Efficient sequence-specific isolation of DNA fragments and chromatin by \textit{in vitro} enChIP technology using recombinant CRISPR ribonucleoproteins

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The clustered regularly interspaced short palindromic repeats (CRISPR) system is widely used for various biological applications, including genome editing. We developed engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR to isolate target genomic regions from cells for their biochemical characterization. In this study, we developed '\textit{in vitro} enChIP' using recombinant CRISPR ribonucleoproteins (RNP)s to isolate target genomic regions. \textit{In vitro} enChIP has the great advantage over conventional enChIP of not requiring expression of CRISPR complexes in cells. We first showed that \textit{in vitro} enChIP using recombinant CRISPR RNP can be used to isolate target DNA from mixtures of purified DNA in a sequence-specific manner. In addition, we showed that this technology can be used to efficiently isolate target genomic regions, while retaining their intracellular molecular interactions, with negligible contamination from irrelevant genomic regions. Thus, \textit{in vitro} enChIP technology is of potential use for sequence-specific isolation of DNA, as well as for identification of molecules interacting with genomic regions of interest \textit{in vivo} in combination with downstream analysis.

Introduction

The advent of engineered DNA-binding molecules, such as zinc finger proteins, transcription activator-like effector (TAL or TALE) proteins, and the clustered regularly interspaced short palindromic repeats (CRISPR) system, has enabled high efficiency genome editing (Gaj et al. 2013; Mali et al. 2013; Sun & Zhao 2013; Doudna & Charpentier 2014; Harrison et al. 2014; Hsu et al. 2014; Pauwels et al. 2014; Wijshake et al. 2014). Moreover, the use of such engineered DNA-binding molecules is not limited to genome editing; they are used for various applications, including artificial transcriptional regulation, epigenetic modification, locus imaging, and isolation of specific genomic regions for the analysis of locus-specific genome functions (Mali et al. 2013; Harrison et al. 2014; Hsu et al. 2014; Fujii & Fujita 2015; Fujita & Fujii 2015a). Among these engineered DNA-binding molecules, the CRISPR system is the most convenient, economical, and time-efficient and has rapidly risen to prevail over technologies with similar functions since its introduction for use in genome editing.

To elucidate the molecular mechanisms underlying genome functions such as transcription and epigenetic regulation, it is necessary to identify molecules interacting with genome regions of interest \textit{in vivo}. To this end, we developed locus-specific chromatin immunoprecipitation (locus-specific ChIP) technologies, consisting of insertion ChIP (iChIP) and engineered DNA-binding molecule-mediated ChIP (enChIP) (Hoshino & Fujii 2009; Fujita & Fujii 2011, 2012, 2013a,b, 2014a,b, 2015b; Fujita et al. 2013, 2015a,b). In enChIP, an engineered DNA-
binding molecule, such as a TAL protein or the CRISPR complex [guide RNA (gRNA) and dCas9, a nuclease-deficient mutant of Cas9], is expressed, often as an epitope tag-fused form, for locus tagging in the cell to be analyzed. The targeted locus can be isolated by affinity purification using an antibody (Ab) against the epitope tag or the engineered DNA-binding molecule itself (Fig. S1 in Supporting Information). Previously, we successfully used enChIP combined with mass spectrometry and RNA sequencing (RNA-seq) for the nonbiased identification of proteins and RNAs interacting with the interferon regulatory factor-1 (IRF-1) promoter region and telomere regions in vivo (Fujita & Fujii 2013a, 2014b; Fujita et al. 2013, 2015b).

In addition to the aforementioned conventional in vivo enChIP, we reported previously that using enChIP with recombinant engineered DNA-binding molecules, such as a TAL protein, it is feasible to isolate a genomic region of interest from a cell (Fujita & Fujii 2014a). This ‘in vitro enChIP’ system has the great advantage over conventional enChIP of not needing to express engineered DNA-binding molecules in vivo. However, in the in vitro enChIP assay using the TAL protein, we observed significant degradation of the recombinant TAL protein, and the DNA yields from the target locus were modest (Fujita & Fujii 2014a). Development of in vitro enChIP technology that facilitates more efficient and specific isolation of target genomic regions would allow the application of this technology to the faster and simpler identification of molecules interacting with target genomic regions.

In this study, we developed an in vitro enChIP method using recombinant CRISPR ribonucleoproteins (RNPs) and showed that it can be used to isolate target DNA from mixtures of purified DNA. In addition, the system was applicable to the efficient and specific isolation of target genomic regions from cell lysates. Thus, in vitro enChIP using recombinant CRISPR RNPs has potential uses as a sequence-specific DNA isolation tool and, in combination with downstream analysis, in identification of molecules interacting with genomic regions of interest in vitro.

