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SUPPRESSION OF LIGASE4 OR XRCC6 ACTIVITIES ENHANCES THE DNA HOMOLOGOUS RECOMBINATION EFFICIENCY IN ZEBRAFISH PRIMORDIAL GERM CELLS

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Abstract: Primordial germ cells (PGCs) give rise to gametes which transmit the genetic information to next generation, therefore PGCs provide us an ideal cell type for genetic manipulation. Homologous recombination (HR) is the most efficient technique to create designed genetic modifications, however, its efficiency is rather low in vertebrates. In this study, by using zebrafish as an *in vivo* model, we aimed to enhance the efficiency of HR in zebrafish PGCs. First, we injected *UAS:mRFP-nos*1 construct into *Tg* (*kop:KalTA*4) embryos to label the transgenic PGCs, and we showed that screening of PGCs-specific mRFP expression led to relatively high-efficient germline transmission of transgene. Then we established an *in vivo* assay to evaluate the HR frequency in PGCs. We further revealed that suppression of the activities of DNA ligase IV (Lig4) and Xrcc6 (previously known as Ku70) could significantly increase the HR efficiency, not only at whole embryo level but also in PGCs. We proposed that the *Tg(kop:KalTA4)* line could be used as an effective platform for HR-mediated gene targeting.

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Gene targeting via homologous recombination (HR) is an efficient genetic technique to create designed genomic modifications. It can be used to induce deletions, insertions or designed mutations of specific sequences, to study the function and regulation of genes. In combination with Cre/loxP system, HR based gene targeting can be used to conditionally knock out certain gene in specific tissues or cell types [1, 2]. The frequency of traditional gene targeting strategy is approximately 10^{-5} to 10^{-7}, which is the most serious problem that limits the wide application of this technique [3, 4]. For a long time, gene targeting was only achieved in a few animals with embryonic stem cells, which could facilitate efficient *in vitro* selection [5]. Fortunately, gene targeting is stimulated two to three orders of magnitude by DNA double-strand break (DSB) at the target locus [6, 7]. In recent years, with the development of customized nucleases, such as ZFNs (Zinc Finger Nucleases), TALENs (Transcription Activator-Like Effector Nucleases) and CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) systems, which can generate sequence-specific DSBs in host genomes, the HR based gene targeting has been rapidly developed in *Drosophila* [8], mouse [9, 10], rat [10], and zebrafish [11].

To repair DSB, organisms from yeast to vertebrates have developed two major mechanisms for survival, non-homologous end-joining (NHEJ) and homologous recombination (HR). HR plays a dominant role in DSB repair in yeast, whereas NHEJ significantly contributes to DSB repair in vertebrates [12, 13]. In zebrafish, the frequency of NHEJ is much higher than the frequency of HR in the repair events in early development [14]. Since there is a balance between HR and NHEJ, suppression of NHEJ may enhance HR activity in vertebrates. NHEJ is the most straightforward way of DSBs repair, and it simply rejoins the broken ends and often results in errors. The DNA breaks are initially recognized by the heterodimeric Ku70/80 complex, which changes conformation and

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promotes the recruitment of the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) [15]. Then a complex of XRCC4 and DNA ligase IV (Lig4) is recruited by the assembled DNA-PK complex to carry out ligation [16]. Deficiency of Ku70/80 or Lig4 has been shown to result in decreased NHEJ and enhanced HR in yeast [17—20], mammalian cells [21, 22], mice[21] and rice [23]. HR repair pathway uses the allelic sequences as a template, and thus it is a precise repair mechanism. The Rad51 recombinase plays a critical role in HR, and other critical proteins include RAD51, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, BRCA1, BRCA2, RAD54, and RPA [16, 24]. Interestingly, overexpression of RAD51 has been shown to play positive or negative roles on DSB-induced HR [25, 26].

As a model animal, the embryos and larvae of zebrafish are optical clarity, which allows easy visualization of developmental processes of primordial germ cells (PGCs) and other organs [27, 28]. In zebrafish and other vertebrates, PGCs give rise to the sperm and eggs to transmit the genetic information to next generation. Although HR may occur in whole embryo, only the HR events that occur in PGCs are of significance for generation of gene targeted lines. Researchers have introduced foreign genes carried by sperms into fish embryos to establish transgenic lines [29]. Therefore, if we can detect HR events in the PGCs of the founder fish (P_0) at early stage of development, it will greatly reduce the workload of screening.

