Proceedings of ICMMGH 2027 Symposium: Transforming Nutrition for Public Health Across the Life Course
DOI: 10.54254/2753-8818/2027.KL39006

Application of CRISPR-Cas Biosensors in Signal Detection

Qinxuan Liu

School of Life Science, Ludong University, Yantai, China
lappy0302@163.com

Abstract. Stem cell differentiation is characterized by complex changes in gene expression and signaling networks, making it hard to capture these changes at the end-point of the process, which are typically assessed by RT-qPCR, western blotting and immunofluorescence. Many of the methods involve cell lysis, making continuous monitoring of cells and characterization of temporal changes in living cells difficult. With programmable target recognition and signal amplification, CRISPR-Cas biosensors are a promising alternative. This review outlines the working principle of Cas9-, Cas12a- and Cas13a-based sensing systems, with a focus on two representative systems for monitoring stem cell differentiation, the CRISPR/Cas13a FRET sensor and the dCas9-SunTag system. They and their respective detection mechanisms are discussed their applications in tracking the dynamics of miRNA and the activity of transcription factors and their potential advantages in dynamic cellular monitoring. The potential broader applications of CRISPR-Cas biosensors in tumor liquid biopsy, pathogen detection and environmental monitoring are also discussed. Such technologies have the promise of offering real-time molecular understanding of differentiation processes but have some drawbacks, such as different performance needed for different application scenarios, as well as a lack of standardized manufacturing and evaluation criteria. In the future, the development of a standardized design framework, systematic performance assessment, and application-specific optimization should be emphasized to enhance the comparability of different works and to translate laboratory results into applications. In summary, CRISPR-Cas biosensors offer a valuable tool for the study of stem cell differentiation and have the potential to aid in optimizing stem cell differentiation protocols and assessing their safety in regenerative medicine applications.

Keywords: biosensors, stem cell differentiation, CRISPR-Cas, signal amplification, real-time monitoring of living cells

1. Introduction

The fundamental problem of stem cell research is learning how a cell with the entire genome is differentiated to a certain type of cell. It is a decision that concerns the activation and deactivation of gene expression over time as well as the dynamic flow of information through signaling networks—sometimes within hours or even minutes. Traditional mainstream methods like RT-qPCR, western blot and immunofluorescence staining involve cell lysis and results in discontinuous data collection.

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There is an intrinsic limitation in the reconstructed differentiation process to represent instantaneous events during the cell differentiation process and to present the difference between the single cell differentiation process and population average. The CRISPR-Cas sensing platform has been thrust into the spotlight in this methodological void. It takes advantage of the collateral cleavage effect of Cas proteins after target recognition, which enables the cleavage of hundreds to thousands of reporter molecules as a result of a single recognition event to amplify changes at the single-molecule level to a level detectable by conventional instruments. The sensor is not dependent on cell lysis, and can be stably expressed in target cells using delivery vectors, allowing continual readings. Hence, the development of the CRISPR-Cas sensing technology is moving differentiation monitoring of stem cells from endpoint imaging to process monitoring. In recent times, the CRISPR-Cas programmable target recognition and trans-cleavage activity have been widely used in clinical point of care testing, environmental monitoring, and food safety. For stem cell differentiation monitoring, the platforms such as CRISPR/Cas13a Förster Resonance Energy Transfer (FRET) and dCas9-SunTag have made it possible to track miRNA dynamics and transcription factor activity continuously in living cells [1-4].

The research presented in this paper primarily focuses on the basic principles and working mechanisms of CRISPR-Cas sensors, as well as their typical applications in the field of stem cell differentiation monitoring. It focuses on two representative platforms: the CRISPR/Cas13a FRET sensor and the dCas9-SunTag system, and their practical applications. It systematically summarizes the current state of the art and the potential scope of this technology and helps to choose the suitable sensing method for various stages of stem cell differentiation and differentiation goals, to shift the monitoring method of stem cell differentiation from “end-point” to “process”, and to give clearer and more accurate research idea directions for the studies of stem cell differentiation in regenerative medicine and developmental biology.

2. The fundamental principles and working mechanism

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The principle of CRISPR-Cas sensors is to couple the recognition with the signal transduction. The sensor consists of two components: one, a complex of Cas proteins with sequence recognition function and the guide RNA; two, a reporter molecule which can be cleaved by Cas proteins to trigger the output signal. If the desired nucleic acid sequence exists in the system, the guide RNA guides the Cas protein to specifically bind to it and thus activates the nucleic acid sequence cleavage activity of the Cas protein. The Cas9, Cas12a and Cas13a proteins have different cleavage properties; Cas9 mainly cleaves double-stranded DNA, Cas12a has a non-specific trans-cleavage activity on single-stranded DNA reporter molecules after activation by the target DNA, and Cas13a has the non-specific trans-cleavage activity on single-stranded RNA reporter molecules after activation by the target RNA. An individual target molecule can activate an individual Cas protein, and an individual activated Cas protein can cut hundreds to thousands of reporter molecules. The trans-cleavage activity can amplify a single-molecule recognition event to create a much more readily detectable signal [5].

