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Live cell imaging of X chromosome reactivation during somatic cell reprogramming

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ABSTRACT

Generation of induced pluripotent stem cells (iPSCs) with naive pluripotency is important for their applications in regenerative medicine. In female iPSCs, acquisition of naive pluripotency is coupled to X chromosome reactivation (XCR) during somatic cell reprogramming, and live cell monitoring of XCR is potentially useful for analyzing how iPSCs acquire naive pluripotency. Here we generated female mouse embryonic stem cells (ESCs) that carry the enhanced green fluorescent protein (EGFP) and humanized Kusabira-Orange (hKO) genes inserted into an intergenic site near either the *Syap1* or *Taf1* gene on both X chromosomes. The ESC clones, which initially expressed both EGFP and hKO, inactivated one of the fluorescent protein genes upon differentiation, indicating that the EGFP and hKO genes are subject to X chromosome inactivation (XCI). When the derived somatic cells carrying the EGFP gene on the inactive X chromosome (Xi) were reprogrammed into iPSCs, the EGFP gene on the Xi was reactivated when pluripotency marker genes were induced. Thus, the fluorescent protein genes inserted into an intergenic locus on both X chromosomes enable live cell monitoring of XCI during ESC differentiation and XCR during reprogramming. This is the first study that succeeded live cell imaging of XCR during reprogramming.

1. Introduction

iPSCs, generated by introduction of defined reprogramming factors into somatic cells [1], hold great promise for regenerative medicine and drug development [2]. However, iPSC generation is beset by inefficiency of reprogramming and heterogeneity of obtained cell populations [3]. It is therefore important to improve the efficiency of somatic cell reprogramming and select for iPSCs with full pluripotency.

iPSCs and ESCs display two distinct phases of pluripotency, the primed and naive states [4]. The ESCs in the naive state show higher ability to differentiate than those in the primed state [4], and generation of iPSCs with the pluripotency equivalent to the naive state is critical for their application in regenerative medicine. The naive state of pluripotency can be distinguished from the primed state by cell morphology, gene expression pattern, dependence on growth factors as well as, in the case of female cells, the presence of two active X chromosomes [4].

In eutherian mammals, female cells possess two X chromosomes, one of which is epigenetically inactivated during the early phase of embryonic development by a dosage compensation mechanism termed XCI [5]. XCI strictly depends on the X-inactive specific transcript (*Xist*) gene encoding a non-coding RNA, which plays a central role in inactivating the X chromosome in *cis*. Female somatic cells possess one active X chromosome (Xa) and one Xi, but once reprogrammed into the fully pluripotent state, female somatic cells reactivate Xi by a reverse process termed XCR [6]. Recent studies showed that XCR is closely coupled to acquisition of pluripotency by iPSCs [6,7]. Thus, monitoring XCR may enable evaluation of pluripotency acquisition by iPSCs during somatic cell reprogramming.

Here we used the CRISPR/Cas9 system [8,9] to generate female ESCs that carry the EGFP gene on one X chromosome and the hKO gene on the other. The obtained ESC clones expressed both EGFP and hKO, one of which was repressed in a random mode upon differentiation, concurrent with up-regulation of the *Xist* expression. The derived

Abbreviations: iPSCs, induced pluripotent stem cells; ESCs, embryonic stem cells; XCR, X chromosome reactivation; XCI, X chromosome inactivation; EGFP, enhanced green fluorescent protein; hKO, humanized Kusabira Orange; Xist, X-inactive specific transcript; Xi, inactive X chromosome; Xa, active X chromosome

