Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome.

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.
Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome

Russell C. DeKelver,1 Vivian M. Choi,1 Erica A. Moehle,1 David E. Paschon,1 Dirk Hockemeyer,2 Sebastian A. H. Meijsing,3,6 Yasemin Sancak,2 Xiaoxia Cui,4 Eveline J. Steine,2 Jeffrey C. Miller,1 Phillip Tam,1 Victor V. Bartsevich,1 Xiangdong Meng,1 Igor Rupniewski,1 Sunita M. Gopalan,1 Helena C. Sun,1 Kathleen J. Pitz,1 Jeremy M. Rock,1 Lei Zhang,1 Gregory D. Davis,4 Edward J. Rebar,1 Iain M. Cheeseman,2,5 Keith R. Yamamoto,3 David M. Sabatini,2 Rudolf Jaenisch,2,5 Philip D. Gregory,1 and Fyodor D. Urnov1,7

1Sangamo BioSciences, Inc., Point Richmond Tech Center, Richmond, California 94804, USA; 2The Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, USA; 3Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94158, USA; 4Sigma-Aldrich Research Biotechnology, St. Louis, Missouri 63103, USA; 5Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

Isogenic settings are routine in model organisms, yet remain elusive for genetic experiments on human cells. We describe the use of designed zinc finger nucleases (ZFNs) for efficient transgenesis without drug selection into the PPP1R12C gene, a “safe harbor” locus known as AA VS1. ZFNs enable targeted transgenesis at a frequency of up to 15% following transient transfection of both transformed and primary human cells, including fibroblasts and hES cells. When added to this locus, transgenes such as expression cassettes for shRNAs, small-molecule-responsive cDNA expression cassettes, and reporter constructs, exhibit consistent expression and sustained function over 50 cell generations. By avoiding random integration and drug selection, this method allows bona fide isogenic settings for high-throughput functional genomics, proteomics, and regulatory DNA analysis in essentially any transformed human cell type and in primary cells.

[Supplemental material is available online at http://www.genome.org.]
A location could be found. Selection of such a locus to act as a “safe harbor” for ZFN-driven gene addition was guided by the observation that integration of adeno-associated virus (AAV) into the human genome disrupts the \( \text{PPP1R12C} \) gene on chromosome 19 (the locus is commonly referred to as \( \text{AAVS1} \)) (Kotin et al. 1992; Tan et al. 2001). AAV infection is not associated with a known pathophysiology (Smith et al. 2008); both hES (Smith et al. 2008; Hockemeyer et al. 2009) and hiPS (Hockemeyer et al. 2009) cells with a disruption of \( \text{PPP1R12C} \) retain pluripotency. Further, this gene is transcribed in all primary human cells studied (http://www.biogps.gnf.org) as well as in commonly used transformed cell lines, i.e., HEK293, K562, HeLa, DU-145, and Hep3B (DeKelver et al. data not shown). Thus, the \( \text{AAVS1} \) locus fits two criteria for a transgene “safe harbor”: (1) no known adverse effect on the cell resulting from its disruption, and (2) transcriptional competence across cell types to maintain expression from an inserted gene cassette(s).

We describe here a method for using ZFNs to achieve rapid, efficient transgenesis into the \( \text{AAVS1} \) locus in many commonly used human transformed cell types (K562, HeLa, HEK293, U2OS, and others) in human fibroblasts and in hES cells. The gene addition process occurs following simple treatment of the cells with the ZFN/donor-carrying plasmid and produces a pool of cells harboring the donor-specified novel DNA at the ZFN-specified location. Both promoterless (i.e., reliant on the native \( \text{PPP1R12C} \) gene promoter) and promoter-containing inserts placed into the \( \text{AAVS1} \) locus exhibit consistent levels of expression over extended passaging in culture.

