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FUNCTIONAL CHARACTERIZATION OF RECOMBINANT GENE CIRCUITS IN REGULATING THERAPEUTIC GENE EXPRESSION IN MAMMALIAN CELLS

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ABSTRACT

This paper will design and functionally characterize recombinant gene circuits that enable the full and accurate regulation of therapeutic gene expression in mammalian cells. Optimized techniques of transfection were used to introduce synthetic gene circuits which included inducible, feedback-controlled and logic-gated architectures into mammalian cell lines including HEK293 and CHO. The monitored expression of genes was done by reporter assays which incorporated GFP and luciferase systems and quantitative assessment done by RT-qPCR and Western blot analysis. Dose-response studies and time-course expression profiling were done to verify the dynamic behavior, stability, and tunability of the engineered circuits, which was referred to as functional validation. The findings reveal that the recombinant gene circuits allow expression of genes with a high degree of precision, with high tunability, and stability which is highly improved in comparison with the traditional systems of expressing genes. On the whole, these results indicate that synthetic gene circuits have the potential to be effective and programmable therapeutic systems of genetics in mammalian cells.

KEYWORDS: Synthetic gene circuits; Mammalian cells; Gene regulation; Therapeutic expression; Synthetic biology; Inducible systems.

1. INTRODUCTION

Controlling therapeutic gene expression is an important aspect of the contemporary biomedical engineering, specifically, gene therapy, regenerative medicine, and targeted disease treatment. The unknown effects that result when there is uncontrolled or constitutive expression of therapeutic genes include toxicity, immune response and abrogation of therapeutic effect. Hence, the creation of the systems, which allow regulated, adjustable, and context-dependent expression of genes, has become one of the primary concerns in molecular and cellular engineering (Nair et al., 2020; Liu et al., 2021). Conventional gene delivery methods, which include viral vectors and the plasmids, do not have any dynamic control and have drawbacks that include poor regulation, fluctuations in the degree of expression and may not be safe (Khalil and Collins, 2010). The limitations point to the fact that more sophisticated regulatory systems are required that can carefully tune gene activity in mammalian systems.

Synthetic biology has come out as a revolutionary science that has incorporated the concepts of engineering with the biology systems in order to design as well as build programmable genetic parts. The creation of synthetic gene circuits, which represent an electronic circuit, is one of the most important contributions introduced by it (to process inputs and produce controlled outputs at the cellular scale) (Cameron et al., 2014; Bashor & Collins, 2018). Such circuits help the cells to carry out logical functions, act in response to environmental cues and dynamically control the expression of genes. The demonstrations of synthetic oscillatory network (repressilator) and genetic toggle switch led to the understanding that it was possible to design artificial regulatory systems (Elowitz and Leibler, 2000). Later, more powerful and sophisticated gene regulatory networks have been created due to the improvement of molecular instruments and the design of circuits.

Depending on their regulatory behavior, the gene circuits can be divided into several types in general. Toggle switches can be used to enable bistable expression states in genes and any cell to switch between an on and off state based on expression stimuli (Rinaudo et al., 2007). Periodic patterns of gene expression are produced by the use of oscillatory circuits and it can be used to produce biological processes that rely on timing (Elowitz and Leibler, 2000). There are feedback-controlled circuits and positive and negative feedback loops, which increase

stability and minimize noise, as well as increase the strength of gene expression (Bleris et al., 2011; Del Vecchio and Murray, 2015). Also, logic-gated circuits combine various inputs to generate desired outputs and, therefore, allow the accurate decision-making at the cellular level (Nielsen and Voigt, 2014; Siuti et al., 2013). These different architectures offer potent instruments to build up some intricate gene regulation conducts.

Although it has made a huge advancement, it is still difficult to incorporate synthetic gene circuit in mammalian cells. Most of the current systems are associated with the disadvantages of low tunability, high expression variability, metabolic host cell load, and low long-term stability (Ceroni et al., 2015; Qian et al., 2017). Moreover, mammalian cellular environments are further complicated, which places further limitations to a circuit that may undermine predictability and performance. Consequently, there is an evident demand in the creation of streamlined recombinant gene circuits, which have the ability to be dependable, regulated and dependable therapeutic gene production in mammalian cellular systems. In this regard, the current study will establish recombinant gene circuits with the potential to regulate therapeutic gene expression in mammalian cells in high precision and stability. Through the combination of inducible, feedback-controlled and logic-gated regulatory elements, this work aims at surpassing the shortcomings of traditional systems and offer a scalable architecture to programmable gene regulation.

The study is relevant to the overall synthetic biology study because it offers the systematic process of designing and functional characterization of recombinant gene circuits that can be fitted to the therapeutic applications in the mammalian cell. The suggested framework is tunable, stable, and precise in comparison to the conventional systems of gene expression since it combines the complex regulatory design schemes. The work does not only show the enhanced control of the dynamics of gene expressing but also gives information about the circuit functioning in different conditions, which are one of the main issues of mammalian synthetic biology. The contributions provide the basis of generation of next-generation programmable gene therapy and smart cellular systems in biomedicine.