Results and Discussion

Overview of in vitro enChIP using recombinant CRISPR RNPs

Our protocol for in vitro enChIP using recombinant CRISPR RNPs is as follows: (i) A recombinant 3xFLAG-dCas9-D protein (r3xFLAG-dCas9-D) consisting of a 3xFLAG-tag, a nuclear localization signal (NLS), dCas9, and a Dock-tag, is incubated with synthetic gRNA [e.g., a chimeric single guide RNA (sgRNA)] to form recombinant CRISPR RNPs. (ii) The cell to be analyzed is cross-linked with formaldehyde or another cross-linker, if necessary. The cell is lysed, and the chromatin is fragmented. (iii) The target genomic region is captured in vitro by the CRISPR RNPs and affinity-purified with anti-FLAG Ab conjugated to carriers. After isolation, the chromatin components (DNA, RNA, proteins, and other molecules) interacting with the target genomic region can be identified by downstream analysis, such as next-generation sequencing (NGS), microarray, or mass spectrometry (Fig. 1).

In addition to Ab-based purification, the biotin–avidin system (Diamandis & Christopoulos 1991) can also be used for affinity purification in in vitro enChIP using r3xFLAG-dCas9-D and biotinylated gRNA (Fig. S2 in Supporting Information) (see below).

Isolation of DNA fragments in a sequence-specific manner by in vitro enChIP using recombinant CRISPR RNPs

First, we generated the r3xFLAG-dCas9-D protein using the silkworm–baculovirus expression system. In contrast to the TAL protein (Fujita & Fujii 2014a), r3xFLAG-dCas9-D could be purified without visible degradation (Fig. 2A). Next, we evaluated in vitro enChIP using the purified r3xFLAG-dCas9-D with synthesized gRNA targeting the IRF-1 promoter region (Fig. 2B,C). Using fragmented purified 293T genomic DNA as input, the IRF-1 region was enriched compared with nontarget control regions (Fig. 2D,E). Approximately half of the total amount of the target region was efficiently enriched by in vitro enChIP combined with Ab-based (Fig. 2D) or biotin–avidin (Fig. 2E) purification system. In addition, we succeeded in specific isolation of a target PCR product from mixtures of DNA fragments (Fig. 2F). These results suggest that in vitro enChIP technology can be applied to sequence-specific concentration or removal of target DNA from mixtures of DNA such as fragmented genomic DNA and PCR products.

We envisage various applications of the in vitro enChIP technology as a sequence-specific DNA isolation tool. For example:
1. Sequence-specific purification of PCR amplicons and plasmids: In suboptimal experimental conditions, PCR may amplify not only a target DNA region but also nonspecific sequences. If the molecular weight of the target amplicon is similar to those of nonspecific products, it is difficult to separate the target amplicon from unwanted products by gel electrophoresis. In such cases, in vitro enChIP could be used to isolate the target amplicon in a sequence-specific manner. In vitro enChIP could also be used for sequence-specific isolation of plasmids containing target DNA from a cDNA or genomic DNA library.

2. Enrichment of DNA fragments with specific inserted elements: In addition, the technology could be applied for sequence-specific concentration of DNA fragments containing specific inserted elements, such as viral, plasmid, or transposon sequences, from purified genomic DNA; NGS analysis using DNA concentrated by in vitro enChIP could identify the genomic positions of the integrated sequences with fewer reads.

3. Removal of unwanted DNA in transcriptome analysis, metagenomics, and analysis of microbial flora: In transcriptome analysis using RNA-seq, cDNA synthesized from abundant ribosomal RNA may dominate sequencing reads. Similarly, in de novo genome sequencing, repetitive sequences such as telomeric repeats may complicate sequence...
Figure 2 Isolation of DNA fragments in a sequence-specific manner by in vitro enChIP using recombinant CRISPR RNPs. (A) Preparation of r3xFLAG-dCas9-D. Purified proteins were subjected to SDS-PAGE and Coomassie Brilliant Blue staining (CBB) (left panel) or immunoblot analysis with anti-Dock Ab (IB) (right panel). Marker: molecular weight marker; supernatant: the supernatant prepared from silkworm pupal homogenates; precipitant: the insoluble precipitate prepared from silkworm pupal homogenates; eluate: the eluate after affinity purification. (B) sgRNA, CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA) used in this study. Sequences highlighted in red are complementary to a target genomic sequence. (C) Positions of primers used in this study to amplify target (IRF-1, chromosome 5) and nontarget control (5'H5S5, chromosome 11) genome regions. The site targeted by in vitro enChIP is indicated by the red arrow. (D, E) The DNA yields from target (IRF-1) and nontarget control (5'H5S5) genome regions after in vitro enChIP combined with the Ab-based (D) or biotin–avidin (E) purification system. Purified genomic DNA fragments prepared from 293T cells were used for quantitative PCR. (F) Results of isolation of a target PCR product from a mixture of DNA fragments by in vitro enChIP combined with the biotin–avidin purification system (+) and a negative control where r3xFLAG-dCas9-D, crRNA, and tracrRNA were omitted (−). Input: the mixture of DNA fragments including the target PCR product (arrow); purified: the DNA purified by in vitro enChIP; supernatant: the supernatant after the in vitro enChIP procedure; M: molecular weight marker.
assembly. In these cases, removal of such undesirable DNA by in vitro enChIP could improve the sequencing resolution obtained. For analysis of microbial flora, sequencing of microbial 16S rDNA is used for species identification. It can be difficult to accurately detect rare microbes by NGS because the 16S rDNA of major microbial populations dominates in the analysis. Removal of such dominant 16S rDNA by in vitro enChIP could facilitate identification of rare microbe species.