In previous study, we have established a transgenic activator line, *Tg*(*kop:KalTA*4), which express the Gal4 activator proteins in PGCs [2]. PGCs can be specifically labeled with high efficiency when we directly inject the *UAS:mRFP-nos*1 construct into the embryos, which allows the *Tg*(*kop:KalTA*4) line to be an ideal host to manipulate the PGCs of zebrafish. In the present study, we used zebrafish as an *in vivo* model to study how to enhance the efficiency of HR in PGCs. We first established an *in vivo* assay to evaluate the HR frequency in PGCs, and then revealed that the efficiency of HR could be significantly increased by inhibition of the activities of Lig4 and Xrcc6 (previously known as Ku70), not only at whole embryo level but also in PGCs.

1 Materials and Methods

1.1 Zebrafish

The *AB* embryos and *Tg*(*kop:KalTA*4)^{ihb8} [2] embryos were obtained from the China Zebrafish Resource Center (Wuhan, China), raised under standard conditions at 28.5℃ and maintained as described [30, 31].

1.2 Plasmids construction

PGC-HR reporter construct: Firstly, a 5′-*mRFP* was amplified from the *UAS:mRFP-nos*1 vector [2] with the primers of 5′-*mRFP*-F and 5′-*mRFP*-R, and cloned into the pMD18-T vector (TaKaRa) as the intermediate construct. Secondly, the *UAS-mutant mRFP-nos*1 fragment was amplified by overlap PCR from the *UAS:mRFP-nos*1 vector with introducing enzyme sites *Hin*dIII and *Sal*I at both ends, as well as two opposite I-*Sce*I sites which substitute 4 bp (from 199 to 202) of *mRFP*. The first round of overlap PCR involved two reactions. One reaction amplified the UAS and 5′-part of the mutant *mRFP* with the primers of UAS-*Hin*dIII-F and Mutant *mRFP*-199-R. The other reaction amplified the 3′-part of the mutant *mRFP* and UTRnos1 with primers of Mut*mRFP*-203-F and nos-*Sal*I-R. The second round of PCR amplified the UAS-Mut*mRFP*-nos1, using the two DNA fragments generated in the first round as templates with two primers: UAS-*Hin*dIII-F and nos-*Sal*I-R. Finally, we inserted the UAS-Mutant *mRFP*-UTRnos1 fragment at the *Hin*dIII and *Sal*I sites of the intermediate construct. 0.5 pg of plasmid digested or undigested by I-*Sce*I was injected into each embryo at single-cell stage.

DN-lig4-SV40, DN-xrcc6-SV40, and rad51-SV40: Dominant negative *lig*4 (DN-*lig*4) fragment was amplified from the cDNA of 1-day-old zebrafish embryos with the primers of DN-lig4-*Bam*HI-F and DN-lig4-*Xho*I-R, and the putative DN-lig4 product contains amino acid residues 658—910 of Ligase4. Dominant negative xrcc6 (DN-xrcc6) fragment was amplified with the primers of DN-xrcc6-*Bam*HI-F and DN-xrcc6-*Xho*I-R, and the putative DN-xrcc6 product contains amino acid residues 62—410 of Xrcc6. *rad*51 fragment was amplified with the primers of rad51-*Bam*HI-F and rad51-*Xho*I-R. Then the three fragments were cloned into the pCS2+ vector, separately. The three vectors were digested with *Not*I, capped mRNA was synthesized with a mMESSAGE mMACHINE SP6 Kit (Ambion, America). 0.25 pg of *DN-lig*4-*SV*40 mRNA, 0.25 pg of *DN-xrcc*6-*SV*40 mRNA or 0.5 pg of *rad*51-*SV*40 mRNA was injected into each embryo at single-cell stage, respectively.

1.3 Morpholino

*Lig*4 morpholino (lig4-MO) (5′-TTGCAGAAGA CACGGAAGAAACTGT-3′) was kindly provided by Prof. Jun Chen, Zhejiang University, China [14] and designed to specifically target the 5′-UTR of lig4. 0.5 nmol of lig4-MO was injected into each embryo at 1-cell stage.

1.4 Microinjection

Different reagents were injected into 1-cell stage embryos for each experiment. The injected embryos were allowed to develop at 28.5℃ for observation of

fluorescent protein expression under a fluorescence microscope (Olympus MVX10) and other analysis.