We describe three applications of this approach by way of illustrating some of the applications of isogenic transgenesis in human cells: (1) We use these ZFNs to construct, in a single step, an hES cell line transheterozygous at the \( \text{AAVS1} \) locus for four distinct genetic entities that together allow inducible gene expression; (2) we construct a panel of isogenic U2OS cell lines, each carrying at \( \text{AAVS1} \)—i.e., in the same chromatin environment—a different reporter responsive to activation by liganded glucocorticoid receptor; (3) we generate a panel of isogenic HEK293 cell lines, each

---

**Figure 1.** ZFN-driven ORF addition to the \( \text{PPP1R12C} \) gene (also known as the \( \text{AAVS1} \) locus) in various transformed cell types: (A) Schematic of the human \( \text{PPP1R12C} \) gene (http://www.genome.ucsc.edu), with the exon/intron structure and the ZFN target site indicated. (B) Schematic of donor construct and of the \( \text{AAVS1} \) locus following GFP marker ORF addition. The first two exons of the \( \text{PPP1R12C} \) gene are shown as open boxes. Also annotated are the locations of the splice acceptor site, the 2A ribosome stuttering signal, and a polyadenylation signal (pA). (C) Southern blotting confirms efficient ZFN-dependent ORF addition to the \( \text{AAVS1} \) locus in K562 cells. The positions of wild-type and transgenic chromatids are indicated to the right of the gel; the percentage of transgenic chromatids in this cell pool is indicated below lane 2. The PhosphorImager traces used for the quantitation are shown in Supplemental Figure 1. The probe used for Southern blotting, which corresponds to positions chr19:55,628,340–55,628,753 (GRCh37/hg19) is indicated as a purple-filled box; “A” indicates recognition sites for AccI that genomic DNA was cut with for this Southern. (D) Efficient ZFN-driven GFP ORF addition to \( \text{AAVS1} \) in K562 cells. Results of a semiquantitative, body-labeled PCR-based assay (see Methods) on cells transfected with the indicated constructs are shown. Primers are located outside of the homology arms and are indicated on the schematic to the right of the gel. The positions of wild-type and transgenic chromatids are indicated to the right of the gel. The frequency of genome-edited chromatids is indicated below each lane. In this assay, when applied to this locus, weak nonspecific incorporation during early PCR cycles produces a band that appears in all samples and migrates above the one generated by the transgenic chromatid. The data below the autoradiograph represent analysis of the frequency of GFP-positive cells by FACS in the same cells genotyped above. (E) As in D, except HEK293T cells were used. (F) As in D, except Hep3B cells were used.
carrying at AA VS1 a distinct shRNA expression cassette directed against a component of the mTOR pathway, and each exhibiting long-term knockdown of the protein targeted by the small hairpin RNA (shRNA); we perform the same experiment—but now using shRNAs directed against TP53 or DNMT1—in hES cells, and observe comparably robust target gene mRNA knockdown by the shRNA.

Results

ZFN-driven open reading frame (ORF) addition to the AA VS1 locus: Use in distinct transformed and primary cell types, and expression stability over time

The PPP1R12C gene is transcribed in all cell types where this issue has been studied, and this allows the use of promoterless donor constructs. We designed a panel of ZFNs against nonrepetitive stretches of its intron 1 using an archive of prevalidated two-finger modules and screened this panel for endogenous gene disruption (RC DeKelver and JM Rock, data not shown). The most active ZFN pair introduces a double-strand break (DSB) ~1800 bp downstream from the transcription start site of the PPP1R12C gene (Fig. 1A). The composite ZFN recognition site is unique in the human genome and is flanked by an extended stretch of single-copy genomic DNA suitable for donor construction.

To trap expression driven by the native PPP1R12C promoter, the donor construct used (Fig. 1B) contained two 800-bp stretches of sequence homologous to the region flanking the ZFN site interrupted by a promoterless green fluorescent protein (GFP) ORF and a polyadenylation signal. Since exon 1 contains the translational start site of AA VS1, the donor included a splice acceptor site, followed by the 2A ribosome-stuttering signal (Fang et al. 2005) upstream of the GFP ORF (Fig. 1B). Addition of this cassette to intron 1 of the PPP1R12C gene yields a single transcript driven by the native promoter (Fig. 1B), translation of which produces the polypeptide encoded by exon 1 of PPP1R12C and, separately, GFP.

