enChIP systems with MSCV-based retroviral plasmids expressing 3xFLAG-Sp-dCas9

Miyuki Yuno
Osaka Daigaku
Toshitsugu Fujita
Hirosaki Daigaku Igakubu Daigakuin Igaku Kenkyuka
Hodaka Fujii (hodaka@hirosaki-u.ac.jp)
Hirosaki University https://orcid.org/0000-0003-1296-4256

Research note

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Abstract

Objective: Engineered DNA-binding molecule–mediated chromatin immunoprecipitation (enChIP) is a technology for purifying specific genomic regions to facilitate identification of their associated molecules, including proteins, RNAs, and other genomic regions. In enChIP, the target genomic region is tagged with engineered DNA-binding molecules, e.g., a variant of the clustered regularly interspaced short palindromic repeats (CRISPR) system consisting of a guide RNA (gRNA) and a catalytically inactive form of Cas9 (dCas9). In this study, to increase the flexibility of enChIP and expand the range of target cells, we generated murine stem cell virus (MSCV)-based retroviral plasmids for expressing dCas9. Results: We constructed MSCV-based retroviral plasmids expressing Streptococcus pyogenes dCas9 fused to a 3xFLAG-tag (3xFLAG-Sp-dCas9) and various drug resistance genes. We showed that it is feasible to purify target genomic regions with high yields using these plasmids. These systems might give enChIP users greater flexibility in choosing optimal systems for drug selection of transduced cells. In addition, they could be used to analyze different types of target cells.

Introduction

To understand the molecular mechanisms underlying regulation of genome functions such as epigenetic regulation and transcription, it is necessary to the identify the regulatory molecules that binding to a genomic region of interest. We recently developed engineered DNA-binding molecule–mediated chromatin immunoprecipitation (enChIP) technology for isolation of genomic regions of interest to facilitate identification of their associated molecules [1, 2]. The engineered DNA-binding molecules that can be used to tag the target locus include transcription activator-like proteins [3] and a variant of the clustered regularly interspaced short palindromic repeats (CRISPR) system [4, 5] consisting of a guide RNA (gRNA) and a catalytically inactive form of Cas9 (dCas9) (see our recent review [6] for the CRISPR-based systems). Locus-tagging can be achieved by expression of engineered DNA-binding molecules (in-cell enChIP) [1, 2, 7-10]. Alternatively, it can be achieved *in vitro* using recombinant or synthetic engineered DNA-binding molecules (*in vitro* enChIP) [11, 12]. After a target locus is tagged using an engineered DNA-binding molecule, it is isolated by affinity purification. Subsequently, associated proteins are identified by mass spectrometry (MS) [1, 2, 7], and associated nucleic acids such as RNAs and other genomic regions are identified by next-generation sequencing (NGS) [8, 10, 12].

Previously, we generated retroviral plasmids expressing *Streptococcus pyogenes* dCas9 fused to a 3xFLAG-tag (3xFLAG-Sp-dCas9), based on pMXs-derived vectors [13]. These plasmids exhibited relatively high recombination rates during expansion in bacterial hosts. To overcome this issue, in this study we generated murine stem cell virus (MSCV)-based retroviral plasmids expressing 3xFLAG-Sp-dCas9 and various drug resistance genes. Using these plasmids, we were able to purify target genomic regions with high yields. These systems might give enChIP users greater flexibility in choosing optimal systems for drug selection of transduced cells. In addition, they could be used to analyze different types of target cells.

Materials And Methods

Plasmids

To construct MSCV expression plasmids expressing 3xFLAG-Sp-dCas9, the pMSCVneo, pMSCVhyg, and pMSCVpuro vectors (631461, Takara Bio, Kusatsu, Japan) were digested with *Bgl*II (1021A, Takara Bio) and *Xho*I (1094A, Takara Bio). After a blunting reaction, the plasmids were treated with bacterial alkaline phosphatase (*E. coli* C75) (2120A, Takara Bio). The cleaved vectors were purified by agarose gel electrophoresis and ligated with the coding sequence of 3xFLAG-Sp-dCas9, which was isolated from 3xFLAG-dCas9/pMXs-puro (Addgene (Watertown, MA, USA) #51240) [13] by digestion with *Pac*I (R0547, New England Biolabs, Ipswich, MA, USA) and *Not*I (1166A, Takara Bio).