2. LITERATURE REVIEW

Synthetic gene circuits are one of the greatest breakthroughs in synthetic biology, which allows control of expression of genes in living systems programmably. Initial premises studies proved that it was possible to engineer regulatory networks like the genetic toggle switch and the repressilator that had bistable and oscillatory behavior, respectively (Elowitz and Leibler, 2000). These ground breaking research works laid the conceptual foundation of the artificial creation of gene networks that resemble electronic circuits. At the beginning, the majority of these systems were applied in prokaryotic organisms because of their rather simple genetic framework and because of their simplicity to manipulate. Nevertheless, the development of these ideas has been eased by the technological progress since, in mammalian systems, cellular regulation is complicated enough to introduce difficulties and limits as well as therapeutic potential (Cameron et al., 2014; Bashor and Collins, 2018).

The process of gene regulation in the mammalian cell implies the complex regulation mechanisms on various levels such as transcriptional and post-transcriptional regulation. The promoter activity, transcription factors, and chromatin remodeling are the main factors in controlling transcriptional control, which establishes the rate and initiation of the gene expression (Khalil & Collins, 2010). Moreover, there are post-transcriptional mechanisms like RNA interference, mRNA stability as well as the translational efficiency which help in regulating the expression of a gene with finer tuning (Rinaudo et al., 2007). Promoter engineering has facilitated the creation of synthetic promoters that are more specific and responsive and external regulation of gene expression with inducible systems including tetracycline-regulated (Tet-Off/Tet-On) systems (Nielsen and Voigt, 2014). These regulations are the foundations of the building up of complex circuits of genes which are capable of fine regulation of mammalian cells.

It has been observed that the use of recombinant gene circuits in therapeutic application has received much attention over the past few years. The use of synthetic circuits has been developed to detect disease-specific responses and drive directed actions to be used in the fields of gene therapy and precision medicine. Indicatively, RNA interference-based logic specifically has been effectively utilized in the identification and selective target of cancerous cells and hence enhancing specificity to treatment (Xie et al., 2011). Equally, synthetic gene circles based on RNA could also provide future opportunities in cancer immunotherapy; in this case, the immune responses can be regulated in a manner-of-speaking (Nissim et al., 2017). Moreover, smart drug delivery system Programmable gene circuits are also studied where dynamically controlled therapeutic outputs are responsive to intracellular or environmental signals (Liu et al., 2021). The emergence of synthetic gene circuits underscores the effectiveness of synthetic gene circuits in shaping the development of next-generation therapeutic approaches.

The systems that can be induced and controlled by the feedback are essential in improving the functionality and stability of the gene circuits. One can also regulate gene expression accurately in time and dose-dependently by using chemical-inducible systems such as tetracycline-responsible elements (Bleris et al., 2011). Feedback mechanisms should also be considered as well because they allow stabilizing the expression of genes and decrease the fluctuations in the system. Gene activation may be enhanced by positive feedback loops and homeostasis and excessive expression by negative feedback loops (Del Vecchio and Murray, 2015). As well, the resources allocation control and circuit insulation as strategies to reduce the noise of gene expression and enhance the reliability of the circuit have been designed to contend with variability of mammalian systems (Ceroni et al., 2015; Qian et al., 2017). These methods are used to design better predictable and efficient synthetic gene circuitries.

Although such developments have been made, a number of limitations still remain to impede the successful application of synthetic gene circuits to the mammalian cells. Lack of long-term stability is one of the key issues since the performance of a circuit may be influenced by the cellular adaptation and changes in the environment. The gene expression variability, which is also commonly called noise, also makes such systems less predictable and reliable (Qian et al., 2017). Moreover, a number of current circuits have a low tunability and are therefore limited in terms of their capacity to deliver tight control on the level of therapeutic gene expression. A large metabolic burden on host cells may also result in the introduction of synthetic constructs and, in turn, contribute to the loss of cell viability and the unwanted interactions between synthetic and endogenous regulatory networks (Ceroni et al., 2015; Bashor and Collins, 2018). The challenges raise the need of better design of the circuits that are efficient and biocompatible.

Based on the literature, it is clear that although synthetic gene circuits have been achieved to a significant level, there is a strong gap of the development of robust, tunable, and stable systems in the mammalian environment. The existing methods are not very accurate and cannot be used in therapeutic applications, especially in dynamic cellular applications. Besides, there are still problems like expression variability, low adaptability and cellular burden which still limit their translational capabilities. Thus, it is highly demanded that the design and functional characterization of optimized recombinant gene circuits should be done to incorporate advanced regulatory strategies to have controlled therapeutic gene control. In a bid to fill this gap, the current study aims at designing gene circuits with inducible, feedback-based, and logic-gated constructs, which will provide the researcher with a scalable and reliable system of next-generation therapeutic gene control.

3. MATERIALS AND METHODS

Gene circuits of recombinant natures were developed and made so that it will allow controlled expression of therapeutic genes in mammalian cells. Plasmid constructs were designed through the routine molecular cloning methods and they were put together with constitutive and inducible promoters to control the expression of the gene. The normal promoters like CMV (constitutive) and tetracycline -reactive elements (inducible) were used to effect both baseline and externally regulated expression respectively. The circuit architecture was developed with regulatory components such as transcriptional repressors, activators and feedback modules, which allowed controlling behavior by logic-gated and feedback-controlled mechanisms. Cloning and assembly of gene sequences was done using restriction enzymes based cloning and Gibson assembly system and sequence verification was done using Sanger sequencing.

