Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases

Melanie Meyer, Martin Hrabé de Angelis, Wolfgang Wurst, and Ralf Kühn

Gene targeting by homologous recombination in embryonic stem cells is extensively used to generate specific mouse mutants. However, most mammalian species lack tools for targeted gene manipulation. Since double-strand breaks strongly increase the rate of homologous recombination at genomic loci, we explored whether gene targeting can be directly performed in zygotes by the use of zinc-finger nucleases. Here we report that gene targeting is achieved in 1.7–4.5% of murine one-cell embryos upon the coinjection of targeting vectors with zinc-finger nucleases, without preselection. These findings enable the manipulation of the mammalian germ line in a single step in zygotes, independent of ES cells.

Zinc-finger nucleases (ZFN) link a DNA binding domain of the zinc-finger type to the nuclease domain of Fok I and enable the induction of double-strand breaks (DSBs) at preselected genomic sites (4). DSBs closed by the error-prone, nonhomologous end-joining (NHEJ) DNA repair pathway frequently exhibit nucleotide deletions and insertions at the cleavage site. This technology has been introduced to introduce knockout mutations into the germ line of rats and zebrafish by the expression of ZFNs. We used a ZFN pair (ZFNRosa) that recognizes 18 nucleotides from the XbaI site in the Rosa26 Locus.

Fig. 1. Principle of ZFN-assisted gene targeting in zygotes. Zygotes collected from wild-type mice are coinjected into the pronucleus and cytoplasm with DSB DNA of a gene targeting vector and mRNAs for the expression of a pair of gene specific zinc-finger nucleases (ZFNRosa). HR of the targeting vector with the target site results in a knockout (KO) or knock-in (KI) allele. Manipulated zygotes are subsequently transferred into pseudopregnant females to recover mutant mice.

Results

We used a ZFN pair (ZFNRosa) that recognizes 18 nucleotides upstream and 12 nucleotides downstream of the XbaI site in the first intron of Rosa26 (Fig. 2A), a locus that enables widespread transgene expression in vivo (17). To confirm the stimulatory power of ZFNRosa-induced homologous recombination, we first transfected murine ES cells with a hygromycin selectable gene targeting vector (pRosa26.11; Fig. 2A) with or without ZFNRosa expression plasmids. Hygromycin-resistant ES cell clones were expanded and analyzed by Southern blotting for their Rosa26 genotype. Thirty-two ES cell clones transfected with pRosa26.11 alone exhibited only the 11.5-kb EcoRV wild-type band (Fig. 2B). The cotransfection of pRosa26.11 with ZFNRosa plasmids strongly enhanced recombination at Rosa26, such that 11 of 34 analyzed clones exhibited the predicted 4.5-kb EcoRV fragment derived from a targeted allele (Fig. 2C). One ES cell clone exhibited only the targeted band and likely represents the targeting of both Rosa26 alleles.

Targeted Integration of a β-Galactosidase Reporter Gene into the Rosa26 Locus. To insert a β-Galactosidase gene into Rosa26 of one-cell embryos, we linked a 4.2-kb reporter cassette to homology regions flanking the target XbaI site (Fig. 3A). This targeting vector was coinjected with ZFNRosa mRNAs in a two-step protocol (Fig. 3B).

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the presence of an additional 4.5-kb EcoRV fragment.

Targeted integration of the resistance gene cassette is indicated by an 8.5-kb XbaI band (Fig. 3). Among the 58 analyzed pups, we found two, #14 and #21, that exhibit unexpected XbaI fragments of 4.7–8.5 kb (Fig. 3B) and were shown by Southern blot (Fig. 3D, #14) and PCR analysis (Fig. S2C) to contain 3’ sequences, but not the coding region of the reporter cassette (Fig. 3D), and represent incomplete or rearranged recombination products.

**Targeted Integration of a Venus Reporter Gene into the Rosa26 Locus.** In a second experiment we used a targeting vector that inserts a 1.1-kb Venus reporter gene into the Rosa26 locus (Fig. 4A). DNA analysis of 22 pups derived from zygote coinjections of this vector with ZFN-Rosa mRNAs revealed the presence of one recombined allele, as shown by a 3.1-kb BamHI and a 5.6-kb XbaI fragment (Fig. 4B, fetus 6, Fig. 4D) detected with the 5’-Rosa probe and by a 3.9-kb BamHI band detected with a Venus probe (Fig. 4C). The Venus reporter cassette was amplified from the genomic DNA of pub #6 and was found by sequence analysis to be entirely intact (Fig. S3). The second Rosa26 allele of pup #6 had lost the XbaI site within the ZFN-Rosa target sequence, as indicated by the presence of a 9-kb XbaI fragment and the absence of the wild-type 4.7-kb band (Fig. 4B), detected with the Rosa26 5’ probe.