Genotyping of K562 cells 48 h following transient transfection with plasmid DNAs encoding the ZFN and donor DNA construct demonstrated that ~10% of all AA VS1 chromatin in the cell population had acquired the donor-specified ORF cassette at the locus as gauged both by genotyping and phenotyping (Supplemental Fig. 4). Differences between cell lines in gene-addition frequency could potentially result from those in ZFN expression levels, in the epigenetic state of the AA VS1 locus, and in cell-type-specific bias for DSB repair pathways. To determine whether these ZFNs could drive gene addition in primary human cells, we generated integration-defective lentiviruses carrying the ZFNs and the donor (Lombardo et al. 2007); infection of hTERT-immortalized human fibroblasts (Rubio et al. 2002) resulted in ~3% ORF addition to the AA VS1 locus as gauged both by genotyping and phenotyping (Supplemental Fig. 5, A and B, respectively).

In the experiment shown in Figure 1 and Supplemental Figure 5, pools of ZFN-edited K562, HEK293, Hep3B cells, and fibroblasts remained marker positive after 1 mo of continuous passaging in culture. To more accurately measure the expression stability over time of a transgene resident in the AA VS1 locus, we used FACS to isolate GFP-positive K562 cells after transfection with the ZFN-donor combination shown in Figure 1B. Approximately 80% of the chromatin in that pool were found to be transgenic for the GFP ORF (Supplemental Fig. 7B; Supplemental Discussion). Limiting dilution without additional sorting for GFP expression generated a panel of single-cell-derived clonal lines exclusively carrying GFP in a monoallelic or diallelic state (RC DeKelver and EA Moehle, data not shown). This analysis showed that the ~20% of nontransgenic chromatin in the GFP-positive pool (Supplemental Fig. 7B) derive from cells with a monoallelic GFP transgene at AA VS1. Representative control K562 cells, the GFP-positive FACS-enriched cell pool, and two single-cell clonal lines, monoallelic and diallelic for a GFP insertion at AA VS1, were grown for 50 cell doublings and assayed for GFP expression level biweekly. This analysis (Fig. 2) revealed: (1) no loss of mean fluorescence intensity over the course of the experiment; (2) consistently higher mean fluorescence intensity of cells diallelic for the GFP cassette at AA VS1 than monoallelic (cf. square- and diamond-annotated lines in Fig. 2). These data showed that ZFN-driven GFP-ORF addition to the AA VS1 gene locus results in stable long-term expression of the introduced transgene in transformed cells.

To demonstrate gene addition to the “safe harbor” in genetically unmodified primary cells, we turned to human embryonic stem cells (hESCs). DNA delivery to hESCs using electroporation is inefficient (~5%), and we included selectable markers in donor constructs solely to enrich for edited cells. In this experiment (Fig. 3) we made use of the fact that AA VS1 is autosomal; hence, a euploid pool (Fig. 1D, bottom right). Less than 1% of the cells treated with the donor plasmid alone expressed GFP (Fig. 1D, bottom left), and no chromatin transgenic for GFP at AA VS1 were detected in that sample by Southern blotting or by PCR (lanes 1 in Fig. 1, C and D, respectively). This showed that ZFN-driven addition of a promoterless GFP ORF to the human AA VS1 locus yields ~10% GFP-positive K562 cells without selection for the desired event.

The same ZFN-donor combination was then used in HEK293 and Hep3B cells. In both cases, ORF addition frequencies as gauged by genotyping (Fig. 1E,F, top) and phenotyping (bottom) were ~3% in the absence of selection and after 1 mo of passaging in culture. We next tested these ZFNs in a larger panel of transformed cells along with a donor DNA plasmid with homology arms flanking a 50-bp heterologous stretch with a novel RFLP (this arrangement allows for accurate measurement of ZFN-driven addition frequency in a nonradioactive assay) (Supplemental Fig. 4). With this approach, editing efficiency comparable to that seen in K562 cells was observed in HCT116 and U2OS cells (Supplemental Fig. 4, lanes 3,11), and greater than 1% transgenic chromatin were detected in A549, DU145, Hela, HepG2, IMR90, and LNCap cells. Differences between cell lines in gene-addition frequency could potentially result from those in ZFN expression levels, in the epigenetic state of the AA VS1 locus, and in cell-type-specific bias for DSB repair pathways. To determine whether these ZFNs could drive gene addition in primary human cells, we generated integration-defective lentiviruses carrying the ZFNs and the donor (Lombardo et al. 2007); infection of hTERT-immortalized human fibroblasts (Rubio et al. 2002) resulted in ~3% ORF addition to the AA VS1 locus as gauged both by genotyping and phenotyping (Supplemental Fig. 5, A and B, respectively).
Functional consequences of deleting or changing the exact sequence to corticosteroids (Hager et al. 2009). Using this approach, the NHR superfamily that forms part of a complex response circuit glucocorticoid receptor (GR), an extensively studied member of the 

