Targeting of the Human Coagulation Factor IX Gene at rDNA Locus of Human Embryonic Stem Cells

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Abstract

Background: Genetic modification is a prerequisite to realizing the full potential of human embryonic stem cells (hESCs) in human genetic research and regenerative medicine. Unfortunately, the random integration methods that have been the primary techniques used keep creating problems, and the primary alternative method, gene targeting, has been effective in manipulating mouse embryonic stem cells (mESCs) but poorly in hESCs.

Methodology/Principal Findings: Human ribosomal DNA (rDNA) repeats are clustered on the short arm of acrocentric chromosomes. They consist of approximately 400 copies of the 45S pre-RNA (rRNA) gene per haploid. In the present study, we targeted a physiological gene, human coagulation factor IX, into the rDNA locus of hESCs via homologous recombination. The relative gene targeting efficiency (>50%) and homologous recombination frequency (>10⁻²) were more than 10-fold higher than those of loci targeted in previous reports. Meanwhile, the targeted clones retained both a normal karyotype and the main characteristics of ES cells. The transgene was found to be stably and ectopically expressed in targeted hESCs.

Conclusion/Significance: This is the first targeting of a human physiological gene at a defined locus on the hESC genome. Our findings indicate that the rDNA locus may serve as an ideal harbor for transgenes in hESCs.

Introduction

Over the past thirty years, the combined use of murine embryonic stem cells (mESCs) and gene targeting, which allows researchers to study gene function in vivo, has revolutionized developmental research [1]. Parallel to mESCs, human embryonic stem cells (hESCs) can also proliferate indefinitely and differentiate into multiple lineages both in vitro and in vivo [2]. More importantly, because they have a human genetic background, they may be powerful tools in the study of human genes and in regenerative medicine. Unfortunately, the gene targeting strategy that is most widely used to manipulate mESCs has worked poorly in hESCs due to their resistance to non-viral transfection and sensitivity to single-cell cloning. So far, only a dozen sites have been successfully targeted in hESCs [3–15]. Random transgene integration is the method of genetic modification most commonly used with hESCs; but the uncertainty of the integration site leads to other problems. A transgene may become silenced if it is integrated into a heterochromatin area, or it may disrupt or activate endogenous genes, leading to apoptosis or otherwise changing the cell’s fate. In addition, because it is thought that random integration is mediated by non-homologous end joining (NHEJ), an imprecise DNA-repair mechanism, integration may be incomplete and plasmid debris may end up integrated into the genome [16,17]. These problems may be solved if the transgene is targeted to an appropriate site where it can be expressed without any serious functional consequences [18].

Human 45S ribosomal DNA (rDNA) is clustered on the short arm of all five acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22). It consists of approximate 400 copies of the 45S pre-RNA (rRNA) gene per haploid [19]. The rRNA gene is transcriptionally active, producing approximately 80% of the total RNA in rapidly dividing cells. Loss or gain of the short arm of acrocentric chromosomes is common in humans and does not usually have any phenotypic effects and can be inherited stably. Balanced translocations between the short arm of an acrocentric chromosome and the other chromosomes are also observed without apparent phenotypic effect. For this reason, we presumed that transgenes targeted into the rDNA locus would be transcriptionally active without any unexpected effects. The rDNA locus is a candidate harbor favorable for effective and safe transgene cell manipulations. A recent report revealed that the rDNA cluster exhibited strikingly variable lengths between and within human individuals and showed high
higher than those of any previous gene targeting experiment and an absolute targeting frequency of 0.01%. These values are based on 39 clones screened by PCR, 12 were identified as homologous recombination events. Finally, 997 resistant clones were obtained (Table S1). Out of the 10 million H1080 cells that were exposed to a single 2000 V, 50 µF pulse with 20 µg linearized pHrnF9 at room temperature, 3.2 × 10^6 cells were highly sensitive to G418, the initial drug concentration of 25 µg/ml was sufficient to kill untransfected cells. Drug-resistant clones were picked and expanded for genotyping two weeks after nucleofection. In a typical experiment, 3.2 × 10^6 cells were nucleofected with 5 µg linearized pHrnF9, and 57 drug-resistant clones were obtained. Of the 22 clones analyzed, 14 targeted clones were identified by PCR (Figure 2d) and Southern blot analysis (Figures 2f and 2g). Relative targeting efficiency was 64% (14/22) and the absolute homologous recombination frequency was 1.13 × 10^-5. Both of these values are higher than those reported in previous studies other than studies of artificial zinc finger nucleases (ZFNs). Generally, dozens of clones were obtained for each case of single nucleofection followed by two weeks of selection. In a series of experiments, similar results were obtained regularly (Table 1).