To construct gRNA-hIRF-1 #12/pSIR-hCD2, pSIR-hCD2 (Addgene #51143) [13] was digested with *Eco*RI (1040A, Takara Bio) and treated with bacterial alkaline phosphatase. The cleaved vector was purified by agarose gel electrophoresis and ligated with the gBlock targeting the *interferon (IFN) regulatory factor (IRF)-1* gene promoter, which was isolated from gRNA-hIRF-1 #12 (Addgene #61079) [1] by digestion with *Eco*RI.

The Addgene plasmid # of the newly made constructs are as follows: 3xFLAG-Sp-dCas9/pMSCVneo: 134982; 3xFLAG-Sp-dCas9/pMSCVhyg: 134323; 3xFLAG-Sp-dCas9/pMSCVpuro: 134983; gRNA-hIRF-1 #12/pSIR-hCD2: 135392.

Cell lines

The 293T cell line was derived by transformation of human embryonic kidney (HEK) 293 cells with the SV40 large T antigen [14]. HT1080 is a human fibrosarcoma cell line purchased from ATCC (Manassas, VA, USA) (CCL-121) [15]. 293T, HT1080, and HT1080-derived cells were cultured in DMEM (FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal calf serum (FCS).

Transduction of retroviral plasmids

For transduction of retroviral plasmids expressing 3xFLAG-Sp-dCas9, 5.5 μg of each plasmid was transfected into 1×10^6 of 293T cells along with 5.5 μg of pPAM3 [16] using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Two days after transfection, viral supernatant was harvested and used for infection of HT1080 cells. Infected cells were selected in culture media containing G418 (Nacalai Tesque, Kyoto, Japan) (0.8 mg/ml), hygromycin (Nacalai Tesque) (75 μg/ml), or puromycin (Nacalai Tesque) (0.75 μg/ml).

For transduction of retroviral plasmids expressing the gRNA, 5.5 μg of gRNA-hIRF-1 #12/pSIR-hCD2 was transfected into 1×10^6 of 293T cells along with 5.5 μg of pPAM3 using Lipofectamine 3000. Two days after transfection, viral supernatant was harvested and used for infection of HT1080 cells expressing 3xFLAG-dCas9. Cells expressing 3xFLAG-Sp-dCas9 and gRNA were sorted by MACS as described below.

Immunoblot analysis

Cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Ten micrograms of cytoplasmic extract was subjected to immunoblot analysis with anti-FLAG M2 antibody (Ab) (F1804, Sigma-Aldrich, Saint Louis, MO, USA) as described previously [1].

MACS sorting and flow cytometry

MACS sorting of human CD2 (hCD2) (+) cells was performed using CD2 Microbeads (130-091-114, Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany). To monitor the results of MACS sorting, cells were stained with phycoerythrin (PE)-conjugated anti-hCD2 Ab (347597, BD Life Sciences, San Jose, CA, USA) for 30 min at 4°C. Flow cytometry was performed on a FACSCalibur (BD Life Sciences), and data were analyzed using the FlowJo software (BD Life Sciences).

enChIP-real-time PCR

enChIP-real-time PCR was performed as previously described [13]. The primers used in this study were reported previously [1].

Results And Discussion

MSCV retroviral expression systems for enChIP

To increase the flexibility of enChIP and expand the range of target cells, we constructed retroviral plasmids expressing *S. pyogenes* dCas9 fused with the 3xFLAG-tag (3xFLAG-Sp-dCas9), using the MSCV system along with various drug selection markers.

To confirm the performance of the system, we transduced the retroviral plasmids into HT1080, a human fibrosarcoma cell line. After drug selection, expression of 3xFLAG-Sp-dCas9 was confirmed by immunoblot analysis with antibody against the FLAG-tag (Fig. 1a). Subsequently, gRNA-hIRF-1 #12/pSIR-hCD2, a self-inactivating retroviral vector expressing a sgRNA targeting human *IRF-1* gene promoter and the human CD2 selection marker protein, was transduced into the HT1080 cells expressing 3xFLAG-Sp-

dCas9. Two days after transduction, cells expressing the sgRNA were selected by MACS sorting. After expansion, 2×10^6 cells were subjected to enChIP analysis. Briefly, the cells were crosslinked with formaldehyde, and the crosslinked chromatin was fragmented by sonication. Subsequently, fragmented chromatin tagged with the CRISPR complex was purified using anti-FLAG antibody. As shown in Fig. 1b, the yields of enChIP were high and comparable to those obtained by other systems. These results revealed that the enChIP system using the MSCV retroviral vectors can be used for downstream applications such as identification of molecules associated with the target genomic regions.