cis in a defined genomic context with predetermined differences in isogenic chromosomal location. In this case, integration at the receptors (NHRs) (Meijsing et al. 2009) can be performed at an allosteric effects of DNA on function by nuclear hormone reporter constructs—that has been used to study, among other things, that functionally interact with the GBS can be assayed. Furthermore, recruitment of GR to such integrated reporters can be determined by chromatin immunoprecipitation (ChIP), an assay that is challenging to perform in transient transfection experiments.

A panel of donor constructs was assembled (Fig. 4A), each harboring a distinct GR response element (GRE) of ∼1000 bp derived from endogenous GR target genes (Gerber et al. 2009) upstream of a basal promoter (FKBPS and TSC22D3, alias GILZ) or harboring its own endogenous promoter (SCNN1A, alias ENAC), driving the expression of a luciferase reporter gene. In all cases, the donor retains the promoterless GFP cassette, such that if site-specific gene addition is successful, GFP expression, which is driven by the native PPIR12C promoter, would be indicative of the presence of the reporter construct at the same locus.

An isogenic panel of U2OS cells expressing GR (Rogatsky et al. 1997) were transiently transfected with AA VS1-targeting ZFNs and each donor construct; GFP-positive cell pools for each donor were isolated, single-cell-derived clones produced without drug selection, and genotyped at the AA VS1 locus. In agreement with data on single-insert cassettes (Fig. 1), of nine randomly chosen single-cell-derived clones that expressed the luciferase reporter, all nine carried the desired donor-specified reporter cassette at the AA VS1 locus in either a monomorphic or diallelic configuration (Fig. 4C). Representative clones carrying each reporter were then treated with dexamethasone, a potent synthetic ligand for the GR. The reporters recapitulated (Fig. 4D) hormone-dependent activation of the endogenous target genes from which the regulatory sequence was derived (Gerber et al. 2009) and displayed distinct magnitudes of transcriptional up-regulation upon ligand treatment (Fig. 4D, cf. “SCNN1A” and “FKBPS” samples). Moreover, ligand-dependent activation was dependent on the presence of an intact GR binding site, as deleting the GR binding site of the SCNN1A GRE (Sayegh et al. 1999) ablated ligand-dependent transcriptional activation (Fig. 4D, sample “SCNN1AΔ”); the same observation was made with a line carrying the TSC22D3 promoter lacking GREs (Fig. 4D, sample “TSC22D3Δ”). Further, hormone-dependent recruitment of GR to the reporter constructs was observed by chromatin immunoprecipitation assays (Supplemental Fig. 6).

Taken together, the data show that the AA VS1 locus can be used to rapidly construct isogenic panels of cells harboring chromosomal reporters of steroid hormone receptor function that recruit the receptor and exhibit a biologically relevant response to hormone.

Isogenic panels of K562, HEK293, and hES cells carrying functional shRNA cassettes

Next, we set out to determine whether the AA VS1 locus would allow the long-term function of shRNA expression cassettes. In budding yeast, genome-wide reverse genetics experiments are conducted by systematic gene knockout in an isogenic background (Giaever et al. 2002). The recent development of several whole-genome collections of expression vectors for shRNAs (Paddison et al. 2004; Root et al. 2006; Berns et al. 2007) has, for the first time, allowed loss-of-function screens in human cells. These are currently performed in transient settings or by the random integration of the shRNA construct via lentivirus transgenesis.

