· Editoral ·

RP research in the era of precision medicine: discovery to translation

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DOI: 10.3760/cma. j. issn. 2095-0160. 2016. 01. 002

[**Abstract**] Retinitis pigmentosa (RP) encompasses many different hereditary retinal degenerations that are caused by a vast array of different gene mutations and have highly variable disease presentations and severities. Work over the past 25 years has resulted in the identification of genes responsible for about 50% of the RP cases, and it´s predicted that most of the remaining disease-causing genes will be identified by the year 2020 or most likelysooner. This marked acceleration is the result of dramatic improvements in DNA-sequencing technologies and the associated analysis. The advent of two recent innovations, induced pluripotent stem cells (iPSCs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease-9 (Cas-9) mediated genome editing, are changing the landscape of RP research, with causative genes being identified at an accelerated rate withgreat potential to translate these discoveries into personalized therapeutic strategies.

[**Key words**] Retinitis pigmentosa/genetics; Individualized medicine/trends; Induced pluripotent stem cells/transplantation; Clustered regularly interspaced short palindromic repeats/genetics

精准医疗时代的 RP 研究:发现与转化　Yang Jing, Xiaowu Gai, Eric Pierce
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DOI:10.3760/cma. j. issn. 2095-0160. 2016. 01. 002

【**摘要**】 视网膜色素上皮变性(RP)包括多种遗传性视网膜变性疾病,这种变性是由一系列不同的基因突变造成的,其临床表现和严重程度具有高度异质性。在过去 25 年来的研究中,半数以上的 RP 病例的致病基因已经确定,预测剩余的致病基因绝大多数将会在 2020 年以前得到鉴定。RP 基因诊断和治疗研究进程的快速进展基于 DNA 测序技术及其相关分析技术的飞速发展。近期的 2 种新技术的开发和应用为 RP 的研究开辟了新的前景,包括诱导多能干细胞研究和规律间隔成簇短回文重复序列(CRISPR)/CRISPR 相关核酸酶-9(Cas-9)介导的基因组编辑技术,使得 RP 致病基因的鉴定结果取得了长足的进步,这种新发现具有转化成 RP 个体化精准治疗策略的潜力。

【**关键词**】 视网膜色素上皮变性/遗传学; 个性化医疗/趋势; 诱导多能干细胞/移植; 规律间隔成簇短回文重复序列/遗传学

President Obama has long expressed a strong conviction that science offers great potential for improving health. Now, president has announced a research initiative that aims to accelerate progress toward a new era of precision medicine. According to the concept of precision medicine, it should ensure that patients get the right treatment at the right dose at the right time, with minimum ill consequences and maximum efficacy[1]. When it comes to the application of precision medicine strategy in ocular diseases retinitis pigmentosa (RP) appears to be the most suitable

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candidate disease.

RP describes a genetically and phenotypically heterogenous group of inherited retinal degenerations that are characterized by initial loss of night vision followed by constriction of visual fields and progressive blindness. Some forms of RP arepresent duringadolescence or even infancy and can therefore impact vision at amuch earlier age. Thus, blindness in the context of RP leads to significantly higher direct costs and a greater number of lost disability-adjusted life years for the patients[2]. The ability to provide early clinical intervention for inherited disorders is heavily dependent on knowledge of a patient´s disease-causing mutations and the resultant pathophysiologic mechanisms. Without knowing a patient´s disease-causing gene, and how gene mutations alter the health and functionality of affected cells, it would be difficult to develop and deliver patient-specific molecular or small molecule therapies. The vast heterogeneity in RP poses a challenge both to the development of therapeutic strategies and to our understanding of the underlying mechanisms and pathophysiology of the disease. Currently, over 3 000 different disease alleles spread across >60 different genes and with variable inheritance patterns are associated with classical forms of RP limited to photoreceptor degenerations only; another 40 genes are associated with various forms of syndromic RP, where vision loss is accompanied by extraocular symptoms, and still others harbor mutations that cause degeneration of the retinal pigment epithelium (RPE)[3]. Whether this great genetic diversity in RP ultimately converges on one or several shared mechanistic pathways leading to retinal cell death is poorly understood at this time, and this lack of knowledge consequently impedes efforts to elucidate universal therapeutic strategies that uniformly treat RP in all of its many different forms. So far, such mutation-independent treatment approaches in humanshave been limited to vitamin supplementation, neurotrophic factor-secreting intraocular implants or electronic retinal prostheses[4-7], although in the absence of mechanism-based therapies that address the underlying molecular defect, these treatments are only minimally effective at best in slowing disease progression or rescuing vision.