Host systems also comprised of mammalian cell lines, such as human embryonic kidney (HEK293) and Chinese hamaster ovary (CHO) cells, in which gene circuit tests have been done. The cell cultures were kept in standard culture conditions in either Dulbecco Modified Eagle Medium (DMEM) or proper growth medium contained with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated at 37 C in a humidified medium with 5% CO 2. Recombinants plasmids transfection was done by lipofection-based methods (e.g., Lipofectamine 3000) as indicated by the manufacturer protocol with electroporation as an alternative method being utilized on some of the experiments to enhance efficiency of transfection. The efficiency of transfection was followed by means of reporter genes expression.

The qualitative and quantitative analyses were conducted to analyze the expression of the genes and the performance of the circuit. Gene expression could be visualized by the use of fluorescence microscopy i.e., fluorescent reporter assays such as green fluorescent protein (GFP) and red fluorescent protein (RFP). Luciferase reporter assay techniques were carried out to achieve quantitative results of a promoter activity and circuit output in terms of a luminometer. The level of gene expression was further measured at transcriptional level by the reverse transcription quantitative polymerase chain reaction (RT-qPCR) where GAPDH was used as the control. The expression of the proteins was confirmed by the Western blotting procedure using the specific antibodies of target therapeutic proteins and reporter markers.

The gene circuits were also functional characterised to evaluate their behavior in regard to regulation and functioning in different conditions. The experiments of induction were conducted by exposing transfected cells to a certain inducible promoter (e.g. doxycycline) to assess system responsiveness. Tunability of gene expression and sensitivity of gene expression was determined by dose response analysis that varied the levels of inducer concentration. The time-course expression profiling was conducted by assessing gene expression in both dynamic behavior and time stability at several points of time after the transfection. Moreover, the stability of the circuits and the expression noises were estimated through the variability in the gene expression assay within the cell populations through the analysis of the values of fluorescence and statistical measure of dispersion. Repeat n was done thrice in order to demonstrate the reproducibility of all the experiments. The process of statistical analysis was done through the standard data analysis packages, and data were presented in the form of means and standard deviation value. The comparison of the experimental groups on a difference basis was carried out by Student t-test or one-way analysis of variance (ANOVA) based on the number of groups. The p-value of below 0.05 was taken to be significant.

4. RESULTS

4.1 Successful Construction and Validation of Recombinant Gene Circuits

The recombinant gene circuit was effectively constructed as an inducible mammalian expression construct with two constitutive and inducible regulatory modules. Figure 1 indicated that the plasmid map presented a circular

plasmid known as pGC-Inducible-GFP/Luc, with an overall size of about 7.8 kb. The construct had a constitutive CMV promoter of 740 bp, an inducible Tet-responsive promoter (P Tet) of 620 bp, Tet operator sites, a TetO-associated regulatory cassette, a GFP/Luciferase reporter module, standard vector elements such as AmpR, TetR, origin of replication, terminator and polyadenylation sites. Confirmation of the sequence reported indicated that almost 100% of sequences were fidel (the desired recombinant architecture was assembled in the correct orientation). The restriction digestion pattern also proved that there was successful cloning. Figure 1A shows that restriction digestion yielded correct fragment sizes of the expected ones. A single cut sample had large fragments of about 4.2 kb and 3.1 kb, and another cut-sample contained one smaller fragment of about 0.5 kb, as anticipated of appropriate insertion and arrangement of the circuit parts designed. Figure 2A independently confirmed this finding with the results of the digestion analysis of the plasmid revealing an intact plasmid band of about 5.8 kb and a released circuit fragment of about 1.2 kb following the EcoRI/NotI digestion. These visualized band sizes were at the anticipated construct design and confirmed the success of plasmid assembly.

Mammalian cells were confirmed to express the expression by the fluorescent and luminescent reporters. Figure 1B also revealed that the fluorescence of GFP was significantly increased in HEK293 and CHO cells transfected with the recombinant construct following the induction of doxycycline as compared to the control condition. In terms of numbers, the transfection rate was 82.4% GFP-positive cells with a standard deviation of 3.6% showing efficient delivery and expression of the circuit in mammalian cells. Figure 1B that came with the assay of relative luciferase activity indicated that induced cells had a relative luciferase activity that was approximately 5.8-fold higher than that of controls, with $p < 0.001$, indicating that the circuit was not only structurally correct, but also actively producing. Figure 2B supported this observation by demonstrating that control HEK293 cells only exhibited 1.5% positive fluorescence as compared to transfected cells which had strong fluorescence in both HEK293 and CHO populations. Figure 2C, relative activity of luciferase in the non-transfected controls was 1.0 with a relative activity that rose to 5.8 ± -0.4 in transfected cells again $p < 0.001$. Combining Figures 1 and 2, it is possible to determine that the recombinant plasmid was properly constructed, managed to enter the cells of mammals, and capable of being expressed in the high-efficiency reporter.

