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·医药生物技术·

雷帕霉素介导的 caspase 9 同源二聚化调控人类多能干细胞自杀

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摘　要：人诱导多能干细胞(human induced pluripotent stem cells, hiPSCs)在再生医学领域具有广阔的应用前景。然而，多能干细胞(pluripotent stem cells, PSCs)具有肿瘤化风险，成为其临床应用最主要的安全性问题。雷帕霉素是一种安全和广泛使用的免疫抑制药物，通过诱导 FKBP12 与 FRB 片段的异源二聚起作用。为了保障 hiPSCs 治疗的安全性，本研究将雷帕霉素诱导的 caspase 9 (*riC9*)基因插入到 AAVS1 安全位点，构建了含有 EF1α 启动子、FRB-FKBP-Caspase9 (CARD 结构域)融合蛋白和嘌呤霉素抗性基因的供体，并与 sgRNA/Cas9 载体共转染 hiPSCs。用嘌呤霉素筛选 2 周后，收集单个克隆进行基因和表型分析。最后，用雷帕霉素诱导 caspase9 同源二聚化，激活工程细胞的凋亡。通过对筛选获得的 5 个 hiPSCs 克隆鉴定，表明供体 DNA 准确敲入内源性 AAVS1 位点。hiPSCs 保持正常的多潜能状态和增殖能力。雷帕霉素可诱导 caspase 9 同源二聚化，并激活细胞凋亡程序。本研究通过药物精确调控 caspase 9 的同源二聚化来启动细胞自杀，实现了可控的 hiPSCs 存活，这为保证 hiPSCs 治疗的安全性提供了新的策略。

关键词：诱导多能干细胞；AAVS1；caspase 9；雷帕霉素；同源二聚化

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Rapamycin mediated caspase 9 homodimerization to safeguard human pluripotent stem cell therapy

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Abstract: Human induced pluripotent stem cells (hiPSCs) are promising in regenerative medicine. However, the pluripotent stem cells (PSCs) may form clumps of cancerous tissue, which is a major safety concern in PSCs therapies. Rapamycin is a safe and widely used immunosuppressive pharmaceutical that acts through heterodimerization of the FKBP12 and FRB fragment. Here, we aimed to insert a rapamycin inducible caspase 9 (*riC9*) gene in a safe harbor AAVS1 site to safeguard hiPSCs therapy by drug induced homodimerization. The donor vector containing an EF1α promoter, a FRB-FKBP-Caspase 9 (CARD domain) fusion protein and a puromycin resistant gene was constructed and co-transfected with sgRNA/Cas9 vector into hiPSCs. After one to two weeks screening with puromycin, single clones were collected for genotype and phenotype analysis. Finally, rapamycin was used to induce the homodimerization of caspase 9 to activate the apoptosis of the engineered cells. After transfection of hiPSCs followed by puromycin screening, five cell clones were collected. Genome amplification and sequencing showed that the donor DNA has been precisely knocked out at the endogenous AAVS1 site. The engineered hiPSCs showed normal pluripotency and proliferative capacity. Rapamycin induced caspase 9 activation, which led to the apoptosis of all engineered hiPSCs and its differentiated cells with different sensitivity to drugs. In conclusion, we generated a rapamycin-controllable hiPSCs survival by homodimerization of caspase 9 to turn on cell apoptosis. It provides a new strategy to guarantee the safety of the hiPSCs therapy.

Keywords: human induced pluripotent stem cells (hiPSCs); AAVS1; caspase 9; rapamycin; homodimerization

Human induced pluripotent stem cells (hiPSCs) have the unique ability to form any types of cells, which gives new hope for regenerative medicine[1-2]. However, hiPSC-based therapy also has raises safety concerns[3-4]. The remaining undifferentiated hiPSCs after transplantation may form teratomas *in vivo*[5]. The genetic and epigenetic abnormalities induced by reprogramming and long-term culture increased the carcinogenic potential[6]. In this connection, the byproducts of hiPSC-derived cells or tissues may cause unforeseen toxicity[7]. A simple and efficient way is required to reduce the risks of the hiPSC therapy.