More recently, the advent of two recent innovations, induced pluripotent stem cells (iPSCs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-9 genome editing, are changing the landscape of RP research, with causative genes being identified at an accelerating rate and great potential to translate these discoveries into patient-centric therapeutic strategies[8-9].

1 New disease-causing gene discovery

RP is genetically very heterogeneous and so far has been associated with mutations in approximately 200 genes (the full list of genes can be found at https://sph.uth.edu/retnet/disease.htm#17.105d). However, it has been estimated that mutations in known RP genes account for only 50%—60% of cases and therefore more effort is still needed to discover the remaining genetic defects. To identify the new RP associated genes, scientists integrate the techniques of whole-exome sequencing, copy number variation (CNV) analysis, linkage and homozygosity mapping using single nucleotide polymorphism (SNP) arrays[10]. Patients that have been previously excluded for mutations in known RP genes by a targeted next generation sequencing and comparative genomic hybridization (CGH) array studies were selected for the analyses. Two strategies of novel gene discovery were conducted: family genetics and cohort based study. In family genetics researchers analyze all available family members and look for rare, likely pathogenic genetic variants that segregate with the phenotype. In the cohort based studies researchers select patients of similar phenotype, for which there are not available family members, and analyze them together as a group. In this scenario researchers are searching for common rare, likely pathogenic variants between the affected individuals.

2 New diseases models

With the efforts of sequencing and bioinformatics analysis, a lot of new disease-causing genes will be discovered. Now the question is, it is well known thatcorrelation is not equal to causation, so how do we validate the functional significance of disease associated genes? Many researchers use the mousemodel system to understand human diseases. However, at the end of the day, mouse is not human. And using amouse model is not adequate to actually recapitulate all human disease phenotypes in many cases. For some diseases, including ocular diseases, researchers have found that mouse experiments can produce misleading results. For

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instance, it is not uncommon that a drug shows promising results when tested in animals but later is found to have little or no effect in a patient trial. So how to solve this problem? In recent years, the discovery of methods to generate stem cells in the laboratory has led to innovative ways of exploring disease mechanisms and identifying potential drugs. Scientists can now isolate a small amount of a person´s skin cell and reprogram them to create iPSCs, which are similar to natural stem cells found in embryos and have the ability to generate almost any type of tissue. The engineered stem cells can be induced to develop into the cell type of choice, such as photoreceptor, RPE, or corneal epithelial cells. These cells can then be used to replicate and study a disease in a petri dish.

Recent studies underline the fact that iPSCs can be instrumental in confirming and studying a patient´s specific disease-causing mutations. Tucker et al[11] used iPSCs from a patient with sporadic RP to verify the pathogenicity of homozygous Alu insertion uncovered through exome sequencing efforts. In this elegant study, the authors found that the insertion of the Alu sequence into exon 9 of the patient´s *MAK* gene prevented the expression of a splice variant of MAK that is normally expressed in retinal precursors. In this case, hiPSCs offered an efficient means not only to confirm the gene defect responsible for disease, but they also concurrently provided insight into its mechanism. Yoshida et al[12] more recently illustrate the same point in using iPSCs derived from an RP patient to confirm the pathogenicity of a suspected rhodopsin disease mutation. They directly demonstrated the pathogenicity of the patient´s E181K rhodopsin mutation by rescuing patient-derived cells using HDAdV-mediated gene correction and precipitating rod photoreceptor death by introducing the mutation into wild-type control iPSCs. Furthermore, they then used their iPSC lines to assay ER-stress modifying drugs for efficacy in reducing in vitro rod photoreceptor death and for effects on various apoptosis and autophagy markers.

