a previously tested targeting construct that contains a Neomycin resistance cassette [4], we electroporated three XENP/HypoSC lines that were derived from three different rat strains and both sexes. When starting with 3–7 million cells, 85–165 G418-resistant colonies resulted, 0.9–3% of which exhibited 6TG resistance (Table 1). These numbers are comparable with those previously obtained with rat ES cells [4].

3.2. The non-targeted HPRT allele in female HypoSC is silent

Each cell line yielded at least one 6TG-resistant colony, but targeting appeared to be somewhat more efficient in line MX13 (Table 1) that shared the strain background (F344) with the HPRT sequences of the targeting construct. On the other hand, that line was male, while the other two cell lines (WKY and BNxF344 background) tested were female, and conceivably the female clones showed higher 6TG sensitivity (because of potential HPRT expression from their non-targeted allele). Indeed, the 6TG-resistant female lines each contained one targeted and one non-targeted allele (Fig. 1A, B); however, their 6TG sensitivity was similar to that of targeted male clone K1 (Fig. 1C). Another male clone (K2) unexpectedly contained two HPRT alleles (presumably because of aneuploidy), one of which was targeted (Fig. 1B); this clone also showed the same 6TG sensitivity. In line with these results, no significant HPRT mRNA level was detected by qRT-PCR in the targeted clones, regardless of sex or ploidy (Fig. 1D), confirming that the non-targeted allele in the female cells and in the aneuploid male cells was not expressed. When we subjected one of the targeted cell lines (the female F344xBN cell line) to HAT selection, no clone was recovered out of 7 million cells seeded, showing that the non-targeted HPRT allele was robustly silenced. We also attempted to generate targeted clones through direct selection with 6TG (i.e., without G418 selection); in this way, we obtained 1 6TG-resistant clone (3 experiments with 3 million cells each) that, however, was negative in the PCR targeting assay.

3.3. Density-dependent differentiation requires a strict selection regimen

Compared to ES cells, gene targeting in XENP/HypoSC was more complicated because XENP/HypoSC colonies show a strong tendency for density-induced differentiation. When primary

Table 1
Efficiency of gene targeting.

Cell line	Strain	Sex	No. of cells electroporated	No. of G418^r colonies	No. of 6-TG^r colonies	Total targeting frequency
MX13	F344	Male	7.0×10^6	121	4	0.57×10^{-6}
MX18	WKY	Female	3.0×10^6	165	1	0.33×10^{-6}
MX14	BNxF344	Female	6.0×10^6	85	1	0.16×10^{-6}

