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Enhancing stem cell survival in an ischemic heart by CRISPR-dCas9-based gene regulation

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Abstract

Ischemic heart disease has remained the number one killer around the world for over the past twenty years. While stem cell therapy has become a promising new frontier to repair the damaged heart, limited stem cell survivability post-transplantation has precluded widespread use of this therapy. Strategies to genetically modify stem cells to activate pro-survival and anti-apoptotic and anti-inflammatory pathways, such as Akt and heme oxygenase-1, have been shown to improve the lifespan of transplanted stem cells within the ischemic myocardium, but constitutive overexpression of these pathways at high levels has been shown to have side effects. Therefore, more specific and controlled gene activation would be necessary. Current techniques used for gene regulation include zinc finger and TALE proteins, but there are still disadvantages to each of these methods, such as ease and cost of use. Also, those methods use synthesized promoters to express synthesized cDNA, which lack regulatory elements, including introns and 3' untranslated regions for microRNA mediated post-transcriptional regulation. A new novel technique, the CRISPR/dCas9 system, was recently developed as a simple and efficient method for endogenous gene regulation. With its use of single guide chimeric RNA's (sgRNA's), this system has been shown to provide a high level of specificity and efficiency. When targeting different loci, past studies have found that the CRISPR/dCas9 system can activate gene expression at varying levels. In addition, this system makes use of the genome's endogenous regulatory elements, such as the aforementioned introns and 3' UTR's, which can help provide a safer method of gene activation. If targeted to a gene promoting cellular survival or decreasing cell death, it could potentially improve stem cell longevity in a more efficient and controllable manner. As a result, our hypothesis is to use the CRISPR/dCas9 system to activate expression of an anti-inflammatory and anti-apoptotic gene, such as heme oxygenase-1 (HO-1), to an optimal level to increase transplanted stem cell survival while also mitigating its cytotoxic effects due to lack of internal regulation, thus prolonging its effects within the ischemic myocardium leading to greater therapeutic benefit.

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Keywords

CRISPR-dCas9; stem cell; Apoptosis; HO-1

Introduction

Cardiovascular diseases cause the highest rates of mortality around the world (1, 2). As of now, the only cure for congestive heart failure has been heart transplantation, but the use of stem cells has become a new potential therapeutic approach since first being reported in 1989. This cell therapy, termed cellular cardiomyoplasty, uses stem cells to regenerate lost myocardium and to reduce the ensuing loss of cardiac function and heart failure status post heart injury (3). Bone marrow stem cells are currently the most studied stem cell for this technique, and early studies have shown improved contractile function as well as the induction of angiogenesis (4–8). However, successful engraftment and survival of stem cells proves to be the most important challenge for stem cell therapy (9). Graft cell death could be due to a number of factors, such as ischemia, host inflammatory responses, or pro-apoptotic and cytotoxic factors (10, 11). Therefore, a successful method that could help increase stem cell survivability within the ischemic myocardium would prove to be critical in improving the outcomes of cellular cardiomyoplasty.

Such studies attempting to increase stem cell longevity have already been performed, and genetic engineering seems to have provided encouraging results. First, Zhang et al. (10) and Mangi et al. (12) studied the effects overexpression of the prosurvival gene *AKT1* would have on improving mesenchymal stem cell survival, and both found that activating the Akt pathway can limit cell death as well as repair the ischemic myocardium and restore some cardiac function. Heat shock treatment, which has been shown to protect cells from various harmful stimuli such as oxidative stress, ischemia, and apoptotic stimuli, was also found to decrease the death rate of implanted cardiomyocytes from 32 to 15% one day after grafting (10). In addition, we and other groups have found that stem cells transduced with the heme oxygenase-1 (HO-1) pathway had increased survivability, reduced infarcted left ventricle remodeling, and improved left ventricular function, all of which further suggest that gene therapy may lead to greater therapeutic benefits of cellular cardiomyoplasty in patients following myocardial damage (13–16).

While the benefits of overexpressing the HO-1 pathway have been shown, it has also been noted that continuous overexpression of this pathway actually diminishes its cytoprotective actions and leads to cytotoxic effects (17, 18), suggesting that a regulated level of HO-1 overexpression would provide the most beneficial and efficient effects for enhancing grafted stem cell survival. Additionally, it may prove prudent to upregulate gene expression whilst keeping their endogenous gene regulatory components, including introns and 3' untranslated regions (UTR), so activating gene expression via their proximal promoter might be a feasible option. Very recent studies have shown that a sequence specific single guide chimeric RNA (sgRNA) can guide a CRISPR/dCas9 VP64 system to specific genome zones, and as a result, this system could be an effective method of gene overexpression by activating endogenous genes.