A stable genetic modification with suicide gene is the most attractive strategy to address the safety concern of the pluripotent stem cells (PSCs) therapy[8]. Inducible caspase-9 (iC9) has become a promising suicide system for pluripotent cell therapy. The recruitment area of caspase-9 is replaced with the dimer binding domain of FK506 binding protein or FRB, the two domains have a strong affinity to form heterodimerization with the chemically inducing drug (CID: rapamycin)[9]. The structural change of FKBP12 caused by F36V mutation (FKBP12-F36V) offers the fusion protein a strong affinity to form homodimerization with the CID (AP1903)[10-11]. The coupled caspase-9 from the induction of CID will activate the downstream signal of caspase-3 and induce more than 90% of iC9-expressing cells[12-14].

Both heterodimerization with rapamycin and homodimerization with AP1903 strategies have been used to engineer the hiPSCs to reduce the risk of clinic therapy[15]. However, heterodimerization strategy requires double gene loads, which increases technical difficulty. Homodimerization requires AP1903, which has not been approved by Food and Drug Administration (FDA) for clinic use.

In this study, we designed a new suicide strategy, homodimerization with rapamycin, to reduce the technical difficulty and promote the clinic use of hiPSCs.

1 Materials and methods

1.1 Human iPSCs culture

Cells were cultured in mTeSR1 media (Stem Cell) on cell-culture plates that had been pre-coated with Matrigel (Corning). Cells were maintained at 37 °C, with 5% CO_2, passaged every 5–7 days using Accutase (Stem Cell) for dissociation. The 10 μmol/L Y-27632 (Selleck Chemicals) was added to the culture medium to improve the cell survival rate from the first day of the generation. The generation of the hiPSC was approved by the Institutional Review Board at Guangzhou Institutes of Biomedicine and Health.

1.2 Vector construction

Generation of CRISPR plasmid containing U6 promoter and single-guide RNA (sgRNA) from pX330-U6-chimeric_BB-CBh-hSpCas9 (Addgene, 42 230) as previously described. sgRNAs was designed to target between the exons 1 and 2 of AAVS1 coding sequence. The genomic sgRNA target sequence, with the protospacer adjacent motif (PAM) sequence shown in bold, was 5′-**GGG**GCCACTAGGGACAGGAT-3′.

Genetic cassettes encoding the safety switch was 2 765 base pairs long and the sequence comprised an EF-1α promoter, an FRB domain, an FKBP domain, an icaspase-9 sequence, a *P2A* gene, a puromycin gene and a BGH poly(A) signal. Homologous arms for homologous recombination were cloned into the pUC19 vector (Addgene, 74 677). Genetic cassettes encoding the safety switch was inserted between the right and left arms.

The pUC19 containing homologous arms was linearized using *Xho* I and purified on a 1.5% agarose gel. The genetic cassettes encoding the safety switch was constructed by PCR amplification using TaKaRa PrimerSTAR Max DNA Polymerase (TaKaRa). The DNA fragment was purified with HiPure Gel Pure DNA Mini Kit (Magen) and then recombined into the backbone by ClonExpress MultiS One Step Cloning Kit (Vazyme).

1.3 Plasmid transfection

hiPSCs grows in a six-hole plate covered with Matrigel™ Matrix (Corning). hiPSCs were cultured until they reached 80% confluence, and then washed with PBS for three times. The cells were dissociated with serum-free dissociation buffer acuutase. Cells were pelleted and centrifuged at 200×*g* for 4 min. The cells were resuspended in 100 μL of electrotransfer buffer, which contained 10 μg of plasmid (sgRNA:donor=1:3), and the cell suspension was placed in an electrotransfer cup. Turn on the LONZA electrometer, set the program to B-016, the cup was placed in the shocking chamber of LONZA Electroporation System. After the start button was pressed, the program ended 3–5 seconds later. Finally, the cells were placed in a six-well

plate containing mTeSR with 10 mmol/L Y-27632 dihydrochloride (Sigma). After 48 h, hiPSCs was attacked with 0.5 μg/mL puromycin, competent colonies were selected.