However, some scientists pointed out that the conclusions from the experiments mentioned above are still not solid enough[13]. If we compare the patient´s genome with normal control population, in addition to the suspected disease associated mutations, there are too many genetic differences between them. It is just like no two people in the world would have the same genetic information, even if we were to see a phenotype, it is difficult to attribute the phenotype to that particular mutation since these two people are already so different. So ideally, what we need to do is to find two individuals where everything is the same, except the mutation existing in the disease associated locus. Of course we know it is next to impossible because there are a lot of differences even between sisters or siblings, but what we can do with the help of iPSCs is to edit the genome. By doing so, we artificially create iPSCs that resemble a person who actually doesn´t even exist. Basically, the only difference between these cells would be the particular mutations that we are interested in. Maybe this sounds relatively simple because all we want to do is to introduce a specific mutation into the genome, but in reality it is incredibly difficult to do in human embryonic stem cells (ESCs)/iPSCs, or in mouse cells and other types of cells for that matter. This kind of precise genome editing has been extremely difficult.

3 CRISPR/Cas-9 mediated genome editing

In 2012 researchers invented a new gene editing tool that makes it possible to manipulate the genetic code of any type of cell with unparalleled efficiency and precision—similar to the way a copy editor might move words around or fix spelling mistakes in a text—causing great excitement among scientists. The new technique, called CRISPR, makes it possible to cut and paste the building blocks of a cell´s DNA with the help of bacterial proteins. The CRISPR/Cas-9 system also can be called DNA scissors and what they essentially do is to introduce a specific DNA double stranded break in the genome. Now some people might be thinking that is incredibly dangerous, are not they? Why would we want to cut our DNA? And the truth is that we actually all live dangerously, so our cells are undergoing a lot of DNA damage on a daily basis.

But the good news is thatthe cells have actually come up with ways to fix the DNA damage. The first way the cells can fix DNA damage is through an error prone mechanism. It is like you to get a quiz and there is a question you do not know the answer to, and you would just fill in something in the hope that you may get it right. As a result, sometimes when the cells do not know what to do, they basically fix the DNA double stranded break through an error prone mechanism which will introduce mutations into the genome. The mutations will be randombut if the

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mutations are in the gene of interest or in any important functional domain of the gene of interest, it is likely to introduce a knock-out or a gene disruption. Cells also have a mechanism to fix double stranded breaks in a way that is precise. Taking advantage of this, what we do is to provide the cells with a template and the template will contain the exact mutation we want the cells to incorporate. Therefore, we are able to modify the genome in a way that is extremely precise, allowing us to make disease mimicking human pluripotent stem cells. Lastly, the third approachis very similar to the second approach where we could also insert a larger piece of DNA into the genome which generates targeted transgenesis, knock-in reporters and knock-outs.

The strong genetic basis of RP provides a significant platform to combine CRISPR/Cas-9 genome editing technology with human pluripotent stem cells to mimic causal mutations of genetic RP and understand the pathogenesis of the disease. With CRISPR/Cas-9 technology, pluripotent stem cells can be efficiently generated through the error prone non-homologous-end-joining (NHEJ) mechanism to create the deletion models or by the HDR (homology-directed-repair) mechanism that combines a mutant donor template with Cas-9 nuclease activity to produce single nucleotide mutation and gene insertion models. We envision the utilization of CRISPR/Cas-9 would greatly accelerate the process.

4 Development of therapeutic strategy

The intersection of iPSCs and CRISPR genome editing holds exciting promises of new tools for the treatment of RP that are at once broadly applicable across the vast heterogeneity of the disease while remaining adaptable to the specific mutations in each individual patient.