In a pilot study (Supplemental Fig. 7), we used a donor construct carrying promoterless GFP upstream of a U6-promoter-driven shRNA expression cassette targeting the cell surface marker CD58 (known to be expressed in K562 cells). Cells were transfected...
with AAVS1-targeting ZFNs and this donor, GFP-positive cells (~8% of total cells; EA Moehle, data not shown) isolated by FACS, and this cell pool was found to contain ~80% chromatids transgenic at AAVS1 (Supplemental Fig. 7B, lane 3; see also Supplemental Discussion). To evaluate the efficacy of the inserted shRNA cassette, we compared control cells and AAVS1 ZFN/donor modified cells by FACS staining for CDS8—the shRNA target molecule (Supplemental Fig. 7C). Cell-surface staining for CDS8 was significantly reduced even after 30 cell population doublings (Supplemental Fig. 7C, last sample) and was comparable in magnitude to that seen 48 h post-transient transfection with the shRNA expression plasmid itself (Supplemental Fig. 7C, third sample); cells carrying GFP at the AAVS1 locus were indistinguishable in their CDS8 levels from control cells (Supplemental Fig. 7C).

We next constructed a panel of donor plasmids (Fig. 5B), each harboring an expression cassette for a shRNA directed against three distinct genes in the mammalian target of rapamycin (mTOR) pathway (Sancak et al. 2008). Plasmids encoding the AAVS1-specific ZFNs and the individual donor DNA constructs were transfected into HEK293 cells, and GFP-positive cells isolated by FACS. Genotyping of each cell pool revealed significant single-step enrichment for cells carrying each shRNA at the AAVS1 locus (RC DeKelver and VM Choi, data not shown).

Western blotting of the pool of GFP-positive cells was performed to assess protein levels for the shRNA-targeted gene products. For two of the three shRNA targets studied (TP53 and RPTOR) we observed lower target protein levels than control cells (Y Sancak and DM Sabatini, data not shown). This was not the case for either of the RRAAGC-targeting shRNA constructs we tested, despite comparable transgenesis levels. We next isolated and genotyped a panel of GFP-positive single-cell-derived clones for each shRNA and identified clonal cell lines monoallelic and diallelic for gene addition at AAVS1 (Fig. 5C, top). In the case of all three gene targets, single-cell-derived clones carrying diallelic gene addition of the relevant shRNA cassette exhibited a significant knockdown of the target protein level (Fig. 5C, bottom). Of note, ~8 wk of continued passaging had elapsed since the cells were initially treated with the ZFNs and the donor cassette. Since ZFN-driven gene addition occurs within 24–48 h of transient transfection, these data indicate that the AAVS1 locus provides a suitable genomic environment for long-term function by shRNA expression cassettes.

In earlier work (Hockemeyer et al. 2009) and in Figure 3 we have demonstrated the feasibility of ZFN-driven addition of transgenes carrying RNA pol II promoters to AAVS1 in hiPS and hES cells. We next asked whether the AAVS1 locus could function as a safe harbor for shRNA expression cassettes in hESCs. Single-cell-derived hESC clones carrying shRNAs directed against TPS3, DNMT1, or control shRNAs were generated using ZFNs and donor constructs (Fig. 5D). In agreement with nucleus-wide data from transformed cells (Supplemental Fig. 3), Southern blotting showed that >90% of hESC clones that carry the desired transgene at AAVS1 lack additional random donor integrants (Supplemental Fig. 8; Supplemental Discussion). Clones were expanded, mRNA isolated, and expression levels of the gene targeted by the shRNAs measured (Fig. 5E,F). In the case of both genes, single-cell-derived clones carrying target-specific, but not control shRNA expression cassettes, exhibited stable long-term knockdown of mRNA levels (Fig. 5E,F); importantly, hESC clones carrying DNMT1-targeted shRNAs transcribed POU5F1, a key marker of pluripotency, to the same level as control cells (Fig. 5E, right).

Taken together, these data reveal the feasibility of using a ZFN-driven “safe harbor” gene addition process to rapidly, and in some
cases without drug selection, obtain a panel of isogenic cells carrying investigator-specified shRNAs at a defined location, and exhibiting robust knockdown of the mRNA (Fig. 5E) or protein (Fig. 5C) encoded by the gene that the shRNA targets.