Following successful gene targeting of hESCs, we analyzed G-band chromosomal DNA from nine targeted clones after genotyping, and all showed normal karyotypes (Figure 2c). To confirm that the targeted clones retained hESC characteristics after gene targeting, we examined hESC marker expression. Immunocytochemistry and alkaline phosphatase (AP) staining revealed that the targeted hESC lines expressed stage-specific embryonic antigen (SSEA)-3 (Figure 3d), SSEA-4 (Figure 3e), tumor-related antigen (TRA)-1-81 (Figure 3g), OCT 3/4 (Figure 3h), and TRA-1-60 (Figure 3i). SSEA-1 was not expressed in undifferentiated cells (Figure 3c). To determine the differentiation potential of the targeted clones, we cultured the cells in suspension to form embryoid bodies (EBs). After 7 days, the EBs were transferred to gelatin-coated plates and cultured for another 7 days. Immunocytochemistry and alkaline phosphatase (AP) staining revealed that the targeted hESC lines expressed stage-specific embryonic antigen (SSEA)-3 (Figure 3d), SSEA-4 (Figure 3e), tumor-related antigen (TRA)-1-81 (Figure 3g), OCT 3/4 (Figure 3h), and TRA-1-60 (Figure 3i). SSEA-1 was not expressed in undifferentiated cells (Figure 3c). To determine the differentiation potential of the targeted clones, we cultured the cells in suspension to form embryoid bodies (EBs). After 7 days, the EBs were transferred to gelatin-coated plates and cultured for another 7 days.

**Results**

We constructed an rDNA-targeting plasmid, pHrnF9, which introduced a promoterless neomycin resistance (neo) cassette and an EF1-α driven human coagulation factor IX (F9) open reading frame into the 45S pre-RNA gene. The two cassettes were flanked by a 5’ long homologous arm (4.5 kb) and a 3’ short homologous arm (1.1 kb). The first cassette contained an encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), which enabled resistant gene expression under the control of endogenous RNA polymerase I (Pol I) promoter upstream after homologous recombination (Figure 1).

Prior to targeting hESCs, we tested the new plasmid construct in HT1080 cells via electroporation. We did two gene targeting experiments on HT1080 cells (Table S1). In one of the experiments, three million HT1080 cells were exposed to a single 2000 V, 50 µF pulse with 20 µg linearized pHrnF9 at room temperature using the BioRad Gene Pulser II (0.4 cm gap cuvette, BioRad, Hercules, CA, U.S.). Up to 400 µg/ml of G418 (Sigma, St Louis, MO, U.S.) was added 72 hours after electroporation. Finally, 997 resistant clones were obtained (Table S1). Out of the 39 clones screened by PCR, 12 were identified as homologous recombinants. This indicated a relative targeting efficiency of 31% and an absolute targeting frequency of 0.01%. These values are higher than those of any previous gene targeting experiment performed on HT1080 cells. The targeted clones secreted F9 protein stably (Table S2).