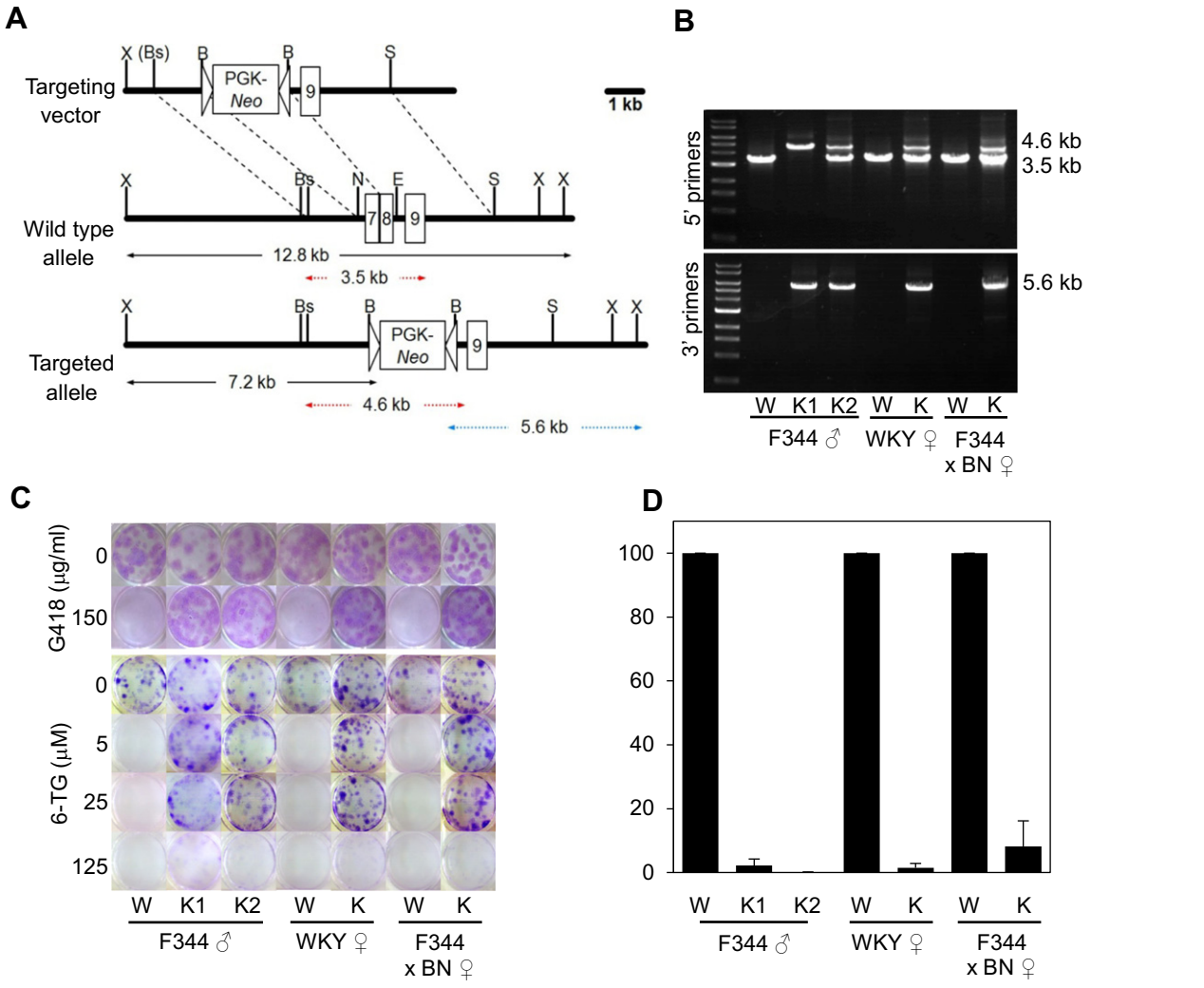

Fig. 1. Targeted inactivation of the HPRT gene. (A) Schematic illustrating targeting procedure. Homologies and amplicons used to verify targeting are shown. (B) Genomic PCR verifying genomic structure of HPRT locus of wild type (W) and targeted (K) cell lines. F344, WKY, and F344xBN denote the strain backgrounds of the wild type and targeted lines. (C) Plating assays (stained with Crystal Violet) after selection with G418 (top) or 6TG (bottom), showing drug resistances of targeted and non-targeted clones. Note identical drug sensitivity for male and female clones. (D) Real-time qRT-PCR results showing HPRT expression (normalized to PGK expression) of the control and targeted cell lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

colonies resulting from selection were not passaged within a week, flat differentiated cells started to accumulate (Fig. S1B). We therefore used an accelerated selection scheme (see Section 2) and employed the expansion phase for "cleaning up" the clones, taking advantage of the fact that the undifferentiated cells proliferate faster.

3.4. Targeted stem cell clones maintain their original identities

In order to clarify whether after selection, the targeted cell lines maintained their original identity, we studied their morphology, growth properties, lineage markers, and ability to differentiate. After expansion, the original characteristic morphology of XENP/HypoSC predominated again (Fig. 2Ai). When the wild type and targeted cell lines were transferred onto feeder cells, they formed characteristic flat colonies with round cells especially at the fringes (Fig. 2Aii) (see [7,8]), and older colonies gave rise to characteristic ductal structures (Fig. 2Aiii). Thus, the undifferentiated morphology and the morphological differentiation patterns were maintained.

Under the routine culture conditions, the expanded targeted cell clones showed an unaltered rate of proliferation (Fig. 2B). Like their parental lines, they required LIF for colony formation, while removal of PDGF had only a very mild effect (Fig. 2C). Thus, their growth characteristics were maintained. The targeted cells also maintained the characteristic molecular signature of XENP/HypoSC, i.e. they expressed the key marker Oct4 and the extraembryonic endoderm markers Gata6, Dab2, and Sparc, while lacking the epiblastic and trophoblastic markers, Nanog and Cdx2 (Figs. 2D, E, and S1C). Furthermore, upon removal of LIF, the wild type and targeted cells showed an increased expression of Hnf4a and a decreased expression of Oct4 (Fig. 2F), reflecting visceral endodermal differentiation [8].

Thus, the targeting procedure did not lead to gross aberrations of the cellular identity, i.e. the inactivation of the non-targeted HPRT locus seen in the female and the aneuploid male cells is not a result of differentiation but a stem cell feature. From the perspective of Xi, HPRT is not privileged, and in fact HPRT gene inactivation is well known to indicate Xi (e.g. [17]); therefore, our results suggest that XENP/HypoSC inactivate additional X-linked genes. To elaborate that further, we currently establish an assay based on expressed X-linked nucleotide polymorphisms. In the future, female XENP/HypoSC with one targeted HPRT gene will be useful for providing a transgene harbor (e.g. [18]) and for using HPRT as a marker for transdifferentiation-associated alterations of the Xi pattern.