1.4 Clonal line characterization

In the process of clonal expansion, a portion of the cells was lysed in 10 μL of lysis buffer (0.45% NP40 plus 0.6% proteinase K) at 56 ℃ for 60 min and then at 95 ℃ for 10 min. Then, the cell lysate was used as a template for PCR amplification. The target sequence was amplified from the genome with specific primers (Table 1). The PCR was run using the following protocol: 5 min at 98 ℃ and followed by 40 cycles at 98 ℃ for 10 s, 58 ℃ for 30 s, and 72 ℃ for 30 s by 2×Rapid *Taq* Master Mix (Vazyme). The PCR products were purified on a 1.5% agarose gel, and then sent to Sanger for sequencing (IGE, Guangzhou).

1.5 Flow cytometry analysis

The clones exposed to 10 nmol/L rapamycin for different times (2, 6 h and 24 h) were harvested and stained with annexin V and PI according to the manufacturer's instruction (Annexin V–FITC/PI Apoptosis Detection Kit, Vazyme). The stained samples were detected by flow cytometry within 10 min. The data for fluorescence-activated cell sorting were collected using an Accuri C6 Plus Flow Cytometer (Biosciences). On the scatter plot of bivariate flow cytometry, the left lower quadrant showed living cells (FITC–/PI–), the upper right quadrant showed non-living cells (necrotic cells) (FITC+/PI+), and the lower right quadrant showed early apoptotic cells (FITC+/PI–).

1.6 Real-time quantitative PCR

Total RNA was extracted using Trizol reagent (Tiangen). 100 ng total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol. cDNA samples were then subjected to PCR amplification with custom-designed primers in the CFX96 Touch Real-time PCR Detection System (Bio-Rad). All experiments were performed in accordance with the manufacturer's instructions, qPCR was performed on three technical replicates for each biological replicate. The primers used for real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) are shown in Table 2.

1.7 Cell viability assay

Cells were inoculated onto 96-well plates at 1×10^4 cells/well and routinely cultured. At the 12nd, 24th, 36th and 48th hour post-inoculation, each well was appended with 20 μL cell CellTiter 96® AQueous One Solution reagent (Promega), with subsequent 1 h cultivation. Next, the optical density (*OD*) value at 490 nm wavelength was then examined using a microplate reader. Three replicate wells were set at each time point to calculate the mean value.

1.8 Rapamycin induced caspase-dependent apoptosis in riC9-hiPSC

Activation of caspase-3 and caspase-9 was determined by Caspase 3/9 Activity Assay Kit (Beyotime) according to the manufacturer's instruction. WT-hiPSC and 5# riC9-hiPSC were incubated with or

表 1 基因鉴定所用引物

Table 1 Primers used for gene identification

Primer name	Primer sequence (5′→3′)
5′ arm-Fw-single	CCAGAGACAGTGACCAACCA
5′ arm-Rev-single	ATGGTGGCTCTAGAACCGGTCCTGTGT
3′ arm-Fw-single	TGCTCGCTGATCAGCCTCGA
3′ arm-Rev-single	CTGGGCTTAGCCACTCTGTG

表 2 RT-qPCR 所用引物

Table 2 Primers used for RT-qPCR

Primer name	Primer sequence (5′→3′)
Oct4-Fw-single	TCGAGAACCGAGTGAGAGG
Oct4-Rev-single	GAACCACACTCGGACCACA
Nanog-Fw-single	ATGCCTCACACGGAGACTGT
Nanog- Rev-single	AAGTGGGTTGTTTGCCTTTG
GAPDH-Fw-single	TGCACCACCAACTGCTTAGC
GAPDH-Rev-single	GGCATGGACTGTGGTCATGAG

without 10 nmol/L rapamycin for 24 h, then harvested and lysed to purify proteins. 50–150 μg total protein were incubated with AcDEVD-*p*NA substrate for caspase-3 assay and AcLEHD-*p*NA substrate for caspase-9 assay, respectively, at 37 °C for 2 h. Samples were analyzed by LB943 (Berthold) at 405 nm wavelength. To inhibit apoptosis, 20 μmol/L of the caspase inhibitor Q-VD-OPh (ApexBio) was added 24 h prior to 10 nmol/L rapamycin exposure.

1.9 Statistical analysis

The GraphPad Prism software (version 8) was used for statistical analysis. The data are presented as $\bar{x} \pm s$. Cell deaths among different concentrations and different time points were compared using two-way analysis of variance (two-way ANOVA) followed by multiple comparison with Tukey correction using GraphPad Prism. $P<0.05$ is determined as significant.