For gene targeting of hESCs, we nucleofected tryptophanase H9 hESCs, using 10 µM rho-associated kinase (ROCK) inhibitor Y-27632 two hours before and during the 24 hours immediately after nucleofection to improve single-cell survival. This method yielded sufficiently high transfection efficiency (Figure 2a). G418 selection was initiated 48–72 hours after transfection, starting at low drug concentrations and slowly building up to 50 µg/ml. Because H9 cells are highly sensitive to G418, the initial drug concentration of 25 µg/ml was sufficient to kill untransfected cells. Drug-resistant clones were picked and expanded for genotyping two weeks after nucleofection. In a typical experiment, 3.2 × 10^6 cells were nucleofected with 5 µg linearized pHrnF9, and 57 drug-resistant clones were obtained. Of the 22 clones analyzed, 14 targeted clones were identified by PCR (Figure 2d) and Southern blot analysis (Figures 2f and 2g). Relative targeting efficiency was 64% (14/22) and the absolute homologous recombination frequency was 1.13 × 10^-5. Both of these values are higher than those reported in previous studies other than studies of artificial zinc finger nucleases (ZFNs). Generally, dozens of clones were obtained for each case of single nucleofection followed by two weeks of selection. In a series of experiments, similar results were obtained regularly (Table 1).

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**Figure 1. Schematic representation of the rDNA unit, targeting vector, and targeted allele after homologous recombination.** White boxes represent the left (GenBank U13369: 937–5467) and right (GenBank U13369: 5468–6523) homologous arms. The neo cassette consisted of an IRES element from the encephalomyocarditis virus, the coding region of the neo gene (neo), and SV40 polyA signal (SpA). Neo lacks a promoter. Expression is activated by the promoter of the rRNA gene after homologous recombination. Knock-in of neo open reading frame (neo ORF) to the rDNA unit caused an addition of PvuII site resulting in fragments of 7.1 kb and 10.3 kb after digestion. The F9 gene is driven by an EF1-α-promoter. Primer 1 (P1) and primer 2 (P2) bind to the bovine growth hormone polyA (BH pA) and the 5.8S RNA coding sequence beyond the homologous sequence respectively, the PCR product will be 1.4 k after homologous recombination.

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fluorescent analysis showed that the EBs can give rise to Tuj1-positive ectoderm (Figure 4b), α-fetoprotein (AFP)-positive endoderm (Figure 4a), and mesoderm positive for smooth muscle actin (SMA) (Figure 4d). Upon directed differentiation, beating clumps emerged (Figure 4c). These consisted of cardiocytes expressing myosin light chain-2a (Figure 4e) and cardiac troponin I (Figure 4f).

To evaluate pluripotency in vivo, we injected the targeted cells subcutaneously into immuno-compromised mice. Two months later, the targeted cells generated various complex teratomas comprising structures and tissues derived from the three embryonic germ layers, including gallbladder (endoderm) (Figure 5a), colon (endoderm) (Figure 5b), respiratory epithelium (endoderm) (Figure 5c), cartilage (mesoderm) (Figure 5d), striated muscle (mesoderm) (Figure 5e), bone (mesoderm), and squamous epithelium (ectoderm) (Figure 5f). Our data indicate that the rDNA-targeted clones retained their ES characteristics.

To determine whether targeted transgene could be expressed at the rDNA locus, reverse transcription PCR (RT-PCR) analysis was carried out, revealing that wild-type H9 did not express endogenous F9 and that the targeted clones did, even after 40 passages of contiguous culture (Figure 6a). Quantitative analysis using enzyme linked immunosorbent assay (ELISA) showed that
the targeted clones expressed the transgene at different levels (Figure 6c), indicating the site-specifically integrated transgene could be expressed in the targeted clones. It also showed that the synthesized protein could be secreted into the supernatant. Western blot analysis showed that the F9 protein could be detected in both the cell lysate and concentrated supernatant (Figure 6b and Figure S1). The different levels of transgene expression observed among different targeted clones may have been caused by the different targeted rDNA copies, which had different transcriptional levels.