**Analysis of Random Vector Integrations.** Pronuclear DNA injection is routinely used for the production of transgenic mice harboring random vector integrations (14). Unexpectedly, using Southern blotting and hybridization probes for the β-Galactosidase and Venus reporter gene coding regions, we did not observe any random integration of targeting vectors among the 80 pups derived from both ZFN coinjection experiments (Figs. 3D and 4C and Fig. S1 B and C).

**Discussion**

Compared to a previous report on homologous recombination in mouse zygotes that described a spontaneous recombination rate below 0.1% (18), our results demonstrate that gene targeting can be achieved in pronuclei of one-cell embryos at a higher frequency (1.7–4.5%) when assisted by ZFNs. By analysis of the XbaI site located within the ZFN-Rosa recognition sequence, we determined that DSBs, which lead to NHEJ-induced loss of nucleotides, occurred in injected zygotes at an even higher rate of 22%. It has been demonstrated in Drosophila embryos that the suppression of NHEJ DNA repair leads to the preferential resolution of ZFN-induced DSBs by HR with a gene targeting vector, resulting in the increased recovery of targeted alleles (19). It is therefore possible that also in mammalian zygotes ZFN-assisted gene targeting can be further improved by the transient inhibition of the NHEJ pathway.

In the only reported case of HR in zygotes, the targeted allele exhibited numerous point mutations that interfered with gene expression, possibly as a result of an error-prone HR process in the pronuclei (18). Here we did not observe mutations in the analyzed regions of both targeted alleles, indicating that ZFN-assisted HR can occur with high fidelity as known for gene targeting in ES cells. As judged by the comparison of Southern blot signals derived from wild-type and mutant Rosa26 loci, both correctly targeted alleles appeared in the heterozygous state indicating that ZFN expression, DSB induction, and HR can occur in injected pronuclei within a few hours, before the completion of DNA replication and the fusion of both pronuclei. Among the 80 pups analyzed, we also observed three genotypes (#14, #15, and #21, Fig. 3B) that exhibit a contribution of mutant Rosa26 alleles below 50%, indicating that a part of the HR and NHEJ repair events occur late, only in a single cell of a 2-cell or 4-cell embryo. Homozygously modified genotypes, which exhibit the loss of the

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**Procedure:**

The targeting vector pRosa26.11 inserted a hygromycin/puromycin resistance cassette into the Rosa26 locus. The structure of the wild-type locus, including the ZFN-Rosa recognition sites that overlap with an intronic XbaI site, and of the recombined Rosa26 allele are shown. The location of the Rosa26 promoter (Pr), first exon, of the 5’-Rosa Southern blot probe and of EcoRV (E) sites and fragments are indicated. Southern blot analysis of EcoRV digested genomic DNA of 32 hygromycin-resistant ES cell clones transfected with pRosa26.11 using the Rosa26 5’ probe. The wild-type Rosa26 locus exhibits a 11.5-kb EcoRV fragment (WT). Southern blot analysis of EcoRV digested genomic DNA of 34 hygromycin-resistant ES cell clones transfected with pRosa26.11 and ZFN-Rosa expression vectors using the Rosa26 5’ probe. Targeted integration of the resistance gene cassette is indicated by the presence of an additional 4.5-kb EcoRV fragment.

**Analysis:**

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To explain the import of cytoplasmic ZFN proteins into both male pronuclei, we focused here on the genetic analysis of fetuses with the reporter gene (5.1). Sequence comparison of cloned PCR products (primers Rosa 5HA/Rosa 3HA), covering the ZFNRosa target region, showed a targeted 8.5-kb band representing the recombined allele. (E) Habitus of fetuses 22 (recombined) and 24 (wild type) and X-Gal staining of liver sections.