2 Results and analysis

2.1 Establishment of hiPSCs with *riC9* KI at AAVS1 locus

The donor was constructed to include 5′ arm and 3′ arm at both sides, and the EF1α promoter was in the middle, which regulates the expression of FRB, FKBP and caspase9 fusion protein. Puromycin resistant gene was also inserted in the vector for cell screening. Cas9/sgRNAs were designed to target the AAVS1 located between exon 1 and 2. The donor would knock-in the AAVS1 locus with the assistance of Cas9/sgRNA in hiPSCs (Figure 1A).

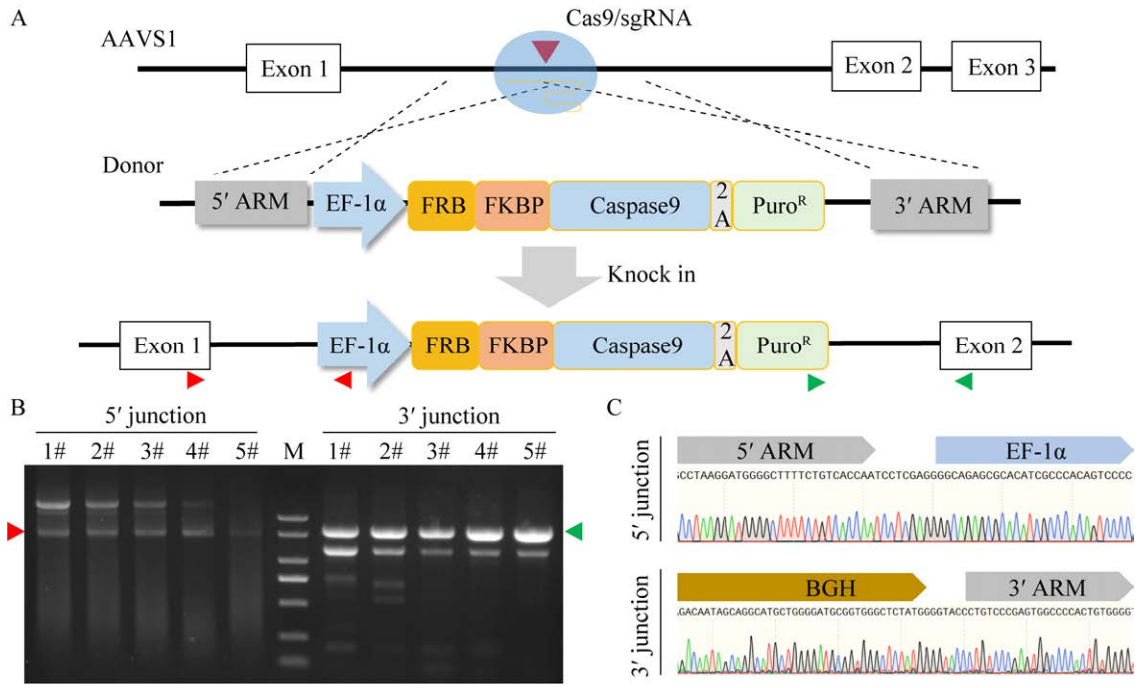

图 1　在人多能干细胞的 AAVS1 基因定点敲入 *riC9* 自杀基因　A：CRISPR/Cas9 的 donor 设计和打靶示意图. B：AAVS1 位点敲入 *riC9* 基因同源臂的 PCR 示意图. C：敲入片段 PCR 产物测序结果

Figure 1　AAVS1 locus knock-in with *riC9* suicide gene in hiPSCs. A: Scheme for generating AAVS1-riC9 clones using CRISPR/Cas9. B: PCR identify the iPSCs clones with *riC9* gene knocked-in the AAVS1 locus. The red and green arrowheads indicate the product bands with precise target insertion. C: Sequencing analysis of hiPSC clone containing the *riC9* gene at the AAVS1 locus.

After the hiPSCs were transfected with the donor and Cas9/sgRNA and selected with puromycin for two weeks, five clones were collected for PCR. The expected bands at both 5′ junction and 3′ junction were identified (Figure 1B). Further sequencing confirmed that *riC9* was precisely inserted into the AAVS1 locus (Figure 1C).