Discussion

In recent years, hESCs have been studied eagerly by thousands of laboratories, but results have been disappointing because of their low rate of transgenesis and homologous recombination relative to mESCs. One of main characteristics of hESCs is the maintenance of normal karyotypes after long-term culture. To achieve this, they must maintain some kind of self-repairing mechanism (such as homologous recombination) through hundreds of mitotic divisions. For this reason, we think the intrinsic frequency of homologous recombination may not be as low as reported and the real barrier to hESC gene targeting is their resistance to clonal expansion. This problem can be solved by using of neurotrophin cocktail or ROCK inhibitor [21,22]. For instance, once treated with Y27632, the survival rate of single hESCs can be improved by more than two orders of magnitude without sacrificing any ES properties [22]. By this means, we achieved homologous recombination rate of more than $10^{-3}$ in hESCs. In other studies, the difficulty of gene targeting has differed from site to site [11]. The addition of Y27632 can only improve clonal expansion, which directly results in increased numbers of drug-resistant clones; but this does not, in theory, markedly alter the ratio of homologous recombinants to non-homologous recombinants. Normally the targeting efficiency of both mESCs and hESCs is about 1%, but in our experiments, it was more than 50%. The gene-trap strategy that we used can partially explain this, especially considering that this strategy has been reported to be more effective than positive-negative selection [3,23]. However, the main reason would seem to be the relatively high intrinsic activity of homologous recombination at the rDNA locus. Although there are about 400 copies of rDNA repeats per haploid, it is, to our knowledge, uncertain whether the high frequency of homologous recombination is due to the copy number of target sites. A previous study proved that targeting does not depend on the number of targets in mammalian cells [24]. However, recent study using ZFNs did obtain higher rate of gene targeting in cells with two target sites than in cells with only one [25].

ZFNs have been widely used to achieve efficient homologous recombination of gene targeting vectors with various endogenous loci in cultured and primary mammalian cells [26], even those with very short homologous arms [27]. The customized artificial nucleases can introduce DNA double strand breaks at target sites and then stimulate the cell’s endogenous homologous recombination machinery. In this system, a donor DNA can replace any lost portion of its corresponding chromosomal segment at high efficiency. In 2009, two research groups successfully performed gene targeting in hESCs using ZFNs. Hockemeyer et al. obtained 40 homologous recombinants among 42 resistant clones (relative targeting efficiency 94%) using their most efficient ZFN pair [28]. An absolute targeting frequency of 0.14–0.24% was achieved by Zou et al. [10]. ZFNs are usually superior when used to generate a mixed population of targeted and untargeted cells without drug selection. However, the use of ZFNs can be compromised by their

| Table 1. Summary of the 4 experiments of gene targeting in H9 cells. |
|----------|------|-----|-----|-----|-----|-----|-----|
| Exp. N | C | S | T | R | ATF | RTE |
| 1 | 1.8 | 32 | 23 | 13 | 10 | 9.9 | 56% |
| 2 | 1.9 | 45 | 21 | 10 | 11 | 11.6 | 48% |
| 3 | 3.2 | 57 | 22 | 14 | 8 | 11.3 | 64% |
| 4 | 2.8 | 40 | 22 | 15 | 7 | 9.8 | 68% |
| Total | 9.7 | 174 | 88 | 52 | 36 | 10.6 | 59% |

Abbreviation: Exp., experiments were performed. N, Number of cells nucleofected ($\times 10^6$). C, total number of resistant clones obtained from each experiment. S, number of clones screened. T, number of clones screened as targeted recombinants. R, number of clones screened as random integrants. ATF, absolute targeting frequency ($\times 10^{-6}$) = TC/NS. RTE, relative targeting efficiency = T/S.

Figure 3. Characterization of targeted clones. (a) Phase-contrast image of a targeted clone. (b) Alkali phosphatase staining of targeted clones. (c-h) Immunocytochemical analysis of targeted clones with SSEA-1, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, and Oct4 antibody. Scale bar = 200 μm for micrographs.

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laborious design process, their toxicity, and other undesired effects. In gene targeting of hESCs with the purpose of generating site-specific edited recombinants, our strategy described here is more effective and safer than the ZFNs method.