1.5 Quantitative PCR (qPCR)

In quantitative assays of HR reporter system in whole body, DNA was extracted from zebrafish embryos with a DNA extraction kit according to the manufacturer's protocol (TianGEN #DP304, China). The amount of injected reporter vector was normalized by normalizing primers with a Bio-Rad Real-time PCR machine. Frequencies of HR repairs were quantified using the pairs of repair primers. Both of the vectors repaired by HR and unrepaired can be used as the templates of normalizing primers, however the PCR product can only be amplified with repaired vector with the repair primers. The primer sequences were shown in Tab. 1.

2 Results

2.1 Screening of PGCs-specific mRFP expression leads to high-efficient germline transmission of transgene

In order to confirm our speculation that the fluorescent PGCs can transmit the transgenic information to next generation, we co-injected 0.25 ng of linearized *UAS:mRFP-nos*1 construct with 0.5 ng of *bucky ball* (*buc*) mRNA [32] into each *Tg*(*kop:KalTA*4) embryo at single-cell stage to label the PGCs of the embryos. As expected, injection of *UAS:mRFP-nos*1 construct into *Tg*(*kop:KalTA*4) embryos could distinctively label the PGCs at 1 day post-fertilization (dpf) (Fig. 1A). After co-injection of *buc* mRNA, we observed an addition *mRFP*-labeled PGCs in the embryos at 1 dpf (Fig. 1b), which indicates that Buc overexpression could significantly induce a few additional germ cells. The ratios of embryos showing PGCs-specific *mRFP* expression continued to decrease before 18 dpf (Fig. 1c, d), suggesting that un-integrated plasmids might contribute to the PGCs-specific expression from 1 dpf to 16 dpf. At 16 dpf, the embryos were divided into two groups, according to whether they showed PGCs-specific *mRFP* expression or not. The ratio of PGCs-labeled embryos was 13.7% (16/122) at 16 dpf (Fig. 1d).

These two groups of embryos were raised to adulthood and the mature transgenic founders were crossed with wild type (WT) fish. The progeny embryos (F1) were collected for analyzing germ line transmission by PCR with the primers of RFP-F and RFP-R. We screened the progeny of 46 PGCs-labeled founder fish and observed the expected PCR products in the progeny of 7 founders, suggesting that the PGCs-labeled founder fish transmitted insertions to their offspring with relatively high efficiency (15.2%) (Fig. 1e), compared to germline transmission efficiency of conventional transgenic technology of 1%—3% in zebrafish [33]. Moreover, only the PGCs-labeled embryos were potential transgenic founders, as all the PGCs-negative embryos derived transgenic founders did not produce transgenic offspring (0/55) (Fig. 1e). Our results demonstrate that the *Tg*(*kop:KalTA*4) line provides us a powerful tool for transgenic screening at germ cell level, and it may be also used for gene targeting screening in germline.

2.2 Injection DN-Lig4 and DN-Xrcc6 mRNA significantly increase homologous recombination in whole embryo

To increase the frequency of HR, we chose DN-Lig4, lig4-MO, DN-Xrcc6, and Rad51 as potential candidates. DN-lig4 is the cDNA for the carboxyl-terminal residues of Lig4 contain BRCT repeat and Xrcc4 binding site, and DN-Xrcc6 contains the cDNA of xrcc6 with 1-61 NH2-terminal residues deletion, as reported in human cultured cells [34, 35] (Fig. 2a). We prepared their mRNA with the expectation that overexpression of these mRNA would induce dominant-negative effects. We also used a lig4-MO to block protein translation of Lig4 to compare the effectiveness with DN-Lig4. Furthermore, we cloned *rad*51 cDNA, synthesized its mRNA and carried out overexpression experiments in zebrafish.

To determine the functional significance of these candidates, we used whole embryo HR (WE-HR) and whole embryo NHEJ (WE-NHEJ) reporter systems that have been established in zebrafish embryos as evaluation systems[14]. The WE-HR construct and WE-NHEJ construct were digested with I-*Sce*I *in vitro* and injected separately into WT zebrafish embryos with the same amount. Strong green fluorescence was observed in the embryos injected with digested WE-NHEJ under a fluorescence microscope at 5hpf, but in contrast, only weak green fluorescence was observed in the embryos injected with digested WE-HR at 9hpf (Fig. 2b, c). The result indicates that the NHEJ activity is much higher than the HR activity in the DSBs repair of zebrafish embryos.