Materials and Methods

Injection of Zygotes. The Fok-I based, heterodimeric ZFNRosa pair targets sequences near the Xbal site (underlined) within the first intron of the mouse Rosa26 gene; the spacer region is shown in bold: 5′-TGG-GCG-GGA-GTC-3′. ZFN expression plasmids were obtained from Sigma. To prepare ZFNRosa mRNA, plasmid DNAs were linearized with NotI, T7 RNA polymerase was added, and mRNA was synthesized with the mMESSAGE mMACHINE® T7 Kit (Ambion) and purified. Messenger RNA was transcribed in vitro using the mMESSAGE mACHINE® T7 Kit (Ambion) and precipitated. Messenger RNA was used to microinject fertilized oocytes, and embryos were cultured until the blastocyst stage.
polyadenylated using the Poly(A) Tailing Kit (Ambion). The resulting mRNA was purified using the MEGAclear™ Kit (Ambion) before resuspension in injection buffer (10 mM Tris, 0.1 mM EDTA, pH 7.2). The mRNA concentrations were determined by absorption and gel analysis, and each ZFN mRNA was diluted in injection buffer to a working concentration of 2.5 ng/μl together with targeting vector and stored at −80 °C.

Targeting vector pRosa26.8 (a derivative of pRosa26.5) (20) consists of the 1-kb 5′ Rosa26 homology arm and the 4-kb 3′ homology arm (17), flanking an adenoviral splice acceptor sequence, followed by the 3.2-kb β-galactosidase coding region and the 220-bp SV40 polycadenylation site from plasmid pCMVβ (Fig. 2A). Targeting vector pRosa26.3 is equal to pRosa26.8, except that it contains a 1-kb reporter cassette for expression of the Venus GFP protein. The homology arms of both targeting vectors were sequenced that it contains a 1.1-kb reporter cassette for expression of the Venus GFP protein. The homology arms of both targeting vectors were sequenced.

Mouse zygotes were obtained by superfusion of Friend virus B (FVB) females and matings to C57BL/6N males (Charles River). The next day zygotes were collected from oviducts and microinjected in M2 embryo medium following standard procedures (21) with a mixture of targeting vector and ZFNiso™ mRNAs (2.5 ng/μl each) loaded into a single microinjection needle. For microinjection a two-step procedure was applied: A first aliquot of the DNA/RNA mixture was injected into the male pronucleus (to deliver the DNA vector, as used for the production of transgenic mice). Upon the withdrawal of the injection needle from the pronucleus, a second aliquot of the DNA/RNA mixture was injected into the cytoplasm to deliver the ZFN mRNA directly to the translation machinery. Injections were performed using a Leica microinjection manipulator and microscope and an Eppendorf FemtoJet injection device. Injected zygotes were transferred into pseudopregnant CD1 female mice and fetuses recovered at day E18 for further analysis. From the transfer of 469 zygotes injected with targeting vector pRosa26.8 we obtained 64 fetuses (14% recovery), from which 58 were further analyzed by Southern blotting. For pRosa26.3 we obtained 22 fetuses from 120 transferred zygotes (18% recovery). Recovered fetuses showed normal development and were viable (Figs. 3E and 4D). Mice were handled according to institutional guidelines and housed in standard cages in a specific pathogen-free facility on a 12 h light/dark cycle with ad libitum access to food and water.

X-Gal Staining. One longitudinal half of E18 fetuses were fixed and stained with X-Gal as described in ref. 21.

Preparation of Genomic DNA. A segment of the other embryo half was used for the isolation of genomic DNA. For this purpose frozen tissue segments were homogenized in lysis buffer (50 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 1% SDS; 100 mM NaCl; 350 μg/μl Proteinase K) and incubated at 55 °C for 2 h under vigorous shaking. Upon tissue lysis phenol/chloroform (1:1) was added, and the mixture was vortexed and centrifugated for 10 min at 16,000 x g. The supernatant was transferred into new tubes and extracted with chloroform. The supernatant was mixed with 0.7 volumes isopropanol to precipitate the genomic DNA. Precipitated DNA was collected by centrifugation, and the pellet was washed with 70% ethanol. The pellet was air-dried, resuspended in 10 mM Tris/1 mM EDTA buffer (pH 7.5), and incubated overnight at room temperature.