Human ROSA26 and ENVY loci are transcriptionally active and considered to be candidate sites for targeted transgene integration [5,29]. But it is uncertain whether the cells will exhibit functional consequences when one of the two copies is disrupted. Another hESC line for gene targeting has been reported. In this line, a LoxP-docking site was introduced to the HPRT locus [12]. This cell line can be used for targeting exogenous sequences using the Cre-Lox system. Because the first round of targeting is favorable to an active X chromosome, disruption of the HPRT locus may lead to a Lesch-Nyhan phenotype. In contrast, the human rRNA cluster consists of hundreds copies of tandemly repeated rDNA units. Variations in rDNA copy number are common among healthy individuals and balanced chromosomal translocation involving the rRNA cluster occurs without any apparent phenotypic effect. These properties indicate that the rDNA locus may hold a high intrinsic homologous recombination activity and facilitate effective transgene expression. In the present study, the rDNA locus was targeted with high efficiency, no undesired effect was detected, and the transgene was expressed stably in the integrated clones.

It has been reported that RNA polymerase II (Pol II)-transcribed genes can be silenced at the rDNA locus in yeast cells, especially under an active Pol I promoter [30,31]. In the

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**Figure 4. In vitro differentiation of targeted clones.** Immunostaining images show cells derived from all three germ layers, including AFP (endoderm), TuJ1 (ectodermal), and SMA (mesodermal) positive cells. Upon directed differentiation, cell clumps started beating rhythmically, and the expression of Mlc-2a and cTn I revealed that the differentiation into cardiomyocytes in these cells had been completed.

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**Figure 5. Teratoma formation in immunodeficiency mice by targeted cells.** H&E staining of teratomas was performed. Derivatives of all three germ layers were observed in the endoderm: (a) gallbladder, (b) intestinal-like epithelium, and (c) respiratory epithelium; in the mesoderm, (d) cartilage and (e) muscle; and in the ectoderm, (f) squamous epithelium. Bar = 200 μm.

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**Figure 6. Expression of the transgene in targeted cells.** (a) RT-PCR analysis of F9 expression in targeted clones. The expected product was amplified from all analyzed homologous recombinants after long-term culture (more than 30 passages), while no transcript was detected in wild-type H9 cells. (b) Western blot of the clone’s lysate using an antibody anti human F9 protein. (c) ELISA analysis of supernatants from recombinant culture. No F9 secretion was detected from wild-type H9 cells. The targeted clones secreted the protein at different levels.
current study, we screened the resistant clones using a promoter-trap strategy. This means that the neo gene could only be expressed under a transcribed Pol I promoter. As a result, the clones cannot be selected unless the transgene is targeted into an active rDNA repeat in a dynamic chromatin structure. However, we found that all the five selected clones expressed the Pol II gene. This differs from the results of a previous study conducted in yeast. The question raised by our results is whether the mechanism of the Pol II gene silencing at the rDNA locus is similar in all eukaryotes, from yeast to human cells. Restructuring of the rDNA clusters has been observed among somatic cells [20]. This raises concerns about the potential instability of any inserted transgene in the rDNA locus and the risk that the transgene might be translocated among the rDNA clusters or lost during mitotic expansion. However, the odds of this happening to one specific targeted copy out of the hundreds of rDNA copies in the human genome are very low. They are rendered even lower by the fact that the restructuring of the rDNA clusters during mitosis is rare.

In summary, using the strategy described here, we integrated human coagulation factor IX into the rDNA locus of hESCs via homologous recombination. Compared to other sites reported, this locus proved to be subject to efficient targeting. The targeting of hESCs at the rDNA locus did not change the main ES characteristics of the cells and the transgene was expressed stably in targeted hESCs. This is the first gene targeting of a human physiological gene at a defined locus on hESCs. Our findings indicate that the rDNA locus may serve as an ideal harbor for transgenes in hESCs.