Conclusions

In this study, we developed enChIP systems using MSCV-based retroviral expression vectors with various selection markers. These systems might give enChIP users greater flexibility in choosing optimal systems for drug selection of transduced cells. In addition, they could be used for analysis of different types of target cells.

Limitations

Further studies might be necessary to assess the utility of these systems in combination with MS and NGS to identify molecules associated with target genomic regions.

List Of Abbreviations

enChIP: engineered DNA-binding molecule-mediated chromatin immunoprecipitation

CRISPR: clustered regularly interspaced short palindromic repeats

dCas9: a catalytically-inactive form of Cas9

gRNA: guide RNA

MS: mass spectrometry

NGS: next-generation sequencing

MSCV: murine stem cell virus

IFN: interferon

IRF-1: interferon regulatory factor-1

HEK: human embryonic kidney

FCS: fetal calf serum

Ab: antibody

hCD2: human CD2

PE: phycoerythrin

NLS: nuclear localization signal

CBB: Coomassie Brilliant Blue

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Competing interests

T.F. and H.F. have patents on enChIP (Patent name: Method for isolating specific genomic region using molecule binding specifically to endogenous DNA sequence; Patent number: Japan 5,954,808; Patent application number: WO2014/125668). T.F. and H.F. are founders of Epigeneron, Inc.

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Authors’ contributions

H.F. conceived the idea of development of enChIP systems using MSCV-based retroviral expression vectors with different selection markers and constructed expression plasmids. T.F. and H.F. designed and performed experiments and wrote the manuscript. M.Y. performed experiments. H.F. directed and supervised the study. All authors read and approved the final manuscript.

Acknowledgement

Not applicable.