Then the digested WE-HR construct was co-injected with *DN-lig*4 mRNA, lig4-MO, *DN-xrcc*6 mRNA, *rad*51 mRNA or control mRNA into embryos, respectively. We observed the green fluorescence under a fluorescence microscope at 10hpf and classified the fluorescent embryos into 3 classes, weak, moderate and strong, according to the fluorescent intensity of the embryos. An obvious increase in green fluorescence intensity was found in the embryos co-injected with *DN-lig*4 mRNA or *DN-xrcc*6 mRNA, compared to those co-injected with the control mRNA (Std-mRNA) (Fig. 2d). The DNA was extracted and subjected to q-PCR assay. We obtained similar result

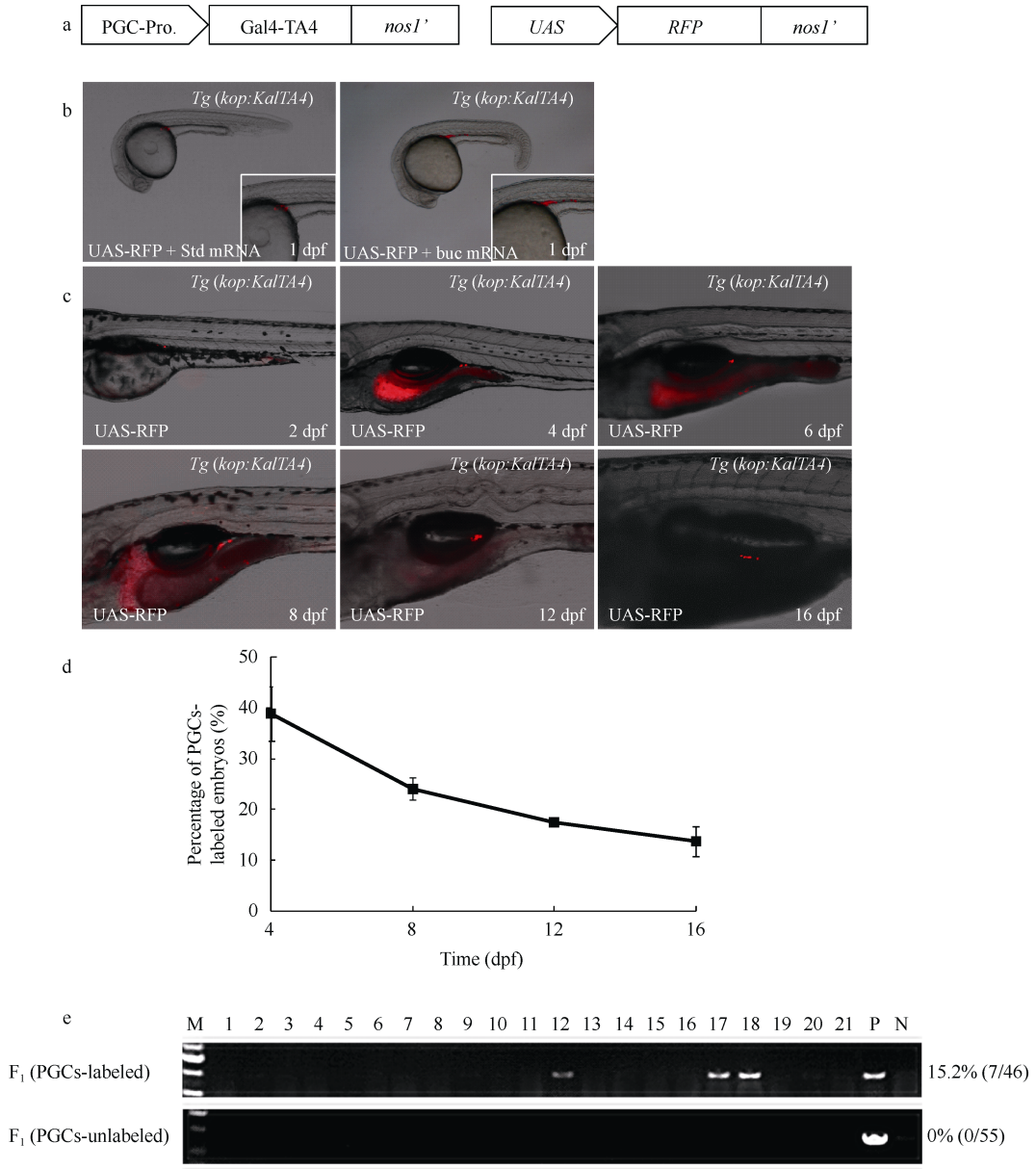

Fig. 1 PGCs-specific mRFP expression and germline transmission of the *Tg* (*kop:KalTA*4) embryos injected with *UAS:mRFP-nos*1 construct a. Diagram of the *kop:KalTA*4 construct and the *UAS:mRFP-nos*1 construct. b. PGCs-specific mRFP expression in the *Tg* (*kop:KalTA*4) embryos injected with *UAS:mRFP-nos*1 construct and Std-mRNA or *buc* mRNA at 1 dpf. c. PGCs-specific mRFP expression was observed in the *Tg*(*kop:KalTA*4) embryos injected with *UAS:mRFP-nos*1 construct at 2 dpf, 4 dpf, 6 dpf, 8 dpf, 12 dpf and 16 dpf. d. The ratios of embryos showing PGCs-specific mRFP expression at 4 dpf, 8 dpf, 12 dpf and 16 dpf. e. The frequency of germline transmission of transgene in the PGCs-unlabeled and PGCs-labeled transgenic founders

with real-time PCR analysis that knockdown of Lig4 or Xrcc6 produced a 31.0% or 59.1% increase in the frequency of HR, respectively (Fig. 2e). These results suggest suppression of Lig4 or Xrcc6 activities have significant effects on the increase of HR frequency. Meanwhile, although lig4-MO was as effective as *DN-lig*4 mRNA, the abnormal development ratio of embryos injected with lig4-MO was the highest in all the injected groups at 1 dpf (data not shown), suggesting that lig4-MO had higher cytotoxicity than other reagents.

2.3 Inhibition of Lig4 and Xrcc6 activities greatly enhance HR in zebrafish PGCs

We then established an *in vivo* model to evaluate HR at PGCs level (PGC-HR reporter construct) (Fig. 3a). The PGC-HR construct was designed on the same principle with the HR reporter system [14]: a four Gal4-binding-site 4×UAS, followed by a mutant mRFP in which the 4 bp (from 199 to 202) of mRFP is substituted by a 14 bp DNA sequence flanked by two I-*Sce*I sites in the opposite direction, a *nanos*1 3′UTR, and (1—418) bp of wild-type mRFP which is used as