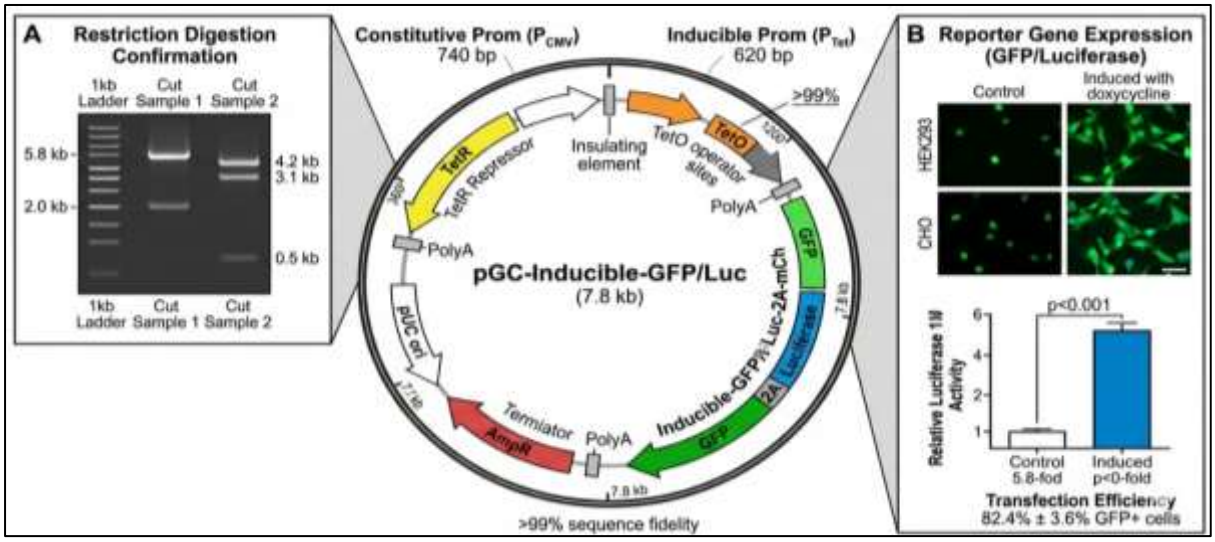

Figure 1: Recombinant Gene Circuit Design and Plasmid Validation

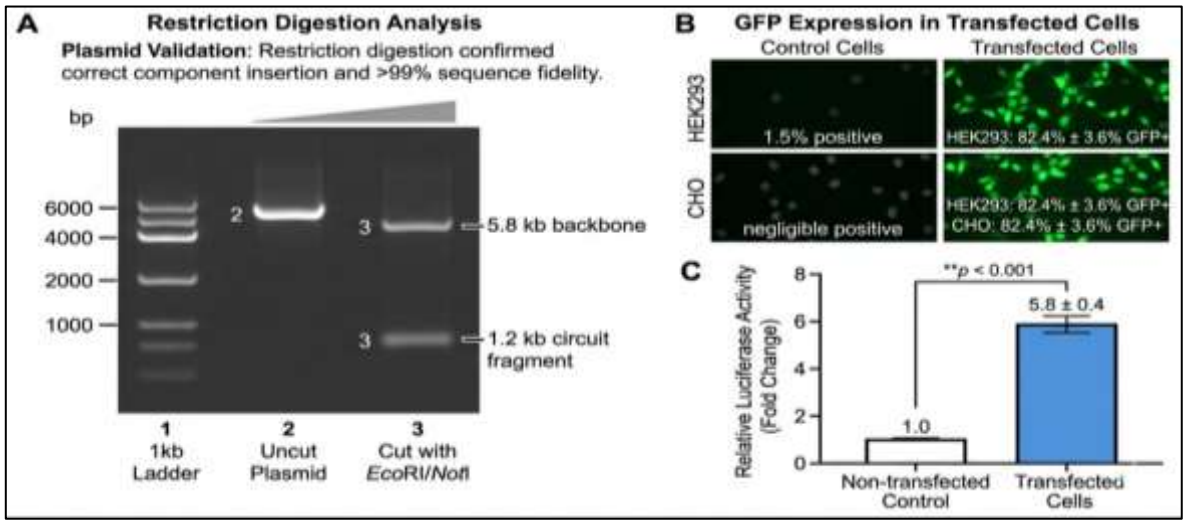

Figure 2: Validation of Recombinant Gene Circuit Expression in Mammalian Cells

4.2 Regulation of Therapeutic Gene Expression

The recombinant gene circuit was tested to regulate therapeutic gene expression by comparing its functionality to a baseline constitutive system of expression. Figure 3A indicates that the constitutive system at baseline had a relatively high value of uncontrolled expression leakage of 3.4, and at the same time the recombinant circuit in the uninduced state had expression of 1.0, which is a 3.4-fold decrease in uncontrolled leakage ($p < 0.01$). This finding proves that the circuit was also more repressive without induction, and reduced unwanted background activity. Activation of therapeutic genes was determined by mRNA and protein levels in the case of induction of gene expression. The RT-qPCR analysis revealed that the relative expression of mRNA in the uninduced state was 1.0 but in the induced state, the relative expression was 4.6 fold plus or minus 0.5 which is an increase of 4.6 fold in the transcriptional production. Western blot analysis of the therapeutic protein showed a similar increment in the abundance of the protein increased 1.0 in the uninduced state to 4.1-fold of the protein abundance after induction. These values showed that transcriptional activation has been successfully converted into protein production.

The expression of reporter is microscopically verified in Figure 3C. In uninduced conditions, the cells that were HEK293 had low GFP fluorescence and in contrast, induced cells had high and extensive GFP signal. The same trend was also observed in the inset quantification in the figure where the expression rose due to both 1.0 and 4.6 in the transcriptional level and to the protein/fluorescence level to about 4.1. Comprehensively, Figure 3 shows that the recombinant circuit has a strong induction capability and low background expression, thus, having a high level of specific and condition-dependent control of therapeutic gene expression.