Hypothesis

Activation of the heme oxygenase-1 pathway through the use of RNA-guided CRISPR/dCas9-based transcription factors would lead to enhanced regulation of the HO-1 pathway and increased stem cell survivability post-transplantation in the ischemic myocardium.

Basis for the hypothesis

Transgenic HO-1/Akt system for stem cell protection

As mentioned above, a number of strategies to attenuate the rate of cell death following stem cell engraftment within the ischemic myocardium have been studied. Of these, activation of the heme oxygenase-1 pathway could prove to be a particularly interesting method to improve stem cell survivability, since the HO-1 pathway has been studied extensively, and its anti-apoptotic, anti-inflammatory, and cardioprotective effects are well-documented (13–17, 19–21). Heme oxygenase-1 is a stress-response protein that helps to attenuate the oxidative damage caused by heme by degrading it into biliverdin, ferrous iron, and carbon monoxide, each of which seem to have their own antioxidant capabilities (19). Its overexpression has been found to improve cardiac function, decrease hypertrophy and stress, and promote neovascularization in the post-infarcted heart (20). It is due to these combined cytoprotective effects that mesenchymal stem cells treated with a hypoxia-activated HO-1 vector appear to have enhanced survival (16).

While inducing HO-1 overexpression, as well as other cell survival mechanisms, in mesenchymal stem cells has proven to be a successful means of improving stem cell survivability in the ischemic myocardium, certain considerations remain. Mesenchymal stem cells transduced with *Akt1* led to a significant decrease in apoptosis as well as ventricular infarct (12); however, constitutive activation of the Akt pathway has been implicated in tumorigenesis (22). Likewise, it has been noted that continuous overexpression of the HO-1 pathway actually diminishes its cytoprotective actions and leads to cytotoxic effects (17, 18), suggesting that a regulated level of HO-1 overexpression would provide the most beneficial and efficient results for enhancing grafted stem cell survival.

Current strategies to control transgenic gene expression

In order to address the complications resulting from a constitutively high level of expression of HO-1, different studies have utilized a hypoxia-regulated HO-1 vector (14, 16). Tang et al. (16) generated a double plasmid system, one acting as a sensor plasmid containing an oxygen-sensitive toggle and the other acting as an effector plasmid containing the HO-1 gene. Mesenchymal stem cells transfected with this double plasmid system exhibited greater protection against apoptosis as well as decreased levels of pro-inflammatory cytokines *in vivo* within the ischemic heart (16). These systems provided preliminary mechanisms for the regulation of HO-1 expression in order to prevent the cytotoxic effects that occur as a result of constitutive overexpression of high levels of HO-1; however, they do present with certain limitations. First, while a hypoxia-regulated system does lend a certain level of regulation to HO-1 expression, it also restricts the activated overexpression of this gene to conditions of ischemic stress. As a result, later treatments would not be able to successfully activate HO-1 and generate a cardioprotective response once collateral circulation and vasculogenesis

occurs within the heart (16). Additionally, a double plasmid system may lead to complications of its own, such as loss of function if either the sensor or effector plasmid fails. A more highly regulated mechanism of controlling the level of HO-1 expression within stem cells combined with a greater degree of specificity could yield a more efficient and effective method of enhancing stem cell survivability within ischemic hearts.

Other methods of gene activation that could potentially be used are through zinc finger proteins and transactivator-like effector (TALE) proteins. Each of these techniques pairs a nonspecific activation domain with a sequence-specific DNA-binding domain. Activation domains can include many different enzymes for DNA modification, such as nucleases, transposases, and transcription factors, depending on what kind of genetic engineering is desired (23). It is the DNA-binding domain, however, that allows these systems their flexibility and customizability in recognizing different DNA sequences. Zinc finger proteins consist of approximately 30 amino acids that form a $\beta\beta\alpha$ domain that is stabilized by hydrophobic interactions, as well as the interactions between a zinc ion and cysteine and histidine residues. Each of these motifs is then able to recognize and bind to a three-nucleotide sequence of DNA (24). TALE proteins usually consist of 33–35 amino acid repeat arrays with two hypervariable residues at positions 12 and 13. It is these two hypervariable amino acids that determine the identity of which base the TALE protein can bind (25, 26). Both zinc finger proteins and TALE proteins provide the potential to target any particular DNA sequence; however, they do have certain limitations. Zinc finger modules have been found to be difficult to assemble and commercial modules are expensive. In addition, it is challenging to perform large fragment (>1 kb) replacements (27). TALE proteins seem to provide a worthy alternative to zinc finger gene activation; however, TALE-mediated gene modification requires the construction of large repetitive sequences to target its specific DNA sequence, which may prove difficult to manipulate (28–30). The high cost and complicated assembly of these proteins have made their use in the laboratory both expensive and challenging. A method requiring a simpler targeting mechanism could be a more efficient method of gene manipulation.