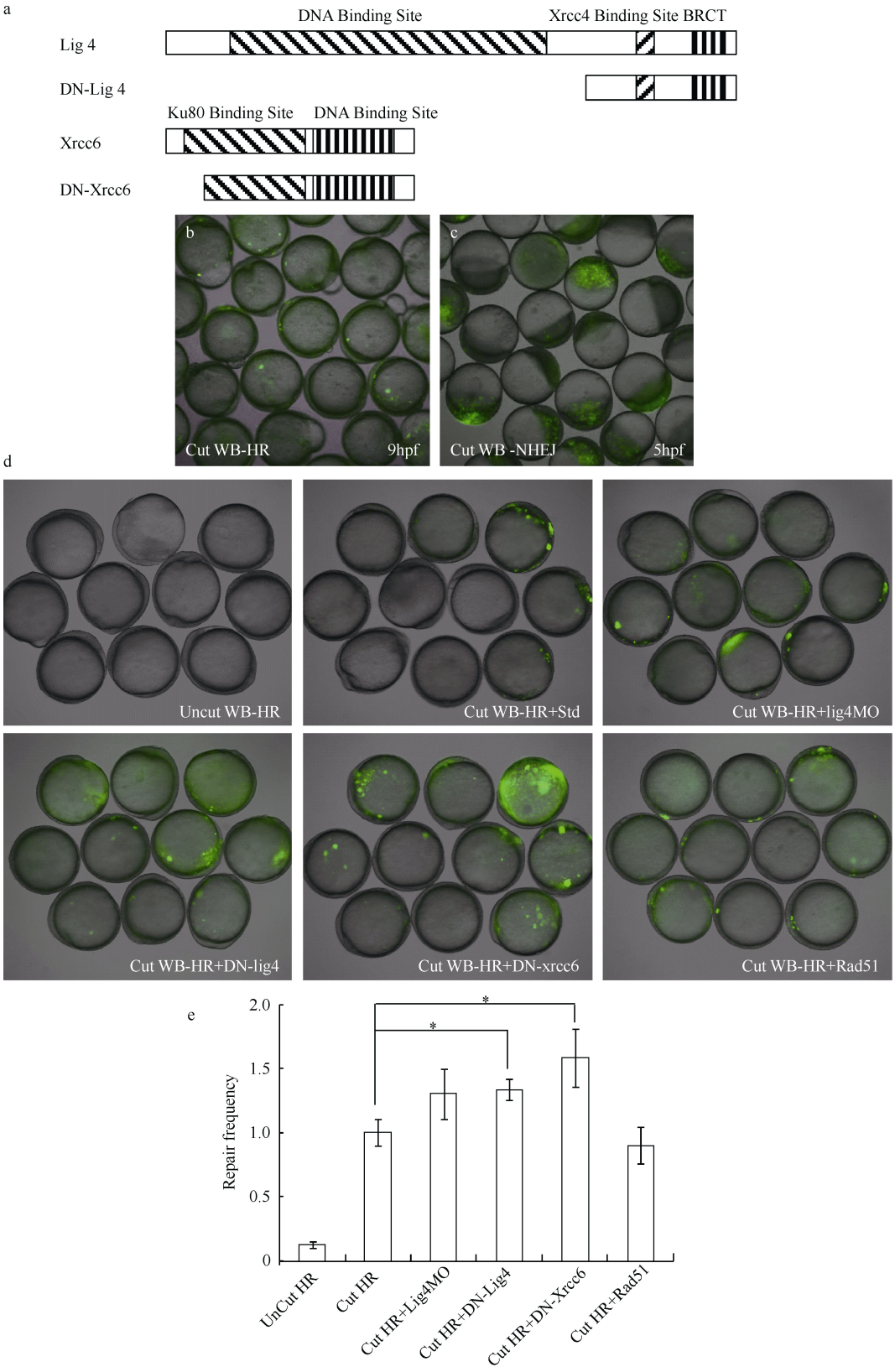

Fig. 2　The HR repair frequency in WE-HR reporter system

a. Diagram of DN-Lig 4 and DN-Xrcc6 proteins. b. Weak expression of EGFP was observed in the wild-type embryos injected with cut WE-HR construct at 9 hpf. c. Strong expression of EGFP was observed in the wild-type embryos injected with cut WE-NHEJ construct at 5 hpf. d. EGFP expression in the wild-type embryos injected with cut WE-HR construct plus *DN-lig*4 mRNA, lig4-MO, *DN-xrcc*6 mRNA, *rad*51 mRNA or the control mRNA. e. the frequencies of HR repairs analyzed by qPCR