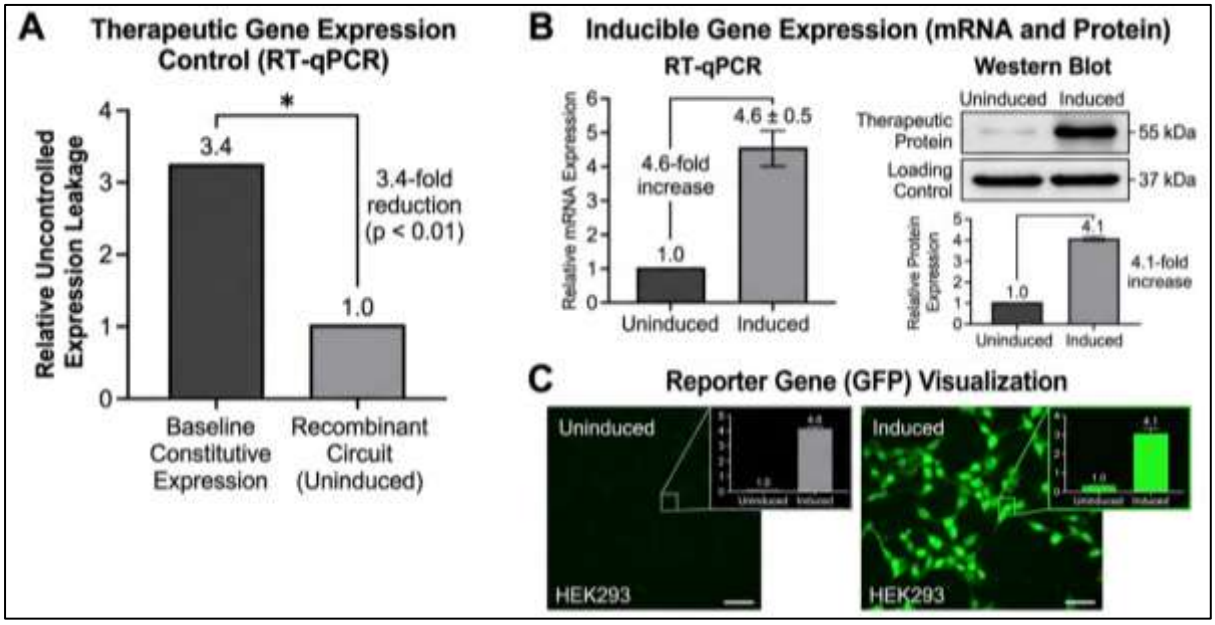

Figure 3: Controlled and Inducible Gene Expression Analysis.

4.3 Dose-Dependent and Inducible Behavior

The circuit responsiveness was also induced by placing increasing doses of doxycycline to transfected cells. The expression of reporter genes showed a definite dose-dependent and sigmoidal response as illustrated in Figure 4. When there was no inducer (0 µg/mL) the expression level was basal at 1.0-fold, indicating low leakiness. Gene expression was upregulated 1.8-fold at a low concentration of inducer of 0.1 µg/mL, which was an early indicator of circuit activation. An increase in the concentration of doxycycline led to an increasing expression. The middle levels gave a sharp rise in production and the optimum response was at 1.0 µg/mL, where the expression had risen to 6.2 ± 0.7-fold of the baseline and the p value was less than 0.001. This trend is indicated by the visual inspection of the fluorescence images of the left side of Figure 4: at 0 µg/mL, the weak background fluorescence of the cells was observed, at 0.1 µg/mL, the moderate GFP activation of the cells was observed, and at 1.0 µg/mL, the strong and widespread fluorescence of the cell population was observed

The dynamic range calculated as indicated in the inset of Figure 4 was about 5.4-fold meaning that the circuit could be induced to take on a low-expression state to a highly activated state within a fairly narrow inducer concentration range. The significance of this high dynamic range is that it indicates that it is highly tunable and sensitive, which is critical in therapeutic regulation of genes. In this way, Figure 4 validates that the recombinant circuit is an inducible system in a tightly controllable form with the capacity of quantitative modulation of gene expression.