The CRISPR/Cas13a FRET system is a typical model of the recognition-cleavage-transduction cascade amplification at the signal amplification strategy level. Once the target miRNA interacts with the Cas13a-crRNA complex, the trans-cleavage activity is triggered causing the FAM-H2

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hairpin probe to be cleaved, thereby reducing the distance between the fluorescent group FAM and the up-conversion nanoparticles (UCNPs), which leads to the elimination of the FRET effect and restoration of the UCL probe fluorescence signal. A single target molecule is able to trigger Cas13a to cleave a large number of reporter molecules in this process. At the same time, the background noise is further reduced by combining cascade DNA circuits with magnetic bead enrichment, which allows the detection limit to be reached at the attomole (am, 10-18 M) level. The key to the CRISPR-Cas13a sensor monitoring is the real-time monitoring of the fluctuation of miRNAs during stem cell differentiation and the activity of core pluripotency transcription factors (OCT4, SOX2, NANOG) and lineage specific transcription factors. Traditional techniques (including RT-qPCR and immunofluorescence staining) involve cell disruption and can't be used to monitor changes in miRNA and transcription factor levels from the same cell population over time. Delivery of Cas13a and crRNA to stem cells is mainly accomplished by lipid nanoparticles (LNPs), electroporation, and viral vectors. In the cell, Cas13a and crRNA self-assemble into a complex that is able to recognise the target, enabling the trans-cleavage activity of Cas13a. For design of sensors, RNA reporter molecules are already present in the cells, and are equipped with fluorescent groups and quencher groups. Once activated, Cas13a starts cutting any single-stranded RNA that is nearby. Cas13a cuts these reporter molecules, releasing the fluorescent groups from the quencher groups and restoring the fluorescent signal. The fluorescent signal can be amplified because an activated Cas13a can cut many reporter molecules and so achieve highly sensitive detection of the target miRNA [5].

3. Other application areas of biosensors

In addition to monitoring stem cell differentiation, CRISPR-Cas sensors also show potential applications in tumor liquid biopsy, pathogen detection, early warning of neurodegenerative diseases, and food safety. In the field of oncology, this platform has been used to detect circulating tumor DNA (ctDNA) and exosomal miRNA, theoretically capturing genetic mutation signals several months before lesions are detected through imaging [3]. In terms of rapid pathogen detection, nucleic acid detection systems based on Cas12a or Cas13a can identify SARS-CoV-2, influenza viruses, and Ebola virus within 30 minutes. Traditional detection methods rely on expensive thermal cycling equipment, but this approach significantly reduces costs and has the advantage of being widely applicable [4]. In addition, Rezaei et al. have used sensors for the quantitative detection of α-synuclein and tau protein in cerebrospinal fluid, exploring early warning indicators for Parkinson's disease and Alzheimer's disease [4]. In food safety and environmental monitoring, this system has also demonstrated the ability to monitor heavy metal ions, antibiotic residues, and pathogenic bacteria.

There are different requirements for the sensors in different application scenarios. Sensitivity and simultaneous identification of multiple reference conditions are required for liquid biopsy; ease of operation and low cost are required for pathogen detection; and high long-term stability and anti-interference are required for environmental monitoring. For example, in the study by Cesar M. Castro and colleagues on bladder cancer, probes need to be designed simultaneously for liquid biopsy based on four bladder cancer-related EV proteins (CD63, EGFR, EpCAM, PD-L1) and three mRNAs (GAPDH, LASS2, GALNT1). While traditional monitoring platforms can only monitor one reference indicator at a time, the CRISPR-Cas13 assays targeting mRNA can simultaneously monitor multiple indicators. The main benefit of CRISPR-Cas sensors, however, is that different applications have varying conditions for activation and challenges for application and none of the existing sensors can address all these conditions [6].

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4. Typical applications in stem cell differentiation monitoring

4.1. CRISPR/Cas13a FRET sensor

The design concept of the paper-based CRISPR/Cas13a PEC sensor can be divided into three functional layers, proposed by Zhou et al. The first layer is the material modification of the paper-based electrode: ZnO nanorods, $CuInS_2$ microtubes, Au nanoparticles and up conversion nanoparticles (UCNPs) are sequentially assembled on the paper-based electrode, and the UCNPs convert the near-infrared light into visible light, which serves as the foundation for subsequent photoelectrical signal output. The second layer is molecular recognition and signal triggering: in the presence of the target miRNA in the system, it will specifically bind to the Cas13a-crRNA complex, which will trigger the trans-cleavage activity of Cas13a to cut the pre-designed FAM-H2 hairpin probe. This cleavage event sets in motion the cascade DNA circuit which ultimately lifts the FRET effect, resulting in the switch from a quenched to a lit fluorescence signal. The third layer is dual-mode quantitative readout, a near infrared photoelectrochemical (NIR-PEC) signal and up conversion luminescence (UCL) signal, both of which will be strongly enhanced upon the presence of the target, resulting in a highly sensitive quantitative detection in the range of zero to measurable [5].