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Figures

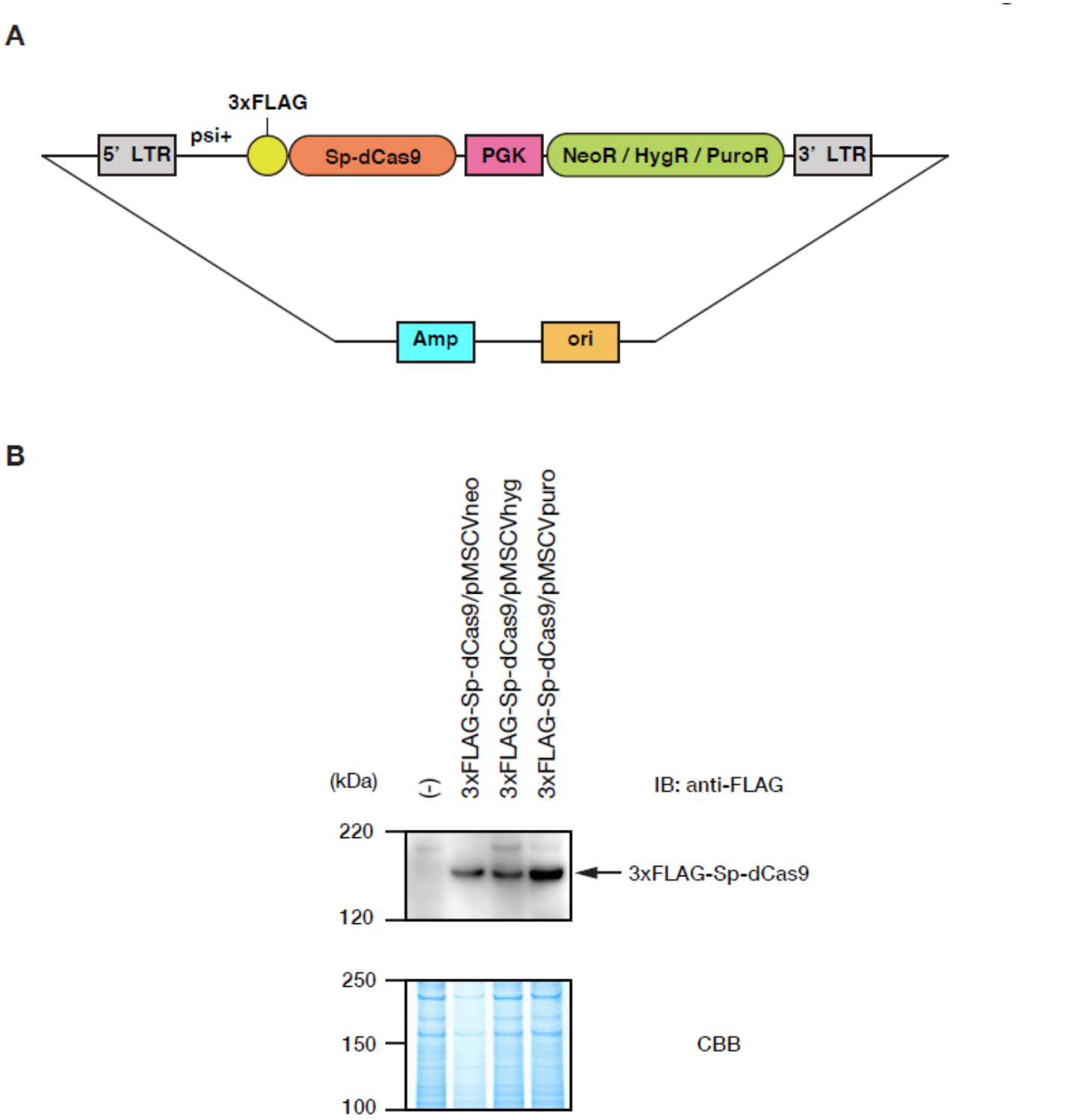

Figure 1

enChIP systems using MSCV-based retroviral expression vectors. (a) Schematics of MSCV-based retroviral expression constructs of 3xFLAG-Sp-dCas9. (b) Expression of 3xFLAG-Sp-dCas9. Immunoblot analysis was performed with anti-FLAG Ab. Coomassie Brilliant Blue (CBB) staining is shown as a protein loading control.

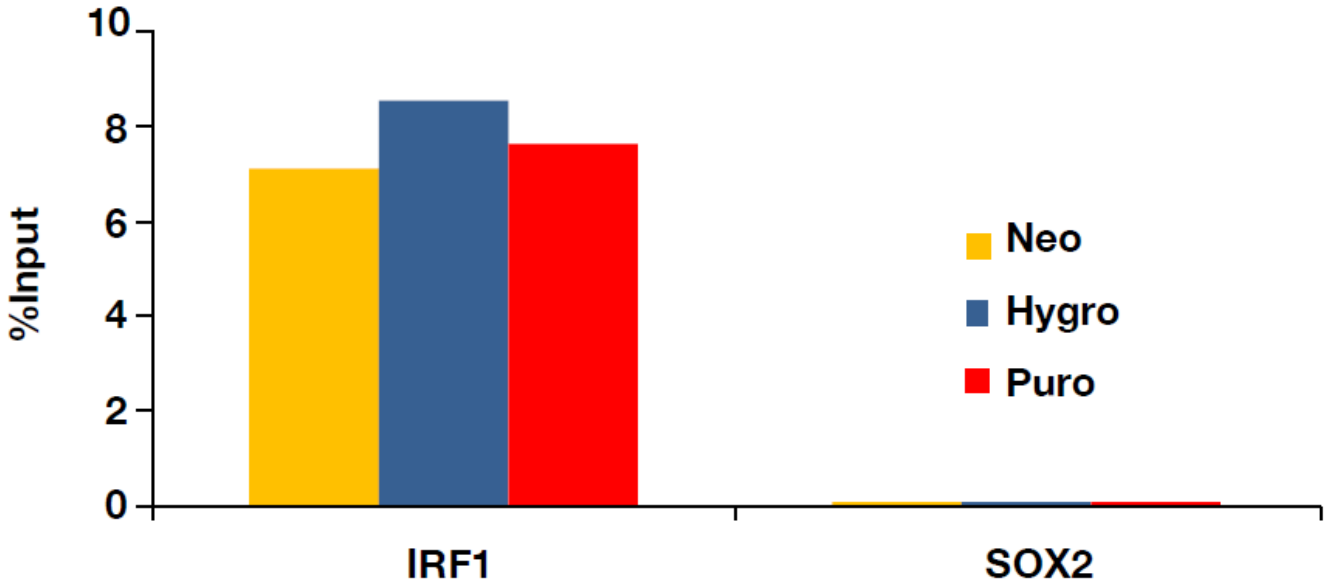

Figure 2

Isolation of the IRF-1 locus by enChIP systems using MSCV-based retroviral expression vectors. Real-time PCR analysis was performed on chromatin complexes isolated by enChIP. An irrelevant locus (SOX2) was analyzed as a negative control.