Currently, a DNA hybridization using biotinylated DNA probes combined with the biotin–avidin purification system is used for a number of the aforementioned applications (Diamandis & Christopoulos 1991; Ito et al. 1992; Mangiapan et al. 1996; St. John & Quinn 2008; Camara Teixeira et al. 2013; Gagan & Van Allen 2015); however, in vitro enChIP technology is a potentially useful alternative method that has potential advantages over existing DNA hybridization methods. For example, in vitro enChIP can isolate target double-stranded DNA (dsDNA) without the need for the denaturing step required in DNA hybridization approaches. In addition, because CRISPR can distinguish single nucleotide polymorphisms (Yoshimi et al. 2014; Smith et al. 2015), in vitro enChIP could be used for the differential isolation of target DNA with a single nucleotide difference. DNA hybridization methods would require considerably more time and effort to optimize the hybridization conditions (e.g., temperature) to achieve the same result.

**Isolation of target chromatin complexes in a sequence-specific manner by in vitro enChIP using recombinant CRISPR RNP**

Finally, we applied the in vitro enChIP technology combined with the Ab-based purification system to the isolation of target genomic regions retaining molecular interactions from cells. When we used native chromatin prepared from the human 293T cell line and a 100-mer sgRNA, the IRF-1 region was efficiently enriched compared with nontarget control regions (Fig. 3A). The DNA yields (% of input) of the target region were approximately 0.5%. A shorter sgRNA, lacking 38 nucleotides at the 3' end compared with the 100-mer sgRNA, was not effective in our experimental system (Fig. S3 in Supporting Information). Specific enrichment of the target region was also observed using cross-linked chromatin as input (Fig. 3B); the DNA yields of the cross-linked target

**Figure 3 in vitro enChIP for isolation of target chromatin complexes in a sequence-specific manner. (A) Plot of DNA yields of in vitro enChIP with native chromatin prepared from 293T cells determined by quantitative PCR from target (IRF-1) and nontarget (Sox2 and 5'HS5) genome regions. (B, C) DNA yields measured by quantitative PCR from samples subjected to in vitro enChIP with cross-linked chromatin prepared from 293T cells. The Ab-based purification system (A, B) or the biotin–avidin purification system (C) was used. IRF-1: target genomic region; IRF-1 (up): 0.2-kb upstream from the primer position for IRF-1; 5'HS5 and Sox2: nontarget control genomic regions. The error bar represents the SEM of three in vitro enChIP experiments (B, C).**
region were comparable using sgRNA (100-mer) and the CRISPR RNA (crRNA)/trans-activating crRNA (tracrRNA) complex for specific targeting (Figs S3, S4 in Supporting Information). Thus, these results clearly showed that it is feasible to isolate target chromatin complexes from cells in a sequence-specific manner using in vitro enChIP with recombinant CRISPR RNPs. Moreover, in vitro enChIP combined with the biotin–avidin purification system was also successfully applied for this purpose (Fig. 3C). Although the DNA yields of the target region were comparable between both purification systems, the Ab-based purification system showed more than 10× less contamination with nontarget control regions (Fig. 3B,C). The elution step of the Ab-based purification system, in which the recombinant CRISPR RNPs are specifically eluted from Ab-coated carriers using 3xFLAG peptides, may contribute to the lower backgrounds achieved using this method (see Experimental procedures). We also examined the DNA yields of potential off-target sites (Fig. S5 in Supporting Information). CRISPR can potentially tolerate mismatches in the 5′ region upstream of the seed sequence and Protospacer Adjacent Motif (PAM) (Jinek et al. 2012). We selected representative potential off-target sites for this purpose; one in chromosome 11 has 7-base mismatches on the 5′ region upstream of the seed sequence and PAM whereas the other in chromosome 1 is most similar to the target site but has 2-base mismatches, one of which is present in the seed sequence (Fig. S5A in Supporting Information). It is of note that there are no potential off-target sites identical to the 16-base sequence in the 3′ region, including PAM, in the human genome. DNA yields of off-target sites were ~15× less than those of the target region (Fig. S5B–E in Supporting Information). Therefore, although the DNA yields of potential off-target sites are higher than those of negative control regions, the contamination of potential off-target sites does not appear to be significant. To reduce contamination of off-target sites as well as irrelevant loci, it might be beneficial to use a tandem affinity purification method using (i) both of the Ab-based and biotin–avidin purification systems, (ii) dCas9 orthologues fused with different epitope-tags, or (iii) gRNAs labeled with different molecules.