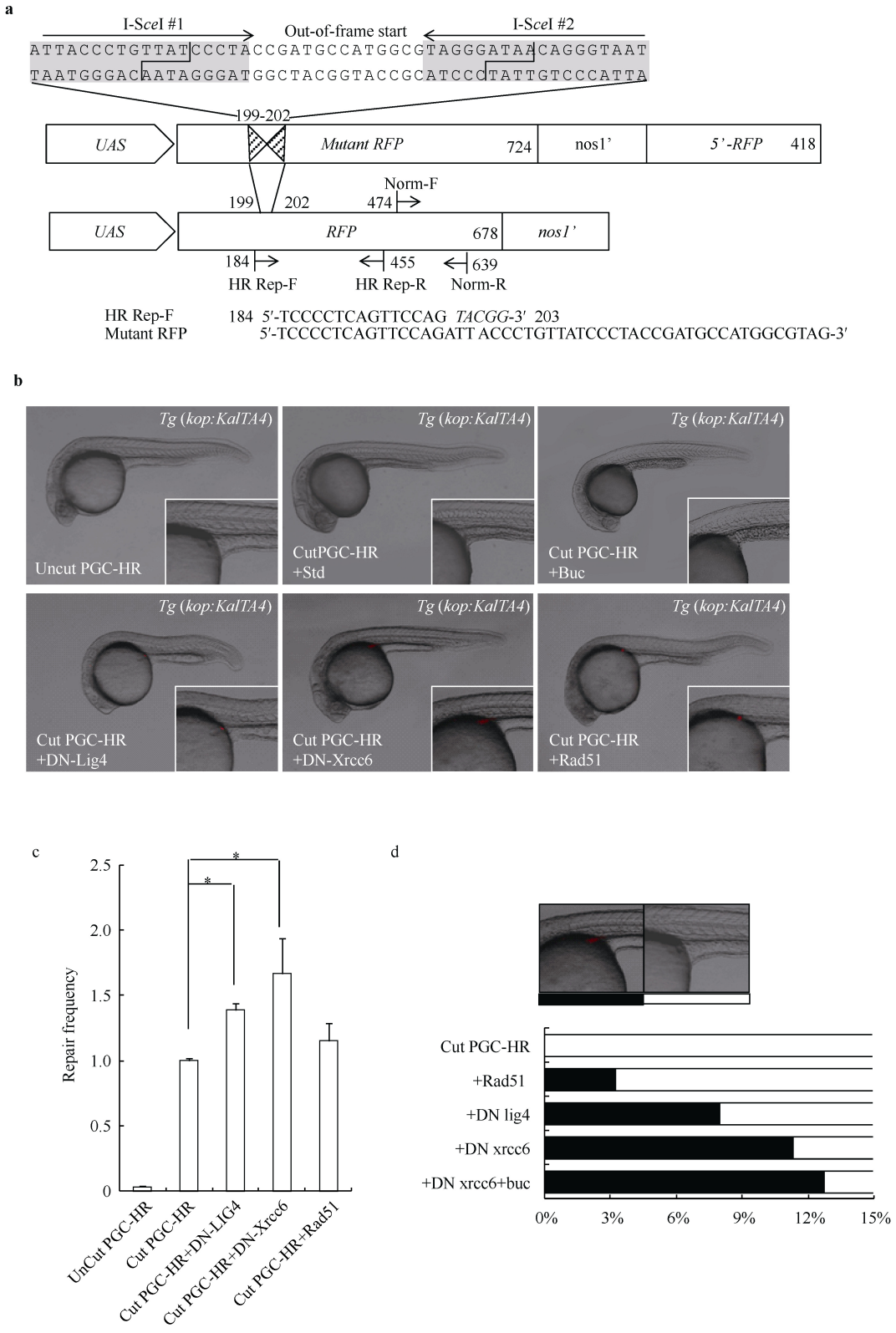

Fig. 3 The HR repair frequency in PGC-HR reporter system

a. Diagram of PGC-HR construct. b. PGCs-specific mRFP expression in the *Tg*(*kop:KalTA4*) embryos injected with cut PGC-HR construct plus *buc* mRNA, *DN-lig*4 mRNA, *DN-xrcc*6 mRNA, *rad*51 mRNA or control mRNA. c. the frequencies of HR repairs in whole embryos analyzed by qPCR. d. The percentage of embryos showing PGCs-specific fluorescence

the donor template. Although the PGC-HR construct could be repaired by HR, NHEJ or SSA mechanism after the initiation of DSB at two I-*Sce*I sites, it will express the wildtype mRFP protein in PGCs only if HR repair occurs.

To determine if the PGC-HR reporter construct works, the construct was digested with I-*Sce*I *in vitro* and injected into the *Tg* (*kop:KalTA*4) embryos. Unexpectedly, none of embryos showed specific mRFP expression in PGCs at 1dpf (Fig. 3b). However, the result from q-PCR assay indicated that the HR repair occurred at the whole embryo level (Fig. 3c). The data suggests that although the digested PGC-HR construct was repaired by HR in whole embryos, the repair frequency in PGCs was too low to accumulate enough mRFP protein at 1dpf.

We used the PGC-HR reporter system to challenge the effectiveness of different reagents that could increase the efficiency of HR in PGCs. Similarly, The PGC-HR reporter construct was digested with I-*Sce*I *in vitro*, and co-injected with *DN-lig*4 mRNA, *DN-xrcc*6 mRNA or *rad*51 mRNA into the *Tg* (*kop:KalTA*4) embryos, respectively. Intriguingly, 12.7% (14/110) of those co-injected with *DN-xrcc*6 mRNA, 8.7% (11/127) of those co-injected with *DN-lig*4 mRNA and 3.3% (4/121) of those co-injected with *rad*51 mRNA showed specific *mRFP* expression in PGCs, compared to 0% (0/100) of those co-injected with Std-mRNA (Fig. 3b, d). Then the total DNA was extracted for q-PCR assay to determine the frequency of HR in the whole body. The result confirmed that the repair frequency of the embryos co-injected linearized PGC-HR construct with *DN-lig*4 mRNA or *DN-xrcc*6 mRNA was significantly increased when compared to the embryos co-injected with Std-mRNA (Fig. 3c).

Since Buc protein has been found to be necessary and sufficient for germ plasm organization in zebrafish [32], we co-injected the PGC-HR reporter construct with *DN-xrcc*6 mRNA and *buc* mRNA into the *Tg* (*kop:KalTA*4) embryos to see the effects. The result showed that 14.5% (15/103) of embryos show specific *mRFP* expression in PGCs (Fig. 3d), which was a little higher than the ratio of the fluorescent embryos without *buc* mRNA injection. Therefore, *DN-xrcc*6 mRNA is the most effective in all candidates to block NHEJ and to increase the frequency of HR in zebrafish, together with *Tg* (*kop:KalTA*4) line and *buc* mRNA, which should provide us a powerful tool for gene targeting screening in zebrafish germline.