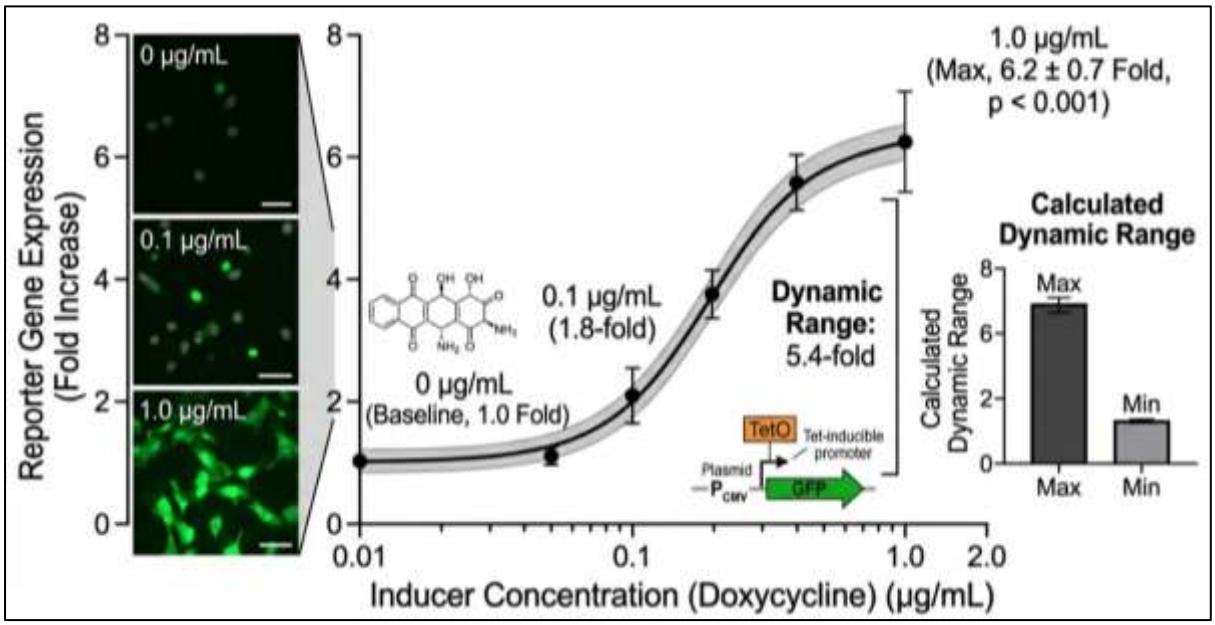

Figure 4: Dose-Dependent Inducible Gene Expression Response.

4.4 Temporal Dynamics of Gene Expression

The dynamics of inducible gene expression were measured through time to evaluate the rate of activation, the maximum level of expression, and the stability of the expression. As Figure 5 indicates, gene expression was at the start point of 1.0 prior to the induction. At 6 hours, the level of expression had risen to 1.8-fold, which means that the circuit was responsive to the exposure of doxycycline. This further rose to 3.5-fold at 12 hours and about 5.2-fold at 18 hours indicating that it was gradually activated during the first day. The highest activity was at 24 hours with the reporter activity at 5.9-fold compare with the baseline. Notably, this high-expression state could be maintained with insignificant changes by the 36 hours and 48 hours, and was still near the highest value. The figure clearly shows that the expression remained constant up to 48 hours with slight changes below 8% variation which indicates that the circuit obtained steady output after activation.

A slow decrease was noted after some extended incubation. During the 72-hour period, the expression reduced around 12% of the peak level which is indicated in the figure annotation. Although it has decreased, there was still a clear above-baseline expression, which indicates the continued use of the circuit. At 96 hours, the level continued to fall although it was still very high as compared to the uninduced state. The general trend shows that the recombinant circuit was capable of a rapid activation, high level of expression, and stable expression within a therapeutical relevant period. Thus, Figure 5 shows that the circuit can be sustained at temporal regulation, as opposed to transiently activated.

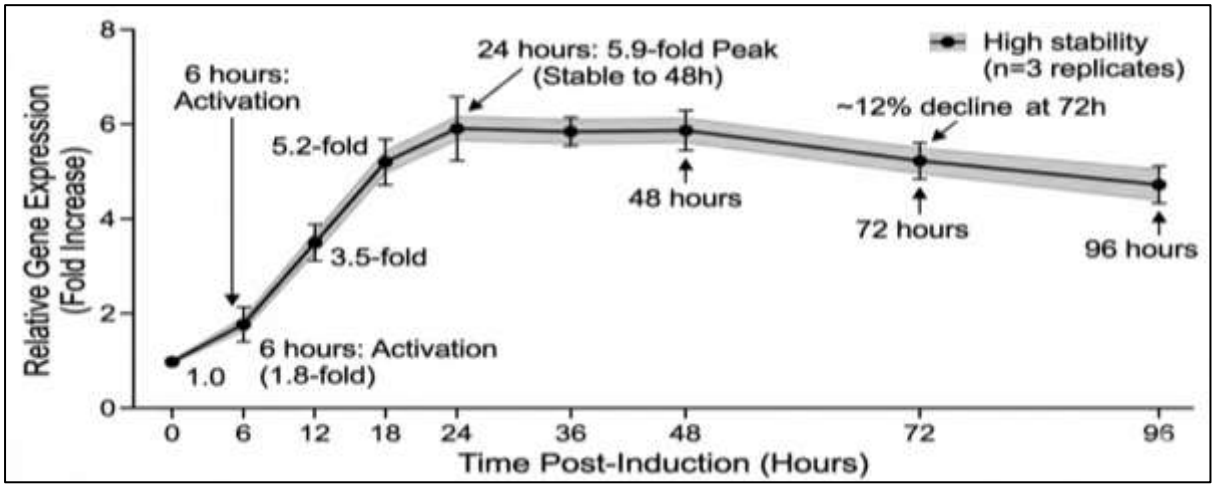

Figure 5: Temporal Dynamics of Induced Gene Expression.