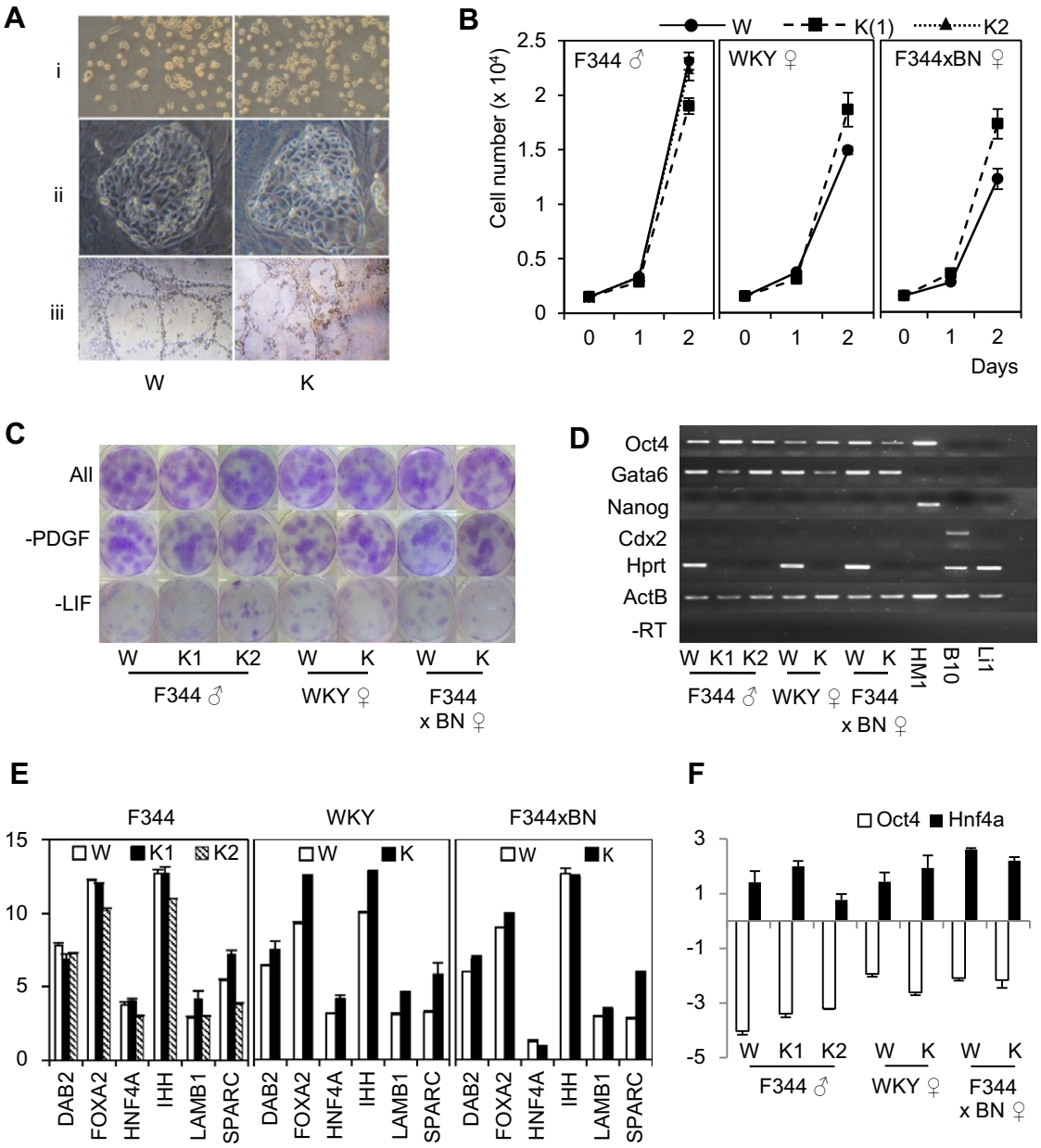

Fig. 2. Characteristics of targeted cells. (A) Representative phase contrast photos of wild type (W) and targeted (K) cells. (i) Routine stem cell culture conditions. (ii) Colonies arising on feeder cell support. (iii) Older colonies on feeder having formed ducts. Orig. magnif. 200× (i, iii) or 100× (ii). (B) Growth curves of wild type (W) and targeted (K, K1, K2) cell lines under routine stem cell culture conditions. (C) Plating assays (stained with Crystal Violet) demonstrating that normal growth factor responsiveness is maintained after targeting. (D) RT-PCR of wild type and targeted cells for selected blastocyst genes (routine stem cell culture conditions). HM1, an ES cell line (positive control for Nanog, negative control for HPRT); B10, a trophoblast cell line (positive control for Cdx2); Li1, a fibroblast cell line (negative control for Oct4, Gata6, Nanog, Cdx2). (E) Comparative expression of selected extraembryonic endoderm marker genes by wild type and targeted cells (qRT-PCR results). Shown are the delta Ct values. (F) Gene expression response (Oct4, Hnf4a) of wild type and targeted cells to LIF removal (qRT-PCR). Shown are the ΔΔCt values (“−LIF” minus “+LIF”). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our results enlarge the still tiny group of cell lines with the demonstrated ability to support a high rate of homologous recombination without the assistance of site-specific nucleases. Perhaps such ability distinguishes truly immortal cell lines. In any case, the successful and efficient targeting of the HPRT gene in XENP/HypoSC will facilitate the manipulation of other genes in this cell type.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.097.

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