CRISPR/dCas system-a unique strategy for promoter regulation

A new novel technique using RNA-guided CRISPR-Cas9-based endonucleases has been reported as a simple and highly specific method for gene activation (31). The CRISPR/Cas system was originally discovered to be an adaptive immune system in bacteria and archaea (32–34). CRISPR, or clustered regularly interspaced short palindromic repeats, works in conjunction with a Cas nuclease to cleave invading foreign DNA into small DNA fragments, which the bacteria then integrates into its CRISPR locus as spacers. When faced with additional viral or phage infections, the bacteria then uses these spacers as transcriptional templates to produce CRISPR RNA (crRNA) which guide the Cas nucleases to target DNA sequences in the invading virus or phage for cleavage (35). To date, the type II CRISPR system, which utilizes a Cas9 protein, has been used as a new novel technique for genome modification and editing. In this synthetic CRISPR/Cas system, Cas9 is targeted to specific DNA sequences through single chimeric guide RNA's (sgRNA), which is a fusion of a precursor crRNA and a transactivating crRNA (tracrRNA) (33). These sgRNA's are capable of recognizing 20 bp target sites that are followed by the protospacer-adjacent motif (PAM)

sequence NGG, N being any nucleotide (30, 31, 33). Now, a Cas9 mutant lacking endonuclease activity (dCas9) has been used for both gene activation and repression when paired with a transcription activation domain (such as the VP64 domain) or transcriptional repressor, respectively (29–31).

The CRISPR/dCas9 system activates endogenous gene expression, so it utilizes not only the exon regions of the gene, but also the introns and 3' untranslated regions (UTR) as well. Previously only considered for alternative splicing use, intron regions have been recently investigated and found to have additional functions. When present in 3' UTR's, introns can target mRNA for degradation by nonsense-mediated decay (NMD) (36). The NMD pathway has long been regarded as a method of degrading mRNA's for quality control purposes, but it has also recently been shown to regulate gene expression through the use of functional 3' UTR introns (36). In addition, 3' UTR's have been considered well-known targets of microRNA's (miRNA) (37). MicroRNA's are short endogenous RNA's that pair to mRNA's to regulate post-transcriptional repression. According to Friedman et al. (38), there are over 45,000 miRNA target sites conserved within 3' UTR's, and more than 60% of human protein-coding genes are "under pressure to maintain pairing to miRNA's." In all, the important functions of introns, 3' UTR's, and miRNA's in regulating gene expression highlight another benefit of using the CRISPR/dCas system to activate endogenous gene expression.

Preliminary evidence

Combining the CRISPR/dCas system with transcriptional activators and repressors has proven effective in gene activation and repression (29–31). Perez-Pinera et al. (31) paired four copies of the V16 transactivation domain (VP64) to the dCas9 protein and used sgRNA's to target four different loci within the promoter region of a number of genes, such as *IL1RN*, *ASCL1*, *NANOG*, *HBG1* and *HBG2*, and *MYOD1*. They found that corresponding mRNA expression varied depending on the specific sgRNA and targeted locus, and they also determined that there is a synergistic effect between the loci: the greatest amount of mRNA expression was observed when all four loci were activated for a gene (31). Likewise, Gilbert et al. not only created a similar dCas9-VP64 construct, but they also paired the p65 activation domain to dCas9. They cotransfected these constructs with an sgRNA targeting the *Gal4* UAS (upstream activation sequence) into HEK293 cells with a *Gal4* UAS-GFP reporter. They found increased GFP expression in cells from both the dCas9-VP64 and dCas9-p65AD constructs, suggesting that dCas9 can pair with various transactivation domains to increase gene expression (29).