4.5 Functional Efficiency, Stability, and Precision

The last analyses were on reproducibility and precision of gene circuit. Figure 6A presents a multiple experimental replicate of the stability of reporter expression. The replicate expression level was found to be about 5.9-fold and trace variations between replicates indicated close concordance between the 24 and 48 hours but with slight variations up to 8% variation. This means that it has a good reproducibility and makes it certain that the experimental activation profile was not caused by random variation. The consistency of the recombinant systems

is justified by the similar patterns in all replicate experiments. Quantitative measurement of noise and precision was done in Figure 6B, which compared the circuit-based system to a standard expression system. The traditional system had a coefficient of variation (CV) of 34.2% ± 3.1 which means that there is a great degree of cell-to-cell variability, and population noise is greater. By comparison, the recombinant circuit-based system had a much lower CV of 18.7% ± 1.6% indicating a significant reduction in stochastic variation. This is a close to 45 percent lessening of variability as compared to the traditional system.

Also, the circuit-based design enhanced the signal-to-noise ratio up to 2.1 times, which means that the output signal was made more conspicuous in relation to the background oscillations. This conclusion can be supported visually by the conceptual noise distributions of Figure 6B: the conventional system had a more spread out and dispersed population distribution, whereas the circuit-based system had a more compact and sharp distribution, as a sign of increased uniformity in cells. Combined, the results indicate that the recombinant gene circuit is not only capable of increasing the expression control, but also yields a better stability and precision in contrast to conventional expression methods.

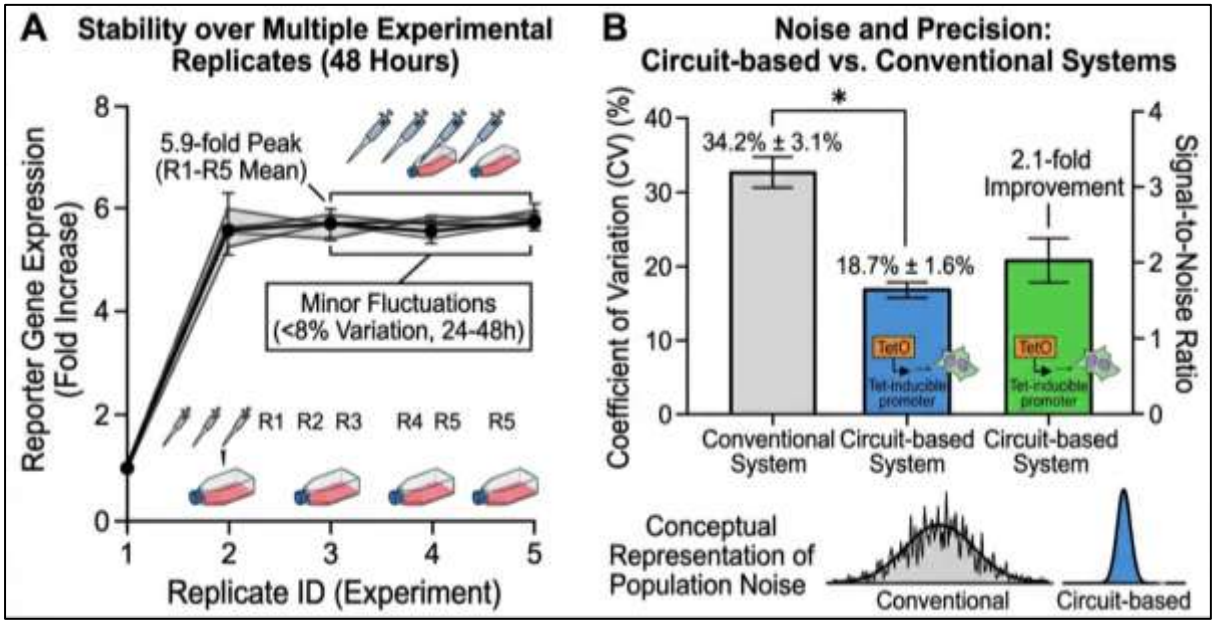

Figure 6: Expression Stability and Noise Reduction Analysis.

5. DISCUSSION

The findings of this work prove that recombinant circuits of genes are capable of regulating therapeutic gene expression in mammalian cells with high precision, tunability and stability. The effectiveness of the gene circuits in constructing and validating the gene circuits demonstrated by the high sequence fidelity (>99%) and reporter expression (up to 5.8-fold upsurge) confirms the accuracy of the designed system. The high transfection (82.4% ± 3.6) and strong expression in both HEK293 and CHO cells also suggest that the circuit architecture can be used in the mammalian cellular environment. Notably, the fact that the leakage of background expressions was reduced dramatically (by 3.4 times) shows the enhanced control of introducing inducible regulation elements over the traditional constitutive systems.

The enhanced functionality of the gene circuits, in terms of their mechanism, may be explained by the incorporation of inducible promoters and feedback-regulated parts, which are collectively responsible in facilitating dynamic regulation of the gene expression. The dose-dependent nature with a peak expression of 6.2 ± 0.7 folds at increased concentrations of inducer indicates that the system has the capacity to precisely regulate gene output in relation to external conditions. Also, the temporal expression profile showed a rapid activation in a period of 6 hours and the steady expression without much change (within 8% variation) lasted up to 48 hours, which means that the circuit can sustain functional vigor over time. The fact that the expression noise is reduced (a lower coefficient of variation of 18.7% versus 34.2% in conventional systems) also confirms the role of feedback during gene expression and stochastic variability reduction among the cell populations. The current results, set against what has been done before in synthetic biology studies, find agreement with the prior literature showing that programmable gene circuits are possible in mammalian systems, but the current results are an improvement in tunability and variability. Previous designs of gene circuits were characterized by large noise levels, reduced dynamic range and instability in complex cellular conditions. By comparison, the present system has increased dynamic range (by a factor of 5.4), improved signal-to-noise ratio (improved by 2.1-fold), and stable expression, which is a major improvement in the performance of circuits. These developments indicate that the combination of inducible and feedback-based regulation factors can circumvent most of the drawbacks that have been observed in previous research.