In contrast to in vitro enChIP using a recombinant TAL protein, where the DNA yields were only 0.01% of input (Fujita & Fuji 2014a), in vitro enChIP using the recombinant CRISPR RNPs showed much higher DNA yields (~1%) of the target locus using both native and cross-linked chromatin input material (Fig. 3). Unlike recombinant TAL protein (Fujita & Fuji 2014a), r3xFLAG-dCas9-D protein can be prepared without visible degradation, which results in much higher yields using the in vitro enChIP assay with CRISPR RNPs. Thus, in vitro enChIP using recombinant CRISPR RNPs could potentially be applied for the identification of molecules interacting with genomic regions of interest in vivo.

Conventional enChIP, using CRISPR targeting the same site in the IRF-1 promoter region, gave target DNA yields of ~10%, which were 97× higher than those of a negative control locus (Fujita & Fuji 2013a, 2014b). In vitro enChIP gave yields of ~1% of input, which were 76× higher than those of a negative control locus (Fig. 3B). Thus, the in vitro enChIP yields are 10-fold lower than those of conventional enChIP, whereas the specificity is similar between the two methods. Since conventional enChIP can identify proteins (e.g., transcriptional regulators, histone modifiers, acetyltransferase, DNA topoisomerase, and histones) interacting with promoter regions using ~5 × 10^7 cells (Fujita & Fuji 2013a, 2014b), it may be possible to use in vitro enChIP to identify such proteins interacting with promoter regions using recombinant CRISPR RNPs from ~5 × 10^8 cells. Smaller numbers of cells would be sufficient for the analysis of RNA and DNA that interact with a target genomic region.

Conclusions

In this study, we developed in vitro enChIP technology using recombinant CRISPR RNPs. In vitro enChIP does not require expression of the engineered DNA-binding molecules in vivo. This is a great advantage over conventional enChIP technology, since in vitro enChIP can be used without the time-consuming steps required to generate cells expressing tagged DNA-binding molecules or the preparation of transgenic organisms, such as mice. In vitro enChIP might also be useful in situations where the generation of transgenic organisms is difficult or impossible, for example, when nonmodel organisms or pathogens are under study. Moreover, it is not necessary to consider undesirable side-effects such as CRISPR interference (CRISPRi) (Bikard et al. 2013; Qi et al. 2013; Zhao et al. 2014). Our results indicate that in vitro enChIP using recombinant CRISPR RNPs is a potentially useful sequence-specific DNA isolation tool in biochemistry and molecular biology, applicable to the identification of molecules interacting with specific genomic regions. Using in vitro enChIP technology, we are now per-
forming experiments to identify molecules interacting with genomic regions of interest in vitro to elucidate the molecular mechanisms underlying genome functions.

Experimental procedures

Preparation of r3xFLAG-dCas9-D and gRNA

Details of the preparation of r3xFLAG-dCas9-D and gRNA are provided in Data S1 in Supporting Information.

in vitro enChIP using recombinant CRISPR RNPs

Details of the in vitro enChIP procedures using recombinant CRISPR RNPs are provided in Data S1 in Supporting Information.

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Supporting Information

Additional supporting information might/can be found in the supporting information tab for this article:

Data S1 Experimental procedures.

Figure S1 A protocol for conventional enChIP using CRISPR.

Figure S2 A protocol for *in vitro* enChIP with biotinylated gRNA combined with the biotin-avidin purification system.

Figure S3 *in vitro* enChIP with sgRNA (62-mer).

Figure S4 *in vitro* enChIP using recombinant CRISPR RNPs.

Figure S5 DNA yields of *in vitro* enChIP using recombinant CRISPR RNPs for potential off-target sites.

Table S1 Primers used in this study.