2.2 Typical pluripotent character of the engineered hiPSC clones

The engineered hiPSC clones exhibited typical morphology of a stem cell colony and normal proliferation ability (Figure 2A, 2B). RT-qPCR showed that these five clones expressed normally pluripotent markers, such as *Oct4* and *Nanog* (Figure 2C).

2.3 Induced apoptosis of riC9-hiPSCs by rapamycin

The riC9-hiPSCs exhibited normal colony morphology before rapamycin treatment. However, the colonies dispersed, suspended and died quickly when treated with 10 nmol/L for 1 day (Figure 3A) To assess the drug sensitivity, three clones (2#, 3# and 5#) were treated with different concentration of rapamycin (0.1−1 000 nmol/L) for 24 h. Nearly all riC9-hiPSCs in three groups died at the high concentration of rapamycin (100 nmol/L). However, difference emerged at low concentration of rapamycin. In 1 nmol/L rapamycin, the apoptosis (Annexin-V positive) rate of 5# riC9-hiPSC reached to 85.28%±1.78%, while the death rates of other two clones were under 25% (Figure 3B). We also treated cells under rapamycin (10 nmol/L) with different processing time, and found 5# clone died after 2 h upon drug treatment. Other two clones also died remarkably, but survived for a longer time (24 h). It indicated that the engineered riC9-hiPSC clones may have different sensitivity to drugs.

To discover whether rapamycin-induced cell death was mediated by caspase-dependent apoptosis, we measured caspase-3 and caspase-9 activation after exposure of iC9-hiPSC to rapamycin. Activation of caspase-9 and subsequently of caspase-3 in 5# riC9-hiPSC was observed 24 h after rapamycin exposure, while the activations of WT-hiPSCs were not observed (Figure 4A). To determine whether blockade of caspase-9 activation inhibited rapamycin-induced apoptosis of 5# riC9-hiPSC, we cultured 5# riC9-hiPSC in combination with 20 μmol/L of the caspase inhibitor Q-VD-OPh and 10 nmol/L of rapamycin. Rapamycin-induced cytotoxicity was completely inhibited by Q-VD-OPh (Figure 4B). Hence, the cell death observed in riC9-hiPSC was mediated by caspase-dependent apoptosis.

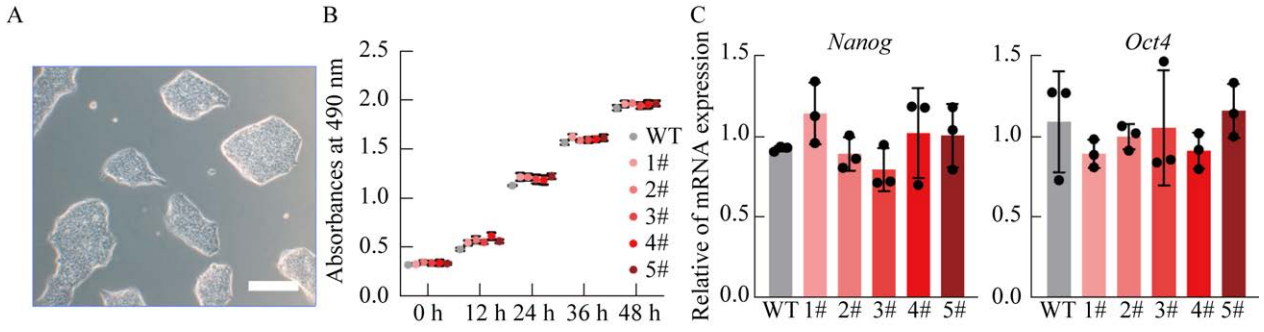

图 2 riC9-hiPSCs 克隆的表征 A：riC9-hiPSCs 克隆的明场图像. B：WT 和 riC9 hiPSCs 体外增殖能力分析. C：WT 和 riC9 hiPSCs 多能性 RT-qPCR 分析

Figure 2 Characters of *riC9* engineered hiPSC clones. A: Bright-field image of riC9-hiPSCs. Scale bars=100 μm. B: The proliferation curve of wild type hiPSCs, and riC9-hiPSC clones, during *in vitro* culture. n=3, values represent mean±SEM. C: RT-qPCR relative mRNA levels of *Nanog* and *Oct4* in the indicated hiPSCs. n=3. Data represent mean±SEM.