Discussion

Isogenic settings are standard in experimental systems such as budding yeast, Drosophila, and laboratory mice, where they can be achieved via well-established protocols (e.g., see Osborne et al. 2009). The work we describe adds transgenesis in human transformed and primary cells to the list of such systems. Gene addition occurs in a single step within 48 h of transient transfection, functions across a range of transformed human cell types commonly used in laboratory practice, in fibroblasts and hES cells (this work) and in induced pluripotent formed human cell types commonly used in laboratory practice, in fibroblasts and hES cells (this work) and in induced pluripotent

Earlier data on gene targeting (Smith et al. 2008) or ZFN-driven gene addition to AAVS1 (Hockemeyer et al. 2009) showed that hES and iPS cells disrupted at that locus retain pluripotency. Further, ZFN-edited human ES cells carrying transgenes at the AAVS1 locus retained a normal karyotype, expression of pluripotency markers, and remained pluripotent as well as wild type at a panel of putative off-target sites (Hockemeyer et al. 2009). In our experiments, K562, Hep3B, HEK293, U2OS, fibroblast, and hES cells carrying transgenes at AAVS1 proliferated indistinguishably from control cells; the hES cells shown growing and dividing normally in Supplemental movie 1, for instance, carry PPP1R12C-disrupting transgenes at both alleles. This said, it remains formally possible that some pathway may be adversely affected by the lack of regulatory subunit 12C of protein phosphatase 1 (which is thought to regulate actomyosin-based contractility) (Mulderr et al. 2004). If such a pathway is found, which could be attempted, for instance, by genome-wide expression analysis of edited cells or by knocking out the orthologous gene in mouse or in rat (Geurts et al. 2009), we have shown that single-cell-derived clones possessing one wild-type and one transgenic AAVS1 allele can be readily isolated (e.g., see Figs. 2, 4, 5) (in a typical experiment, 60%–70% of edited cells fall into that category). We are unaware of evidence that PPP1R12C is haploinsufficient.

We describe three applications for isogenic transgenesis into a “safe harbor.” We show that four distinct coding regions—two promoterless and two with their own promoters—can be introduced site specifically in allelic positions at the AAVS1 locus to generate an inducible expression system (Fig. 3). In the present work, we describe hES cells carrying a histone H2B-eGFP fusion in such an inducible setting. Studies by Grunstein and colleagues (Kayne et al. 1988) launched an ever-growing line of genetic investigation into histone structure and function; the system we describe allows the
rapid construction of a panel of isogenic cells carrying, for instance, an allelic series of histone genes (see also Goldberg et al. 2010). More generally, high-throughput proteomic (e.g., structure-function) studies can be performed using this approach in a setting that is normalized across different cell lines for expression of the transgene and its integration site in the genome. The native

Figure 5. Use of ZFNs to generate a panel of isogenic HEK293 cells and hESCs carrying distinct shRNA expression cassettes at the AAVS1 locus. (A) Experimental outline. (B) Schematic of donor construct for the HEK293 experiment. Gene elements are represented in the same way as in Figure 1B. ShRNA cassettes are driven by a pol III promoter, which is annotated as a white box with an arrow, followed by a red box. (C) Genotypes of the AAVS1 locus (top) and protein expression (bottom) of a panel of single-cell derived HEK293 cell clones. The clones were obtained by FACS and genotyped using primers that lie outside the region of homology with the donor construct (schematic of PCR to the left of the autoradiograph). In all cases, the upper band corresponds to the transgenic, and the lower to the wild-type chromatid, respectively. In a small subset of cases (indicated by asterisks), the clone contains an additional allele of the AAVS1 locus, most likely the result of a DSB-induced deletion. The indicated clones were assayed by Western blot (bottom) for levels of proteins encoded by genes targeted by the indicated shRNAs. A Western blot for a loading control (α-tubulin) is shown at the bottom. (D) Schematic overview depicting the editing strategy for adding shRNA expression cassettes to the AAVS1 locus in hES cells. Annotations are as in Figure 1B. (E) Real-time PCR data (normalized to GAPDH mRNA) for DNMT1 (left) or POUSF1 (right) in pools of single-cell-derived hES clones carrying control shRNAs or three distinct shRNAs directed against DNMT1. See Supplemental Figure 8 for Southern blot clone genotyping data. (F) As in panel E, but with TP53 as the shRNA target. Note that in E and F, for shRNA construct no. 2 for each gene target, a single-cell-derived hES clone was analyzed in this experiment.
PP1RI2C promoter is active in all cells where we and others have studied this issue; in our hands, K562 and HeLa cells carrying promoterless GFP transgenes at AAVS1 show lower mean fluorescence intensity than such cells carrying the same gene driven by the PGK promoter (VM Choi and EA Moehle, data not shown). While weaker by this criterion, the native PP1RI2C promoter drives sufficient transcription to allow FACS or selection-based isolation of pools and clones of cells with correctly added promoterless markers (e.g., Figs. 2–5).