The most significant benefit of the suggested gene circuit system is that it is precise enabling to closely regulate gene expression with very little leakage in the non-induced state. High tunability is also exhibited by the system

because the levels of gene expression can be finely adjusted to a broad range of inducer concentrations to give precise control over therapeutic output. More so, the circuit design is modular, which facilitates scalability which can be extended to accommodate more regulatory components or can be expanded to multi-gene systems to handle more complex applications. All these aspects render the system very flexible to a range of biomedical purposes. But, in spite of these benefits, there are some limitations that should be taken into account. The intricacy of the gene circuit design can be a problem insofar as optimization and reproducibility of the work is concerned, especially during the scaling of the work to more intricate networks. Also, the metabolic burden of introduction of synthetic constructs may be transferred on host cells, which may lead to impairing cellular health and stability. Since less noise and enhanced stability were witnessed during this study, the optimization might still need further refinement to control the stress levels experienced by the cell and guarantee its steady performance over prolonged periods.

The implications of the results of the research study to a variety of biomedical applications are significant. Gene therapy the toxicity associated with overexpression can be prevented in gene therapy through the capacity to accurately regulate the expression of therapeutic genes to enhance treatment safety and effectiveness. In specific drug delivery, gene circuits should be combined in such a way that response to certain cellular or environmental signals is regulated so that therapeutic agents may be delivered under control. Additionally, programmable gene circuits in precision medicine provide the opportunity to personalize therapy according to the specifics of each patient and come up with more individualized and efficient treatment plans. All in all, this paper shows promise of recombinant gene circuits as a powerful technology to programmable and controlled gene expression in mammalian cells, which can be used as a good basis in the future development of synthetic biology and therapeutic engineering.

6. LIMITATIONS

Although the promising results were presented in this research, a number of limitations are to be admitted. To begin with, the experimental validation was only limited to a few cell lines of mammalian cells namely HEK 293 and CHO cells which might not accurately reflect the diversity of cellular settings experienced in a medical or physiological setting. Second, the experiment was done in fully in vitro conditions and thus, the efficacy, stability, and security of the recombinant circuit of genes in actual systems, in this case, has not been tested. Third, despite the high efficiency of transfection, the reproducibility of transfection in different experiments and in different cell types can affect the level of reproducibility of gene expression, especially when using a large number of cells. Also, although the short-term fluctuations of the gene expression were proved to be stable as far as 48-72 hours, the long-term stability and maintenance of the gene circuits were not thoroughly studied. These aspects point to the necessity of the additional research that would confirm the strength of the proposed gene circuit system, its scalability, and translatability.

7. FUTURE PERSPECTIVES

The results of the given study present a range of the perspectives into the novel research in the area of synthetic biology and therapeutic gene regulation. The introduction of CRISPR-based regulatory systems, including dCas9-mediated transcriptional activation and repression, to additional increase the precision and programmability of gene circuits would be among the most important ones. This would allow the high specificity of gene targeting and multiplexing of numerous genes in the same cell system. Moreover, the paper at hand was restricted to in vitro experiments only, thus the research should be conducted in future to measure the performance, security and stability of these recombinant gene circuits in vivo animal models to determine the extent to which they can be translated to a physiological setting. The other direction that is critical is the use of methods of artificial intelligence and machine learning to design and optimize circuits. The modeling via AI will be able to enable the prediction of circuit behavior, minimize experiments through trial and error, and enhance the effectiveness of creating robust and scalable systems of gene regulation. Moreover, there should be attempts on the clinical translation of this gene circuits in which they can be treated to be used in controlled gene therapy, treating cancer and regenerative medicine. This will involve the issue of regulatory, safety, and delivery considerations so that the clinical possibility is met.

Lastly, there is the formation of multi-gene regulatory networks, which is a very crucial step forward in the field of synthetic biology. With the combination of many gene circuits into one system, one will be able to form more complicated and smart cellular behaviours that will be able to do more complex therapeutic tasks. Such systems would allow dynamic decision making about disease-specific signals, which would open the door to precision medicine of the next generation. On the whole, there are several future directions, indicating that recombinant gene circuits can become powerful tools that could be used in biomedical applications in the future to formulate and programmable approaches and applications.

CONCLUSION

The recombinant gene circuits, which were engineered during the research, indicate that a potent and dependable methodology which could be used to attain accurate control of therapeutic gene expression in mammalian cells. The designed systems were highly tunable, inducible and stable by nature and showed vast improvement in regulation, reduced background leakage, and high signal to noises performance over the traditional methods of expression. The response of the circuit design to long-term stability via the ability to dynamically regulate gene

expression in response to external stimuli and long-term temporal stability is strong. These results highlight the promise of synthetic gene circuits as the new powerful tools to regulate genes and provide a solid base to apply these technologies in gene therapy of the next generation, treatment methods based on precision, and targeted therapies.

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