In a similar manner, dCas9 can also be fused with transcription repressor domains to silence gene expression. This time, Gilbert et al. paired dCas9 with the chromatin modifier domain KRAB (Krüppel-associated box) of Kox1 and transfected this construct into GFP-positive HEK293 cells. When sgRNA's targeting *GFP* were later transfected into these cells, a substantial decrease in GFP expression was evident three days post-transfection with strong silencing occurring by the sixth day (29). Qi et al. obtained similar results by simply using a dCas9 protein targeted to the mRFP sequence of a red fluorescent protein (mRFP)-based reporter system in *Escherichia coli*. They found that by binding dCas9 to a DNA sequence

or promoter sequence, it can block transcriptional machinery from binding to the DNA and thereby silencing the gene (30). Therefore, these methods could be used to not only activate anti-apoptotic and anti-inflammatory genes but also repress apoptotic and inflammatory genes in order to enhance stem cell survival. In fact, previous studies have demonstrated the ability of CRISPR/Cas9 to perform multiplexed gene editing (30, 39–41). Qi et al. (30) showed that the CRISPR/dCas9 system can silence two different fluorescent protein reporters without crosstalk, while Cong et al. (40) used a single CRISPR array to target both the *EMX1* and *PVALB* loci. Wang et al. (39) managed to target five different genes (*Tet1*, 2, 3, *SRY*, and *Uty*) with high specificity, while Cheng et al. (41) targeted three (*IL1RN*, *SOX2*, and *OCT4*). These studies provide further evidence of the expanded capabilities of the CRISPR/Cas system in gene manipulation.

Evaluation of hypothesis

By using the CRISPR/dCas9 system to specifically target a number of loci within the promoter region of the *HO-1* gene, we will compare the toxicity of varying HO-1 levels as well as 3' UTR RNA expression. We may examine the levels of HO-1 expression from the different loci and select those that may be the most optimal for achieving HO-1's cytoprotective effects while limiting its cytotoxic effects due to constitutively high levels of overexpression. Also, this system induces the HO-1 pathway through overexpression of the endogenous *HO-1* gene, thus making use of its 3' UTR intron regions for gene control, so we will check for 3' UTR RNA expression.

The CRISPR/dCas9 system would potentially be a more rapid mechanism for overexpressing HO-1 since it already contains a transactivation domain in VP64, eliminating the need to produce additional endogenous transcription factors. Current mechanisms for genome manipulation through DNA-binding proteins, such as zinc fingers or TALE proteins, are more time-consuming and expensive in comparison with the flexibility of the RNA-guided CRISPR/Cas system (29). While it has proven to be successful in activating genes amongst different cell lineages, this specific CRISPR/dCas9 based gene activation system has not yet been performed in cardiac stem cells. Therefore, this project would be innovative in that regard and applying it to promote anti-apoptotic pathways to protect the transplanted stem cells from programmed cell death could prove significant in stem cell survival and ultimately, in patient clinical outcomes.

Implications of the hypothesis

Our hypothesis provides further support of the cytoprotective properties of heme oxygenase-1 and its ability to increase stem cell survival in the ischemic heart. By using the CRISPR/dCas9 technique to activate the gene, we can specifically target whichever locus produces the optimal amount of protein inducing HO-1 activity in a more specific and regulated manner. In doing so, the HO-1 pathway may be activated at specific time points that are most efficient in attenuating the harmful and damaging effects of myocardial infarction. Also, the added level of expressional regulation may mitigate the potential cytotoxic effects of constitutive HO-1 activation. In the future, we hope to extend the use of

the CRISPR/dCas9 system to activate other protective genes, such as Bcl-2, for anti-apoptosis, as well as in other cell lines.

In conclusion, the CRISPR/dCas9 system may provide a more efficient and effective regulator of HO-1 gene activation within stem cells. With its use of sgRNA's to target specific loci within the promoter region of the gene, this system will allow us to choose which particular HO-1 promoter locus to activate in order to overexpress enough HO-1 protein to enhance stem cell survivability, but not to the degree that would produce cytotoxic effects. In addition, this system activates overexpression of endogenous *HO-1*, a safer and more efficient method of introducing additional HO-1 into the cell. Finally, the CRISPR/dCas9 system could prove to be a quicker method of inducing the HO-1 pathway. With these benefits in mind, we believe that the novel CRISPR/dCas9 technique for gene activation could be appropriately applied to enhancing transplanted stem cell survivability within ischemic myocardia in order to better therapeutic outcomes for patients with ischemic heart disease.

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