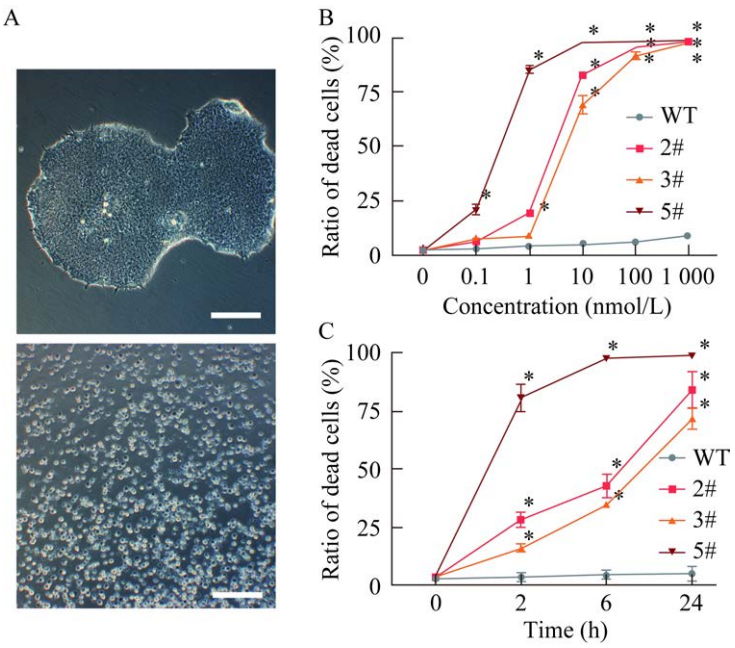

图 3　riC9-hiPSCs 在体外被雷帕霉素清除　A：雷帕霉素给药前后 riC9-hiPSCs 细胞的明场图像. B：不同浓度雷帕霉素诱导 riC9-hiPSCs 细胞的凋亡情况. C：不同时间段的雷帕霉素诱导 riC9-hiPSCs 细胞的凋亡情况

Figure 3　Induced apoptosis of riC9-hiPSCs by rapamycin. A: Bright-field images of riC9-hiPSCs before and after rapamycin administration. Scale bars=100 μm. B: The death rate of riC9-hiPSC clones with increasing concentrations of rapamycin (0.1–1 000 nmol/L) treatment. The percentage of killing reported was the percentage of transduced live cells relative to the untreated control for each condition. n=3. Data represent $\bar{x} \pm s$. C: Time-dependent drug effect of riC9-hiPSC clones. n=3. Data represent $\bar{x} \pm s$. *: P<0.05, represents a significant difference between WT and other clones.

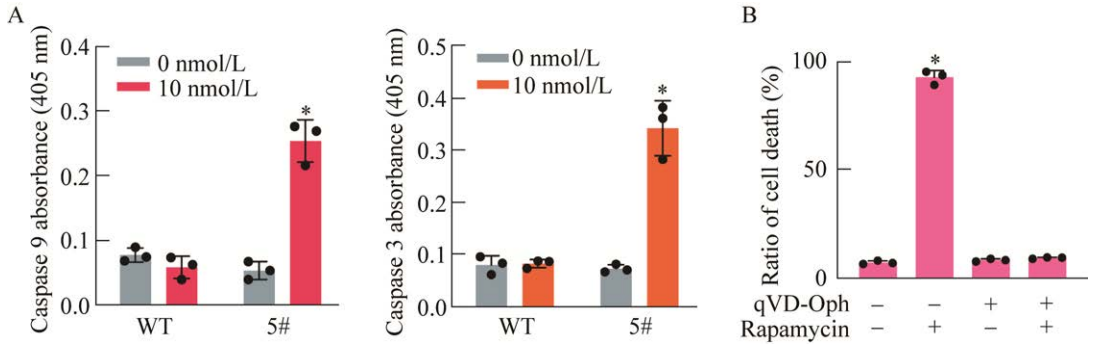

图 4　雷帕霉素诱导 riC9-hiPSCs 中 caspase 蛋白依赖性凋亡　A：比色法测定 caspase 9 和 caspase 3 蛋白的活化情况. B：Q-VD-OPh 与雷帕霉素联合处理 riC9-hiPSCs 的凋亡情况