3 Discussion

The ultimate goal of present study is to establish a highly efficient gene targeting technology in PGCs of zebrafish embryos. The researchers have so far generated transgenic lines to label the PGCs in live embryos of zebrafish [36—39]. However, none of them is suitable for gene targeting technology by using a positive selection strategy to label the PGCs, because PGCs could not be distinctly labeled by direct injection of DNA constructs into embryos[2]. Fortunately, we can utilize the *Tg* (*kop:KalTA*4) line to solve the problem. PGCs can be specifically labeled with high efficiency by directly injection of an expression cassette *UAS:mRFP-nos*1 into the embryos. We further proved that the PGCs-labeled *Tg (kop:KalTA*4*)* founders transmit insertions to their offspring with relatively high efficiency (15.2%).

Therefore, the *Tg* (*kop:KalTA*4) line provides us an effective platform to establish an effective model system for study of gene targeting in PGCs, and we could further investigate how to enhance the efficiency of HR in zebrafish PGCs. Although recombination can be induced at least 1000-fold above background following the introduction of DSB using ZFNs, TALENs or CRISPRs system, the frequency of gene targeting still requires improvement in zebrafish. For instance, Zu, *et al.* [11] showed that only 4 of 275 founder fish transmitted homologous recombination events through the germline in zebrafish. In our work, although we examined potential targeting products by PCR in the embryos 1 day after injection, we could not detect the targeting events in the progeny (F1) (unpublished data). In the past decades, most studies have shown that defects in Ku70/80, Lig4 could lead to decreased NHEJ and enhanced HR activities [19, 20, 40, 41]. Therefore, DN-lig4 mRNA co-injection has been widely used to increase the efficiency of ZFN-mediated gene targeting [8, 42]. Our present study showed that injection of *DN-lig*4 mRNA or *DN-xrcc*6 mRNA could significantly increase the HR frequency not only at whole embryo level but also in PGCs. Interaction with ssDNA-binding protein RPA, BRCA2, PALB2 Rad52 and other proteins, Rad51 recombinase plays a central role in HR pathway. In mammalian cells, overexpression of Rad51 protein can stimulate general intrachromosomal mitotic recombination, whereas overexpression of Rad51 inhibits double-strand break-induced HR [25, 26, 43, 44]. In our work, however, there was no significant difference in repair frequency between the embryos co-injected with *rad*51 mRNA and control group, although 2 embryos co-injected with *rad*51 mRNA showed PGCs-specific *mRFP* expression in the PGCs targeting assay. These results suggested that overexpression of Rad51 was less potent than attenuation of Lig4 or Xrcc6 activities to induce higher HR frequency.

In conclusion, our study demonstrated that the *Tg*(*kop:KalTA*4) line can be used as ideal platform for efficient transgene and gene targeting in PGCs, and that DN-Xrcc6 is most effective in increasing HR frequency in zebrafish embryos. We proposed that co-injection of the gene targeting vector with *DN-xrcc*6 mRNA and *buc* mRNA to zebrafish embryos would greatly improve the frequency for successful gene targeting in germ lines.

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抑制 Ligase4 或 Xrcc6 活性增强斑马鱼原始生殖细胞中 DNA 同源重组的效率

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摘要: 利用斑马鱼作为体内模型, 研究旨在提高斑马鱼原始生殖细胞(Primordial germ cells, PGCs)中同源重组(Homologous recombination, HR)的效率。首先, 将 *UAS:mRFP-nos*1 载体显微注射到 *Tg* (*kop:KalTA*4) 转基因胚胎中标记转基因 PGCs, 结果表明筛选 PGCs 特异表达 mRFP 的胚胎能够相对提高转基因的生殖系传递效率。随后建立了 PGCs 中 HR 效率的评估体系, 并且证明抑制 DNA ligase IV(Lig4)和 Xrcc6(曾用名 Ku70)的活性不但在全胚胎水平, 而且在 PGCs 水平都能够显著提高 HR 的效率。研究表明 *Tg* (*kop:KalTA*4) 转基因品系是开展 HR 介导的基因打靶的一个有效平台。

关键词: 斑马鱼; 原始生殖细胞; 基因打靶; 同源重组; Ligase 4; Xrcc6