Materials and Methods

Cell Culture

Human fibrosarcoma cells (HT1080) were purchased from ATCC and cultured in high-glucose Dulbecco’s modified Eagle’s medium (4.5 g/l) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 unit/ml penicillin, and 50 mg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. H9 cells (WiCell Research Institute, Madison, WI, U.S.) were maintained on mitomycin-C treated mouse embryonic fibroblasts in hESC medium containing 20% knockout serum replacement. The Factor IX was performed using primers 5’-GGG TGG GGC AGG ACA GCA AGG GGG AGG AT-3’ and 5’-GCC GAT CCG AGG GCC GTT ACG AGA-3’. The Factor IX open reading frame and the 3’ untranslated region were generated using PNS-FIX as templates with the following primers: 5’-GGG GCT CGC CGC GCT CTA-3’ and 5’-GCC GAT CCG AGG GCC GTT ACG AGA-3’. These introduced a BamHI site into the product. Neo open reading frame, 3’ untranslated region and SV40 Poly A sequence were generated using pCDNA3.1 as a template with primers 5’-GGC GTC TCA CAT GAT TGA ACA AGA-3’/5’-CCA TGG CTA GCT CTA GAC GGT CAA CCC TAG AGC CCC AGG-3’.

For gene targeting, one passage prior to transfection, hESCs were detached by 1 mg/ml dispase and replate on dishes coated with MatrigelTM (BD Biosciences, San Jose, CA, U.S.) for three days in MEF conditioned hESC medium. Two hours before transfection, 10 μM Y27632 was added to the medium. For targeting, the hESCs were incubated at 37°C with trypLE™ Select (Invitrogen, Carlsbad, CA, U.S.) for 3 minutes. Then the cells were immediately collected and counted. The centrifuged cells were resuspended with 100 μL Human Stem Cell Nucleofector Kit 2 (Lonza, Basel, Switzerland) and 5 μg linearized pHmF9 and nuleofected using Nucleofector II (Lonza, Basel, Switzerland) using program A023. After recovery in 500 μL RPMI-1640 (HyClone, Beijing, China) for 5 minutes at room temperature, the transfected cells were plated on PMEF-NL (Millipore, Bedford, MA, U.S.) in hESC medium containing 10 μM Y27632 (Sigma, St Louis, MO, U.S.). G418 selection was initiated 48–72 hours after transfection, depending on the cell density. The final concentration of G418 was 50 μg/ml. About two weeks after transfection, a portion of resistant clones was picked and the remaining clones were fixed and stained with Giemsa stain. Clones with diameters of ≥2 mm were considered resistant.

PCR and RT-PCR

RT-PCR

Total RNA was extracted using Trizol reagent (Sigma, St Louis, MO, U.S.) and reverse transcribed using Promega’s transcription system according to the manufacturers’ instructions. RT-PCR for F9 was performed using primers 5’-ATG CAC GCG GTG AAC ATG A-3’ and 5’-TAG CTC TTT GGC GGA TTC AGA-3’.

Southern Blotting

After digested with pvull restriction enzyme (New England Biolabs, Ipswich, MA, U.S.) overnight, 5 μg genomic DNA were electrophoresed on a 0.8% agarose gel over night then transferred to positively charged nylon membranes (Roche Diagnostics, Indianapolis, IN, U.S.). A DNA Hind III (Takara, Dalian, China) was used as molecular weight marker. The blots were hybridized with DIG-DUTP labeled probes overnight at 42°C. After incubation with AP-conjugated DIG-Abtoody (Roche Diagnostics, Indianapolis, IN, U.S.) and appropriate washing, the signals were detected using CDP-Star (Roche Diagnostics, Indianapolis, IN, U.S.) as a substrate for chemiluminescence. Probes were generated by PCR DIG Probe Synthesis Kit (Roche Diagnostics, Indianapolis, IN, U.S.) using the primers: 5’ probe 5’-CCC GGA...
metaphase chromosome spreads were prepared using air drying. Cells were trypsinized, centrifuged, and incubated in 0.075 M KCl colcemid (Sigma, St Louis, MO, U.S.) for 2.5 hours. Then the stained with hematoxylin and eosin. All procedures regarding the later, the formed teratomas were removed and fixed in 4% formaldehyde. After being washed with DMEM/F12, cells were resuspended in 0.05% trypsin/EDTA (Invitrogen) and collected in DMEM/F12.