The design idea behind Zhou and colleagues' scheme is that the Cas13a is pre-assembled with the FRET reporter molecule and, when binding to the target miRNA (in this case, neuronal differentiation marker miR-124), Cas13a is activated and cleaves the FRET reporter molecule, increasing the spatial separation between the donor and acceptor, which switches the fluorescent signal from a quenching to an illumination one [5].

Compared with conventional detection methods, this system improves sensitivity by approximately one order of magnitude. When combined with microfluidic chips, the detection window can be reduced to less than 15 minutes. This system has successfully captured transient fluctuations of miR-124 during neuronal differentiation. Tang and colleagues further upgraded it to a CRISPR/Cas13a-FRET-PAINT triple fusion platform, achieving ultra-sensitive detection at the single-molecule level for schistosome-derived miRNAs. However, such Cas13a-based sensing strategies are currently still primarily applicable to RNA targets, and their applicability to proteins or other non-nucleic acid molecules remains significantly limited.

4.2. dCas9-SunTag system

Another technical solution is the inactive variant of Cas9, dCas9. The inactive cleavage activity of dCas9 makes it an ideal pure sensor element since it does not have auto-cleavage activity and therefore does not require an additional component. Tanenbaum et al. have developed the dCas9-SunTag system, which can be described on three levels with respect to the core mechanism. The first layer is targeted localization, which entails the ability of the dCas9-sgRNA complex to be guided by sgRNA to the target gene site (PAM-adjacent region), thus anchoring the whole sensing system on the genomic site to be detected. The second layer is signal amplification: N-terminus of dCas9 protein is fused with a SunTag array of 24 tandem GCN4 peptide repeats, each of which can individually recruit an scFv-GFP10 fusion protein. Thus, by binding to a single dCas9 molecule, up to 24 GFP10 molecules can be recruited simultaneously. The third is self-completion fluorescence assembly and readout of the recruited GFP10 fragments with the free GFP1-9 fragments in the system, with strong GFP signal to enable high signal-to-noise ratio imaging at a single gene locus.

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Moreover, an auxiliary module consisting of a complex made from 12 MBS sequences, MCP protein, mCherry fluorescent protein and GFP11 fragment is available which can label nascent RNA transcripts and synergistically enhance the fluorescence signal for multi colour real-time tracking [7, 8].

In mouse embryonic stem cells, Kofman and colleagues demonstrated that the expression of miR-335 is completely regulated by the promoter of its host gene, Mest. This discovery is significant in terms of understanding regulation of stem cell differentiation. Li et al. also developed a miRNA-inducible dCas9-VPR system to induce the expression of reporter genes by miRNA-mediated sgRNA release strategy, which has been successfully applied to monitor real-time changes in miRNA activity during stem cell differentiation. More recently, Chen et al. continued to expand the dCas13a-SunTag-BiFC system to the realm of live-cell RNA imaging and provided real-time monitoring of RNA localization and splicing processes [9-11].

The above two typical applications are different in terms of the range of application because of the difference in their operating principles. The benefit of the Cas13a FRET system is that it quantifies low-abundance RNA targets, which can be valuable for imaging signals that change rapidly in early stages of differentiation; the dCas9-SunTag is specifically useful for imaging transcription factor binding events to achieve high-contrast images, which has the advantage of the spatial resolution [5, 9].

5. Conclusion

This paper deals with the use of CRISPR-Cas biosensors in signal detection, explaining the mechanism of three CRISPR effector proteins – Cas9, Cas12a and Cas13a. It emphasizes a review of two typical applications in monitoring stem cell differentiation: the CRISPR/Cas13a FRET sensor and the dCas9-SunTag system. Further, the perspective of this technology in the fields of tumor liquid biopsy, pathogen detection, and environmental monitoring are discussed. The amplified signal generated by the trans-cleavage activity of CRISPR-Cas sensors enables observing, for the first time, the dynamics of the levels of miRNAs and transcription factors during differentiation in living cells. This technology gives a new dynamic view of how stem cells differentiate and acts as a reference for optimizing stem cell differentiation protocols and safety monitoring in regenerative medicine. At the same time, there is a problem in the field that the requirements for sensor performance vary for different application scenarios, and there are no unified manufacturing and evaluation standards to make it easy to make horizontal comparisons between platforms and promote them on a large scale. Future studies need to be directed towards developing standardized design frameworks and evaluation systems for various scenarios and the application of this technology from laboratory to practical applications.

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