We also show that the AAAS1 locus can be used to carry a panel of reporter constructs for the study of transcriptional cis-regulatory elements (Fig. 4). In the wake of initial studies in the 1980’s (Zaret and Yamamoto 1984), a significant body of evidence has been accumulated that connects targeted chromatin remodeling and modification to action by nuclear hormone receptors (Stallcup et al. 2003; Hager et al. 2009) and most transcription factors where this issue has been studied. The ability to place investigator-specified reporters into the AAAS1 locus offers an opportunity to study genome control on templates chromatinized via normal physiological pathways; preliminary studies (SH Meijsing, unpubl.) show the reporter transgenes exhibit kinetics of induction by hormone and epigenetic marks similar to those observed at native GR gene targets.

Finally, we demonstrate that the AAAS1 locus serves as a safe harbor for shRNA expression cassettes in transformed and hES cells (Fig. 5). Functional genomics using RNAi is an important research tool and is commonly performed in transient settings where RNAi hairpins or transient transfection approaches suffice. The data we show demonstrate the feasibility of rapidly obtaining single-cell-derived clones isogenic except for a construct expressing a shRNA hairpin against the gene of choice. Robust target gene knockdown is still observed 2 mo after transgenesis. In transformed cells, linear donors with short homology arms produced by PCR may be used (Orlando et al. 2010), or ZFNs and a donor construct can be combined onto the same plasmid without significant loss of efficiency (Supplemental Fig. 9), illustrating two simple paths to establishing genome-wide screening with shRNA constructs stably integrated into the AAAS1 locus.

These data, therefore, expand the toolbox of human somatic cell genetics to include transgenesis in isogenic settings.

Methods

Zinc finger nucleases and donor constructs

ZFNs against the AAAS1 locus (Hockemeyer et al. 2009) were used in their obligate heterodimer, high-fidelity FokI (Miller et al. 2007) form cloned into pVAX as a 2A fusion construct (Perez et al. 2008). Donor constructs for RFLP addition and gene addition were assembled exactly as described (Moehle et al. 2005; Moehle et al. 2007; see also below); shRNA expression constructs for the experiment shown in Figure 5A were obtained from Sigma-Aldrich.

Cell culture

Cell culture techniques for K562, HeLa, and HEK293 cells (Moehle et al. 2007), U2OS cells (Meijsing et al. 2009), hTERT fibroblasts (Lombardo et al. 2007), and hESCs (Hockemeyer et al. 2008) have been described previously. All other cell types were cultured as per ATCC guidelines.

Genome editing in transformed cells and in fibroblasts

ZFN expression and donor constructs, assembled using standard recombinant DNA techniques (detailed protocols are available from the authors upon request) were introduced into transformed cells by nucleofection (Amaxa) exactly as described (Urnov et al. 2005; Moehle et al. 2007) and into hTERT fibroblasts using integration-defective lentivirus generated exactly as described (Lombardo et al. 2007). Genomic DNA was harvested using QuickExtract (Epitentre) for PCR-based assays and DNeasy (Qiagen) for Southern blotting performed exactly as described (Urnov et al. 2005; Moehle et al. 2007); for the Southern blot shown in Figure 1, genomic DNA was digested with Accl. Targeted integration frequency was measured using a body-labeled PCR assay as described (Moehle et al. 2007) with minor modifications. One hundred nanograms of genomic DNA was amplified (forward primer: CCGAATCTGCCTCTAAGCG; reverse primer, CTGGGAT ACCCCGAAAGTG—both anneal to the chromosome outside the region of homology with the donor) in the presence of μCi each of [α-32P]dCTP and [α-32P]dATP using Accuprime HiFi Taq polymerase (Invitrogen) and the following PCR settings: 95°C, 3 min; 24 cycles of 95°C, 30 sec; 62°C, 30 sec; 68°C, 4 min; 68°C, 7 min. The radiolabeled PCR and products were then purified using a column-based kit (Qiagen) and resolved on a 5% nondenaturing PAGE; the gel was dried and analyzed using a PhosphorImager (Molecular Dynamics). Cells were phenotyped for fluorescence using a Guava benchtop flow cytometer exactly as described (Moehle et al. 2007).