Karyotyping
Three-day old cell clumps were treated with 0.08 μg/ml colcemid (Sigma, St Louis, MO, U.S.) for 2.5 hours. Then the cells were trypsinized, centrifuged, and incubated in 0.075 M KCl for 30 minutes at 37°C. After fixing with Carnoy fixative, metaphase chromosome spreads were prepared using air drying.

Alkaline Phosphatase Staining and Immunofluorescence
Three day old cell clumps were fixed with 4% paraformaldehyde. After washing in TBST, alkaline phosphatase was stained with 0.375 mg/ml nitroblue tetrazolium chloride and 0.188 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Roche, Indianapolis, IN, U.S.). The suspension was injected subcutaneously into the hind legs of immunocompromised mice. Eight to twelve weeks after injection, the formed teratomas were removed and fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton-X 100. After blocking with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, U.S.), the cells were incubated with the first antibody against Oct4 (1:200, Millipore, Bedford, MA, U.S.), SSEA-1 (1:100, Millipore, Bedford, MA, U.S.), SSEA-3 (1:100, Millipore, Bedford, MA, U.S.), SSEA-4 (1:200, Millipore, Bedford, MA), Tra-1-60 (1:100, Millipore, Bedford, MA, U.S.), Tra-1-81 (1:100, Millipore, Bedford, MA, U.S.), SMA (1:200, Millipore, Bedford, MA, U.S.), AFP (1:100, Millipore, Bedford, MA, U.S.), Tuj1 (1:300, Sigma, St Louis, MO, U.S.), c-Tn I (1:100, Millipore, Bedford, MA, U.S.), and Mlc-2a (1:200, Synaptic Systems, Goettingen, Germany) at room temperature for 1 hour. The samples were incubated with appropriate secondary antibodies after triple washing. DNA was visualized using DAPI (Sigma, St Louis, MO, U.S.).

In vitro Differentiation
Three day old cell clumps were incubated with 1 mg/ml dispase for 10 minutes at 37°C then washed with DMEM/F12. After culture on ultra-low attachment plates for 7 days in hESC medium without bFGF, EBs were transferred to gelatin-coated coverslips and cultured for another 7 days. Differentiated cells were analyzed by immunofluorescence. Directed differentiation was performed according to the protocol of the National Stem Cell Bank of America (https://www.wicell.org/index.php?option=com_docman&task=doc_download&gid=1064).

Teratomaformation and Analysis
Targeted cells from one 10 cm dish were dissociated with 0.05% trypsin/EDTA (Invitrogen) and collected in DMEM/F12. After being washed with DMEM/F12, cells were resuspended in 140 μL DMEM/F12 and 70 μL Matrigel™ (BD Bioscience, San Jose, CA, U.S.). The suspension was injected subcutaneously into the hind legs of immunocompromised mice. Eight to twelve weeks later, the formed teratomas were removed and fixed in 4% paraformaldehyde overnight. The fixed tissues were sectioned and stained with hematoxylin and eosin. All procedures regarding the care and use of animals are in accordance with institutional guidelines. This study was approved by the Ethics Committee of State Key Laboratory of Medical Genetics of China, No. 2008-ANIMAL-004.

Western Blot
Protein samples were electrophoresed and electrotransferred onto PVDF membranes (Millipore, Bedford, MA, U.S.). Blots were incubated with a primary antibody anti-human Factor IX (Affinity Biologicals, Canada) overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies(Sigma, St Louis, MO, U.S.) for 1 hour at room temperature and detected with an ECL system (Amersham Biosciences, Piscataway, NJ, U.S.). Prestained molecular weight standards (Fermentas, Glen Burnie, MA, U.S.) were used to estimate the apparent molecular weight.