Currently, a great deal of attention has been justifiably focused on the direct therapeutic applications of iPSCs. For translational applications like discovering novel pharmaco therapeutics, human stem cell-based assays represent a physiologically relevant and high-throughput means to assess the efficacy and toxicity early on in the drug development pipeline. Combining the power of differentiated ocular cells with high-throughput screens will likely prove effective in discovering new pharmacologic treatments for delaying the progression of retinal degenerative diseases.

To date there have been a limited number of reports looking at the effects of drugs on iPSC-derived retinal cells. Gamm et al[14] reported the restoration of ornithine-δ-aminotransferase (OAT) activity in iPSC-derived RPE from a patient with gyrate atrophy following treatment with vitamin B6. It has been known for some time that vitamin B6 is beneficial for patients with gyrate atrophy. However, the patient of interest in this study was previously unresponsive to vitamin B6 supplementation. This study highlighted the use of iPSC-derived RPE to test the specific cellular population targeted in gyrate atrophy to more accurately test vitamin B6 efficacy. In another study, the lab of Masayo Takahashi generated patient-specific iPSC-derived rod photoreceptor-like cells harboring mutations in several genes that cause RP such as *RP1*, *PRPH2*, *RHODOPSIN*, and *RP9*[15]. This study showed that diseased RHODOPSIN-positive iPSC-derived retinal cells perished after 120—150 days in the *in vitro* culture condition. The authors then treated cells with 3 antioxidant vitamins, α-tocopherol, ascorbic acid and β-carotene. They observed that cells treated with α-tocopherol led to a statistically significant increase in survival of diseased iPSC-derived photoreceptor cells, providing proof-of-concept for drug screening of iPSC-derived retinal cells.

However, for numerous other diseases, especially those involving significant neurodegeneration or other types of organ failure, drug intervention alone will not suffice. In these conditions, cellular replacement will be required to rescue, refurbish and preserve tissue function. iPSCs offer an autologous alternative to ESCs transplantation that would likely not require lifelong immunosuppressive therapy since the graft would contain the patient´s own cells expressing self-antigens and would therefore largely preclude the possibility of rejection. This point is relevant to cell transplantation therapies for RP, despite the immunoprivileged space established by the blood-retinal barrier (BRB), because previous studies have found that degenerative conditions and surgical trauma can compromise the BRB and lead to loss of immune privilege, microglial infiltration and destruction of allogenic retinal grafts. Although there are no ongoing human treatment trials involving iPSCs, transplantation of human iPSC-derived RPE cells into a mouse retinal degeneration model rescued electroretinogram function and could be detected by histology[16]. Likewise, preclinical studies of iPSC-

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derived photoreceptor precursors reproduce many of the findings first seen in studies of ESC-based transplants, including synaptic integration between graft and host, maturation of outer segments and some restoration of functional vision[17-20]. Generally, these and other studies of iPSC-based retinal transplantation indicate that iPSCs possess many of the same capabilities, characteristics and therapeutic promises of ESCs. In some cases even without correction of the underlying mutation, iPSCs can be therapeutically useful to RP patients if the degeneration timeline is sufficiently protracted, as it is for many autosomal dominant, and some autosomal recessive, conditions[21]. Although unaltered transplant cells would also eventually degenerate, resetting the degeneration timeline with new cells, at least in cases of slowly progressive disease, may be sufficient to preserve useful vision for the remainder of a patient´s life.