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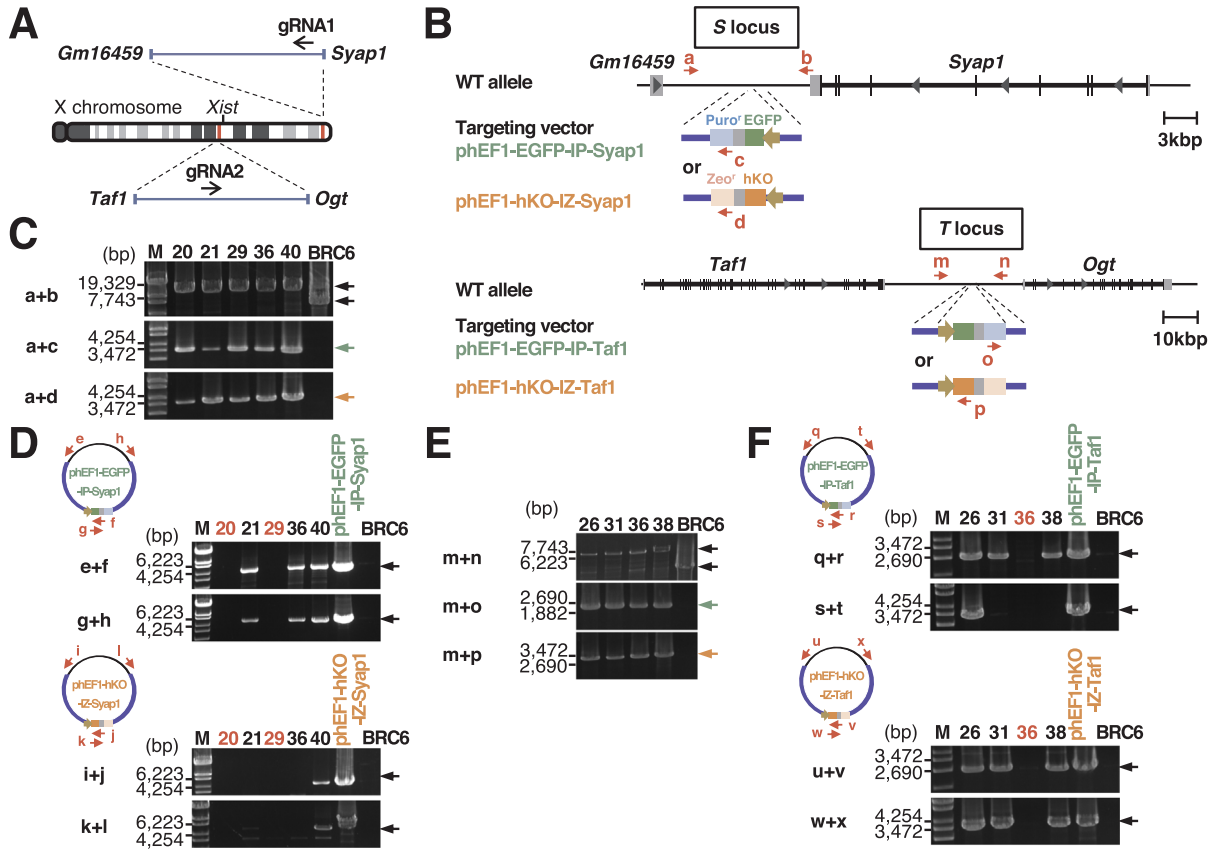