Live cell imaging

Targeted hESCs (BG01 [NIH Code: BG01; BresaGen, Inc.]) were cultured for the duration of imaging on mitomycin C inactivated mouse embryonic fibroblast (MEF) feeder layers and on glass-bottom dishes (MatTek Corporation) in CO2 independent medium (Gibco supplemented with 15% fetal bovine serum (FBS) (Hyclone), 5% KnockOut Serum Replacement (Invitrogen), 1 mM glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 4 ng/mL FGF2 (R&D systems). Cells were cultured and maintained in the presence of 2 μg/mL doxycycline for more than 2 wk prior to imaging to induce and maintain H2B-eGFP expression. Cells were imaged over a period of 20 h using a Detavision microscope (40× objective) and a HQ2 camera. Time lap intervals were 4 min, and seven 1.5 μm Z-stack images were taken for each time point with an exposure time of 0.25 sec and 10% transmittance.

Luciferase reporter assays and Western blotting

For reporter activity assays, single-cell derived clonal lines were seeded into 24-well plates in DMEM/5% FBS at ~20,000 cells per well. Cells were cultured and maintained in the presence of 2 μg/mL doxycycline for 20 h before imaging to induce and maintain H2B-eGFP expression. For Western blotting, hESC extracts were electrophoresed on a 7.5% gel, which was then electrotransferred to nitrocellulose (Bio-Rad). After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS), the blots were probed with primary and secondary antibodies. After washing in TBS containing 0.1% Tween 20 (TBST), the blots were exposed to an X-ray film. The signal was quantified with the NIH Image J program.
well and treated the next day with either vehicle (ethanol) or 100 nM dexamethasone. After treatment for ~13 h, cells were lysed in 100 μL per well of 1× lysis buffer (PharMingen) and assayed for luciferase activity, which was normalized for cell number. Western blotting was performed as described (Sancak et al. 2008).

Reverse transcription of total RNA and real-time PCR

RNA was isolated from hESCs, which were mechanically separated from feeder cells using TRizol extraction and subsequent precipitation. Reverse transcription was performed on 1 μg of total RNA using oligo dT priming and thermostable reverse transcriptase at 50°C (Invitrogen). Real-time PCR was performed in an ABI Prism 7900 (Applied Biosystems) with Platinum SYBR green pPCR SuperMIX-UDG with ROX (Invitrogen). Primers for the analysis of endogenous gene expressions were: hrdnmt1_F ggttcagcaaaaccaactcatgacca. The latter two from Boyle et al. (2000). Gene expression was normalized using GAPDH primers: hrgapdh_F cagtcttctgggtggcagtga; hrgapdh_R cgtggaaggaggatgtggtcc; hrtoct4_R cgttgtgcatagtcgctgct; hrtp53_F gcccccagggctctatgatg; hrtdnmt1_R gccaagatttttgccattaacac; hrtoct4_F gctcgaga.

Acknowledgments

We thank Judith Campisi for the gracious gift of hTERT fibroblasts, William Hahn for proposing the experiment that resulted in the data shown in Figure 5, Edward Weinstein for shRNA reagents, and Edda Einfeldt for technical assistance. We deeply appreciate suggestions provided by Aaron Klug, Jasper Rine, and Michael Holmes, and thank the three anonymous referees for comments on the manuscript. D.H. is a Merck Fellow of the Life Science Research Foundation. R.J. was supported by US National Institutes of Health grants R37-CA084198, RO1-CA087869, and RO1-HD045022 and by the Howard Hughes Medical Institute. R.J. is an adviser to Stemgen and a cofounder of Fate Therapeutics. S.H.M. and K.R.Y. received research support from NIH grants, K.R.Y. is a paid consultant with Merck and Company. Y.S. and D.M.S. were supported by non-homologous recombination. EMBO J 11: 5071–5078.

References


B引起了教授的热烈反响。华威大学化学学院的教授表示，这项研究为理解细胞分化和基因表达提供了新的视角。


Received February 18, 2010; accepted in revised form May 11, 2010.