Figure 4　Rapamycin-induced caspase-dependent apoptosis in riC9-hiPSC. A: Activation of caspase-9 and -3 was measured by colorimetric assay. n=3. Data represent $\bar{x} \pm s$. *: P<0.05, represents that riC9-hiPSC had a significant difference compared to other groups, after 24 h of exposure to rapamycin. B: riC9-hiPSCs were treated with rapamycin in combination with 20 μmol/L of Q-VD-OPh, and the ratio of cell death was measured by Annexin V/PI staining by flow cytometry. n=3. Data represent $\bar{x} \pm s$. *: P<0.05, riC9-hiPSCs only exposed to rapamycin showed significant difference compared to other groups.

3 Discussion

In this study, we established a *riC9* suicide system in human iPSCs by precisely inserting the *riC9* gene in the AAVS1 safe harbor locus. The riC9-iPSCs were eliminated within 24 h upon rapamycin administration. In addition, this modification of iPSCs did not show adverse effect on pluripotency. The caspase 9 directly activates downstream components of the intrinsic apoptosis pathway, including caspase-3[16]. We found the engineered *riC9* activated by rapamycin could also initiate caspase-dependent apoptosis of hiPSC rapidly (Figure 5). Although both FRB and FKBP fused with caspase 9, the space steric effect ensures intermolecular dimerization instead of intramolecular dimerization after CID administration, resulted in dimerization of caspase 9 and activation of apoptosis.

To date, three common suicide gene systems, hrpes simplex virus thymidine kinase (HSV-tk), yeast cytosine deaminase (yCD) and inducible caspase-9 (iC9), have been applied as a safety switch for iPSC[17-18]. The iC9 system is the most widely used due to its following advantages: First, it can extend its suicidal function to their progeny, and the differentiated cells still have this function. Second, the suicide strategy can rapidly kill the divided undifferentiated hiPSC and their slower mitotic or post-mitotic progeny. The activated prodrug is non-toxic and biologically inert. Finally, this system is non-immunogenic and immune cells would not attack the cell products[19-21].

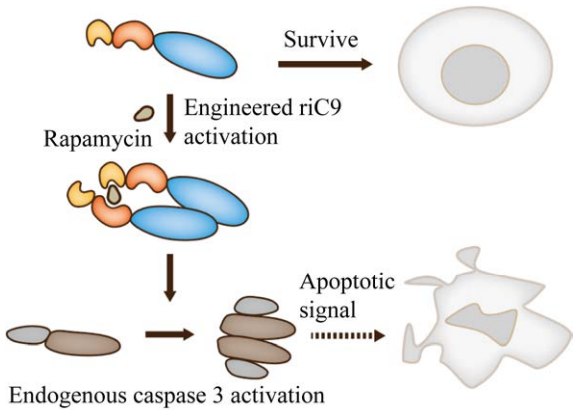

图 5 *riC9* 基因介导的可调控细胞自杀示意图

Figure 5 Schematic diagram for summarizing the *riC9* mediated controllable cell suicide.

The safe harbor AAVS1 locus have been deeply studied[22-24]. Engineering of AAVS1 locus has not been reported to cause abnormal proliferation or differentiation of iPSCs[25-26]. Driven by the endogenous promoter of PPP1R12C gene, the transgenic expression of this site is stable in many cell types. The drug sensitivity of the clones was different, mainly due to the heterogeneity of pluripotent stem cells.

In previous research, we reduced risks of PSCs by administration of drug AP1903 to eliminate undifferentiated hiPSCs *in vivo*[15]. Although the intervention can improve safety of stem cell therapy, the adverse effects of hiPSCs still exists after its differentiation. To maximize safety, suicide gene should play a key role in whole life of stem cells. Thus, integrated the suicide gene the FRB-FKBP-iC9 system into the universal safe site AAVS1 with the assistance help of the CRISPR/Cas9 system.

In summary, we generated riC9-hiPSCs by integration of *riC9* at the AAVS1 locus, which provides an effective and safe strategy for hiPSC-based therapies.

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