ELISA
After culturing in hESC medium for three days, 24-hour-old supernatants were collected from six-well plates. Total cells (together with MEFs) and MEFs from parallel wells (subtracting the number of MEFs from the total number of cells) were trypsinized and counted. All supernatants were collected in triplicate. ELISA was performed using Paired Antibodies for ELISA-Factor IX (Cedarlane Laboratories, Ltd., Burlington, Canada) according to the manufacturer’s instructions. Reference curves were constructed using serial dilutions of normal pooled plasma (Pacific Hemostasis, Cape Town, South Africa), with correlation coefficient (R2) of at least 0.990 using a 5-parameter logistic curve fit algorithm.

Supporting Information

Figure S1 Western blot analysis of the concentrated supernatant from targeted hES clones.

Table S1 Summary of the 2 experiments of gene targeting in HT1080 cells.

Table S2 FIX levels in cultured supernatant from targeted HT1080 clones.

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Author Contributions
Conceived and designed the experiments: XL YW ZL J. Xia LW DL. Performed the experiments: XL YW ZL J. Xia LW DL. Analyzed the data: XL YW ZL J. Xia LW DL. Contributed reagents/materials/analysis tools: WN QY ML. Wrote the paper: XL YW ZL J. Xia LW DL.

References
1. Mak TW (2007) Gene targeting in embryonic stem cells scores a knockout in Stockholmm. Cell 131: 1027–1031.
2. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282: 1145–1147.
3. Zwaka TP, Thomson JA (2003) Homologous recombination in human embryonic stem cells. Nat Biotechnol 21: 319–321.
4. Khan IF, Hirata RK, Wang PR, Li Y, Xia LW, DL. et al. (2004) Engineering of human pluripotent stem cells by AAV-mediated gene targeting. Mol Ther 22: 635–641.
5. Irion S, Luche H, Gadue P, Fehling HJ, Kennedy M, et al. (2007) Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat Biotechnol 25: 1477–1482.

6. Suzuki K, Mitus K, Aizawa E, Hasegawa K, Kawase E, et al. (2008) Highly efficient transient gene expression and gene targeting in primate embryonic stem cells with helper-dependent adenoviral vectors. Proc Natl Acad Sci USA 105: 13781–13786.

7. Di Domenico AI, Christodoulou I, Pells SC, McWhir J, Thomson AJ (2008) Identification of a novel tandem repeat sequence. Genomics 26: 521–526.

8. Davis RP, Ng ES, Costa M, Mosman AK, Sourris K, et al. (2008) Targeting a transgene into a defined locus in human embryonic stem cells. Cell Stem Cell 5: 97–110.

9. Xu X, Wu S, Papadeas ST, Spusta S, Swistowska AM, et al. (2009) A targeted neural progenitor line generated by homologous recombination in human embryonic stem cells. Stem Cells 27: 1829–1840.

10. Irion S, Luche H, Gadue P, Fehling HJ, Kennedy M, et al. (2007) Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat Biotechnol 25: 1477–1482.

11. Xue H, Wu S, Papadeas ST, Spusta S, Swistowska AM, et al. (2009) A targeted neural progenitor line generated by homologous recombination in human embryonic stem cells. Stem Cells 27: 1829–1840.

12. Sakurai K, Shimoji M, Tahimic CG, Aiba K, Kawase E, et al. (2010) Efficient BAC-based homologous recombination system. Cell Stem Cell 6: 535–546.

13. Sakurai K, Shimoji M, Tahimic CG, Aiba K, Kawase E, et al. (2010) Efficient BAC-based homologous recombination system. Cell Stem Cell 6: 535–546.

14. Buecker C, Chen H, Polo JM, Daheron L, Bu L, et al. (2010) A murine ESC-like embryonic stem cell model of human disease. Cell Stem Cell 8: 217–230.

15. Bu L, Gao X, Jiang X, Chien KR, Wang Z (2010) Targeted conditional gene knockout in human embryonic stem cells. Cell Res 20: 379–382.

16. Gonzalez F, Monasterio MB, Tiscornia G, Montserrat Pulido N, Vassena R, et al. (2008) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Rev Genet 11: 636–646.