Southern Blot Analysis. Genomic DNA (6 μg) was digested overnight with 30 units restriction enzyme in a volume of 30 μl and then digested with 10 units enzyme for 2–3 h. Samples were loaded on 0.8% agarose gels in Tris (89 mM)/borate (89 mM)/EDTA (2 mM), pH 8.0 buffer and run at 55 V overnight. The gels were then denatured for 1 h in 1.5 M NaCl; 0.5 M NaOH, neutralized for 1 h in 0.1 M Tris-HCl pH 7.5; 0.5 M NaCl, washed with 2× SSC, and blotted for 48 h with 20× SSC on Hybond N⁺ membranes (GE Healthcare). The membranes were then washed with 2× SSC, UV-cross-linked, and stored at −20 °C. For hybridization the membranes were preincubated in Church buffer (1% BSA, 1 mM EDTA, 0.5 M phosphate buffer, 7% SDS) for 1 h at
65 °C under rotation. The Rosa26 S′ probe was isolated as a 460-bp EcoRI fragment from plasmid pCRRI-RosaS′ probe; the Rosa26 S′ probe was isolated as 660-bp EcoRI fragment from the plasmid pCRRI-RosaS′ probe, as described (17, 20). As β-Galactosidase probe we used the 1,250-bp SacI/EcoRV coding fragment from pCMVβ and as Venus probe we used the venus coding region, isolated as a 730-bp BamHI/EcoRI fragment from pCS2-venus, which was used. DNA fragments used as hybridization probes were heat denatured and labeled with PI2 marked dCTP (Perkin Elmer) using the high-prime DNA labeling kit (Roche). Labeled probe DNA was purified on MicroSpin™ S-200 HR columns (GE Healthcare), heat denatured, added to the hybridization buffer and membranes were rotated overnight at 65 °C. The washing buffer (2x SSC, 0.5% SDS) was prewarmed to 65 °C, and the membranes were washed three times (5 min, 30 min, and 15 min) at 65 °C under shaking. Next, the membranes were exposed at −80 °C to Biomax M51 films and enhancing screens (Kodak) for 1–5 days until development. Photos of autoradiographs were taken with a digital camera on a transmitting light table, and segments were excised with the Adobe Photoshop software.

PCR and Sequence Analysis. To analyze the Rosa26 alleles recombinated with pRosa26.8 we amplified DNA from fetuses 22, 21, and 14 (coinjected with ZFNrosa and pRosa26.8) using the primer pairs Rosa S′HA (5′-aaaactgctgcttcgaattc-3′) and SA1 (5′-gtcatcccagttgcttg-3′) or SA2 (5′-tgcggctttcgctgcgg-3′) to amplify the S′-homology arm/splice acceptor junction and the primer pairs Rosa 3HA (5′-ccacaggtagttgctttc-3′) or pA1 (5′-tcgcttgattgctgcggg-3′) or pA2 (5′-aagaactatagctgctgctg-3′) to amplify the junction of the SV40 polyA′-homology arm. Amplification was performed using Herculase polymerase (Invitrogen) in 100-μl reactions with 38 cycles of 95 °C – 30 s; 59 °C – 1 min; 72 °C – 1 min. In embryos with recombination events PCR products have the expected sizes: Rosa S′HA/pA1: 346 bp; Rosa S′HA/pA2: 365 bp; Rosa 3HA/pA1: 467 bp; Rosa 3HA/pA2: 625 bp. PCR products from embryos exhibiting homologous recombination events were directly sequenced (Sequiserve) and compared to the pRosa26.8 vector sequence using the Vector NTI software (Invitrogen).

Genomic DNA from fetuses 9, 14, and 21, which lost the XbaI site in the ZFN target region as found by Southern blotting, and of wild-type controls were further analyzed for NHEJ-induced mutations by PCR amplification using the primer pair Rosa S′HA and Rosa 3HA (wild-type PCR product: 252 bp). These PCR products were cloned using the Stratagene Blunt PCR Cloning Kit (Stratagene). Plasmid DNA was isolated from the Qiagen Spin Miniprep Kit (Qiagen) and analyzed by digestion with EcoRI to confirm the presence of vector inserts and digested with XbaI to analyze for the loss of the ZFNrosa target XbaI site. Selected clones were sequenced using the T3 forward primer (Sequiserve) and analyzed for mutations in comparison to the Rosa26 wild-type sequence using the Vector NTI software.

To analyze Rosa26 homologous recombinated alleles with pRosa26.3, we amplified DNA from fetus 6 (coinjected with ZFNrosa and pRosa26.3) using the primer pair Rosa S′HA (5′-aaagtcgcttgagttgttat-3′) and Rosa 3HA (5′-caacaggttagctttaagcc-3′). The PCR product (SHA/3HA: 1,402 bp) was directly sequenced with a single 950-bp read (Sequiserve) and compared to the pRosa26.3 vector sequence using the Vector NTI software.

Note Added in Proof. While this article was in print we obtained also live adult heterozygous targeted mice from coinjections of ZFNsosa and pRosa26.3.

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