For those cases with more rapid degenerations or where a definitive cure is desired, the CRISPR/Cas-9 system can be tailored to precisely and specifically target the patient´s particular mutation[22-23]. Application of CRISPR/Cas-9 in this treatment scenario can occur either in conjunction with or independent of iPSC-derived retinal cell transplantation. Adeno associated virus (AAV) vectors used in gene supplementation therapy are also optimal vehicles for genome editing machinery and can deliver components directly to the organ or cells of interest. AAV-mediated delivery of CRISPR/Cas-9 components was successful *in vivo*, achieving low but not insignificant editing efficiency in mouse hepatocytes following tail vein injection. Given the successes of sub-retinal injection in gene supplementation therapy, it is reasonable to expect that AAV-CRISPR can also be delivered directly and locally to treat the diseased retina. This hypothetical, novel viral vector treatment would potentially have broader applications than conventional gene supplementation therapy for several reasons. Firstly, it can address dominant mutations via the same gene correction mechanism that is used for recessive mutations. Secondly, it is not limited by size constraints with regard to the gene of interest. Lastly, CRISPR editing can maintain the endogenous gene expression stoichiometry within a cell as long as it doesn´t introduce mutations to knockout the gene. Non-compliantly, gene supplementation results in variable increases in gene copy number and expression that could be deleterious to the treated cell. Especially, for some genes whose expression patterns fall within a narrow physiologic window, the excessive production of functional protein may be just as harmful as underproduction.

All these advantages of CRISPR/Cas-9 editing give it a wide range of possible clinical applications. For RP patients, the precision with which CRISPR/Cas-9 can correct mutations without leaving a genetic footprint, combined with the regenerative capacities of autologous iPSC-derived retinal cell transplantation, could realize the first platform for precision medicine- one that can be adaptable to any patient´s mutation and effective at any stage of the disease. In this treatment model, a patient´s dermal fibroblasts would be reprogrammed back into iPSCs, which would then be differentiated into photoreceptor precursor cells and/or RPE cells. CRISPR editing of the disease mutation could occur either on the iPSCs prior to retinal differentiation or on the precursor cells prior to transplantation. And because the graft would contain the patient´s own cells expressing self-antigens, immunosuppression would not be needed following transplantation.

5 Challenges and future directions

Like any tools, however, both iPSCs and CRISPR/Cas-9 system have limitations that must be taken into account when choosing a genetic disease to study. Indeed, the greatest asset of pluripotent stem cells-the ability to produce a diverse array of cell types derived from all three germ lineages-can bedetrimental unless desired cell types can be identified and isolated, or at least greatly enriched, in culture condition. Otherwise, subsequent experiments are at risk of being confounded by the variable influences of unknown or unwanted cell types that occupy the same culture plate. Once adequate enrichment canbe achieved, it is also necessary to expand and reseed cells onto a variety of surfaces and differentiate them to a uniformly mature state. The latter task is particularly important, since pluripotent stem cells are, at their core, dynamic mini-developmental model systems, As such, it is easy to envision differences in maturation levels between or within hiPSCs cultures, which in turn will affect physiological behavior and experimental outcomes. Finally, there is the question of disease complexity. More specifically, what is the a priori likelihood that a disease phenotype can be

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recapitulated in a dish with a single cell type? It goes without saying that diseases that require involvement of multiple cell types or organ systems and perhaps environmental influences pose greater, although not insurmountable, challenges[24].

CRISPR/Cas-9 system also currently has limitations, especially with regards to off-target effects and unintended mutations introduced into normal parts of the genome. Another major challenge of broad application of CRISPR/Cas-9 in genetic research appears to be its requirement of a PAM motif, which lowers its design density and may be a potential problem for small-range precise mutations like single nucleotide substitution because there could not be a PAM motif nearby[25]. Since its emergence just a few years ago, CRISPR/Cas-9 technology has shown great promise to transform biomedical research and to be developed as a new type of treatment-based genome editing for a wide range of genetic disorders.

In summary, iPSCs and CRISPR/Cas-9 genome editing are remarkable innovations that have many applications in the research and potential treatment of RP. The goal of combining these tools to establish a personalized therapy is possibly within reach for degenerations affecting the RPE layer. For RP patients with primary defects in photoreceptors, realizing this therapy may be more difficult, however it is important to point out that these two are still very nascent technologies that in a short period have continually yielded significant and unprecedented advancements.

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（收稿日期：2015-11-04）

（本文编辑：尹卫靖　杜娟）

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