Fig. 1. Knock-in of the EGFP and hKO genes driven by the human EF-1α promoter into the X chromosomes of mouse female ESCs. (A) Locations of the *S* and *T* loci on the mouse X chromosome are indicated by red bars. The location of the *Xist* gene is also indicated. The black arrows indicate the position and orientation of the guide RNAs (gRNA1 and gRNA2) used for the CRISPR/Cas9 system. (B) The intergenic sites between the *Gm16459* and *Syap1* genes ("*S* locus" in this study) and between the *Taf1* and *Ogt* genes ("*T* locus" in this study) on the mouse X chromosome were chosen for insertion of the EGFP-IRES-Puro^r or hKO-IRES-Zeo^r cassette, which is driven by the human EF-1α promoter to express EGFP or hKO, respectively. The positions of the primers for genomic PCR are indicated by red arrows (a-d, m-p). (C) Genomic PCR analyses of the inserted fluorescent protein genes at the *S* locus in isolated ESC clones. BRC6 indicates the original female mouse ESCs used to insert the fluorescent protein genes. The primer sets used for PCR analyses are shown on the left. (D) Detection of random integration of the targeting vectors in the genome of isolated clones. The positions of primers for PCR analyses are indicated by red arrows (e-h for phEF1-EGFP-IP-Syap1, i-l for phEF1-hKO-IZ-Syap1). (E) Genomic PCR analyses of the inserted fluorescent protein genes at the *T* locus in isolated ESC clones. (F) Detection of random integration of the targeting vectors in the genome of isolated ESC clones. The positions of primers for PCR are indicated by red arrows (q-t for phEF1-EGFP-IP-Taf1, u-x for phEF1-hKO-IZ-Taf1).

somatic cells that expressed only hKO were reprogrammed by Sendai virus expressing OCT4, SOX2, KLF4, and c-MYC [10], and found to initiate expression of EGFP when pluripotency marker genes were induced.

2. Materials and methods

2.1. Plasmids and guide RNAs

pX330-U6-Chimeric_BB-CBh-hSpCas9 (#42230) was purchased from Addgene. pPyCAG-EGFP-IP and pPyCAG-EGFP-IZ were generous gifts from Dr. Hitoshi Niwa (RIKEN CDB). Guide RNAs (gRNAs) were designed using CRISPRdirect (https://crispr.dbcls.jp), and the gRNAs that had the minimum potential off-target effects were chosen for the *S* and *T* locus (Fig. 1A, B and Supplementary Table 1). The B6N mouse Bac clones B6Ng01-177J10 (for the *S* locus) and B6Ng01-316J16 (for the *T* locus) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan.

2.2. Construction of plasmids

Complementary pairs of oligonucleotides encoding the gRNAs were annealed and inserted into the *BbsI* site of pX330-U6-Chimeric_BB-CBh-hSpCas9 to prepare Cas9/gRNA-expression vectors. The targeting vectors to knock-in the fluorescent protein genes into the *S* or *T* locus were constructed using pPyCAG-EGFP-IP and pPyCAG-EGFP-IZ. The CAG promoters were replaced by the human elongation factor-1α (EF-1α) promoter, and the EGFP gene in pPyCAG-EGFP-IZ was replaced by the hKO gene. The DNA fragments spanning the target site of gRNA1 (*S* locus) or gRNA2 (*T* locus) were isolated from the B6N mouse Bac clones and inserted into the upstream of the fluorescent protein gene and downstream of the drug-resistant gene (Supplementary Table 2).

2.3. Transfection of female mouse ESCs

Female mouse ESCs, BRC6 (RIKEN BRC, AES0010), were seeded at 5×10^5 cells/well on SNL feeder cells harboring the puromycin-resistant gene in a 6-well plate and cultured at 37 °C under 5% CO_2 for 5 h in DMEM supplemented with 1 mM sodium pyruvate (Nacalai tesque, Inc.), 15% KnockOut Serum Replacement (KSR) (Thermo Fisher Scientific Inc.), nonessential amino acids (NEAA) (Wako Pure Chemical Industries, Ltd.), 0.1 mM 2-mercaptoethanol (2-ME) (Thermo Fisher Scientific Inc.) and 1000 U/mL LIF (Wako Pure Chemical Industries, Ltd.). Two micrograms each of pX330-Cas9/gRNA expression vectors and two different targeting vectors (phEF1-EGFP-IP-Syap1 and phEF1-hKO-IZ-Syap1, or phEF1-EGFP-IP-Taf1 and phEF1-hKO-IZ-Taf1) were mixed with 10 μl of Lipofectamine 2000, and the mixture was added to the ESCs. The cells were treated with 1 μg/mL puromycin for 5 days