Lack of RNA-DNA oligonucleotide (chimeraplast) mutagenic activity in mouse embryos.

Aristides D. Tagalakis[*], James S. Owen[†§] and J. Paul Simons[*§#].

[*] Department of Anatomy & Developmental Biology,
Royal Free Campus,
University College London,
London NW3 2PF, UK

[†] Department of Medicine,
Royal Free and University College Medical School,
University College London,
London NW3 2PF

[§] Royal Free Centre for Biomedical Science,
Royal Free and University College Medical School,
Rowland Hill Street,
London NW3 2PF

[#] To whom correspondence should be addressed

Email address simons@rfc.ucl.ac.uk; Fax no: <+44> (0)20 7830 2917

Abstract

There are numerous reports of the use of RNA-DNA oligonucleotides (chimeraplasts) to correct point mutations *in vitro* and *in vivo*, including the human apolipoprotein E gene. Despite the absence of selection for targeting, high efficiency conversion has been reported. Although mainly used to revert deleterious mutations for gene therapy applications, successful use of this approach would have the potential to greatly facilitate the production of defined mutations in mice and other species. We have attempted to create a point mutation in the mouse apolipoprotein E gene by microinjection of chimeraplast into the pronuclei of 1-cell mouse eggs. Following transfer of microinjected eggs we analysed 139 E12.5 embryos, but obtained no evidence for successful conversion.

Keywords: gene targeting, pronuclear injection, chimeraplast, oligonucleotide, mutagenesis, SNP

Introduction

With the availability of the finished human genome sequence and the draft mouse genome sequence, the priority is now the analysis of gene function. Important goals are the determination of the phenotypes of animals that have deficiencies in every gene (Nadeau et al, 2001) and, at another level, determination of the functional significance of naturally occurring DNA sequence variation, most of which is in the form of single nucleotide polymorphisms. For these ambitions to be realised, the efficiency of the generation and analysis of mutants is critical. Mice are the pre-eminent system for experimental mammalian genetics, and various strategies are in place for high-throughput mutagenesis of mice (Zambrowicz et al, 1998; Chen et al, 2000; Munroe et al, 2000; Hrabe de Angelis, 2000; Nolan et al, 2000; Wiles et al, 2000). These approaches are very powerful but they all have drawbacks: not least, they are all based on random events and there is no possibility of designing the mutation. Conventional gene targeting in ES cells is directed, permitting precisely designed mutations to be made in mice. Various refinements have streamlined the creation of mutants by gene targeting (Eggan et al, 2002; Seibler et al, 2003; Valenzuela et al, 2003), but nevertheless, the process is technically demanding and remains relatively inefficient. The ideal approach to complement these established techniques would be highly efficient directed mutagenesis of embryos, applicable not only to mice, but also to other species.

A number of laboratories have reported success in correcting single base mutations in a variety of target genes using synthetic oligonucleotides (e.g. Alexeev et al., 1998, 2000; Bandyopadhyay et al., 1999; Cole-Strauss et al.,1996; Kren et al., 1997, 1998, 1999; Tagalakis et al., 2001). These studies have been undertaken with the ultimate goal of gene therapy applications, and typically the mutations targeted were reversion mutations. In most of these studies, the effective reagents used were synthetic self-complementary oligonucleotides known as RNA-DNA oligonucleotides (RDOs) or chimeraplasts, that are composed of DNA and 2'-O-methyl RNA segments, and which have terminal hairpins and a GC clamp to aid stability. Such synthetic oligonucleotide reagents have been found to be capable of targeting specific mutations in a variety of systems, including a variety of mammalian cells *in vitro*; liver, muscle and melanocytes *in vivo* as well as plants, and yeast. We have used chimeraplasts to target changes to human apolipoprotein E (Tagalakis et al, 2001). In cultured cells, we have converted (at best) over 50% of gene copies and have rigorously validated these conversions showing that they are specific, stable, heritable and functional.

Successful use of synthetic oligonucleotides to target mutations directly in mouse embryos would dramatically speed up the production of specific mutant strains of mice because it would remove the need to build targeting vectors, ES cell culture and much else. For these reasons we have tested whether gene targeting can be accomplished in mouse embryos by microinjection of a chimeraplast.

Materials and Methods

Pronuclear microinjection

Microinjection was done using standard procedures (Hogan et al, 1994). C57BL/6 x CBA/Ca F_1 mice were used throughout, apart from CD1 vasectomised males. Females were superovulated with 5IU PMS and 5IU hCG; embryos were recovered in M2 medium and cultured in M16 in 5% CO_2, under oil. The chimeraplast was dissolved in 10mM Tris-HCl pH8.0, 0.1mM EDTA. One pronucleus of each embryo was microinjected, and any embryo in which the injected pronucleus was not observed to swell was discarded. Following microinjection the embryos were cultured overnight. For assessment of chimeraplast toxicity, 2-cell embryos were transferred to fresh M16 and cultured for a further three days, at which time they were evaluated. Normal blastocysts were those which had not fragmented, which had a single discrete blastocoel and which had expanded to at least fill the zona pellucida. To test for chimeraplast-mediated mutagenesis, 2-cell embryos were transferred (average 28 per

mouse, range 18-35); they were transferred to one oviduct of pseudopregnant foster mothers under avertin anaesthesia (Papaioannou & Fox, 1993). These experiments were performed fully in compliance with current UK legislation.

Screening for the mutation

The region of the mouse ApoE gene that includes the two sites that are most polymorphic in man was amplified by PCR using primers ccgacatggaggatctacgc and gcgaccttgctccaccagag. PCR conditions were 75mM Tris-HCl (pH8.8 at 25℃), 20mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5mM $MgCl_2$, 0.1mM dNTPs, 10% DMSO, 0.6μM each primer and 0.04u/μl Taq polymerase. Following an initial denaturation for 5 mins at 95℃, 36 cycles of 94℃, 62℃ and 72℃ (40 sec each) were performed with a final extension of 8mins at 72℃. We sequenced PCR products from this region of the ApoE gene from wild-type mice from our colony, and the sequences obtained corresponded to the published sequence.

DNA was prepared from E12.5 embryos by digesting embryo fragments (whole tail) in 100μl of 50mM KCl, 10mM Tris-HCl pH8.3 at 25℃ 10μg/ml gelatine, 0.045% Igepal CA-630, 0.045% Tween 20, 0.5mg/ml proteinase K at 55℃ overnight. 7μl of the crude lysate was used per 25μl PCR reaction after heat inactivation of the proteinase K. For digestion with HinP1I, 7μl of unpurified PCR products were mixed with an equal volume of H_2O and 1μl of HinP1I (10u/μl) was added. Following overnight digestion, the DNA was run on 20% polyacrylamide gels (Novex) in TBE and stained with SYBR green I.

Results

We selected apolipoprotein E (ApoE) as a test target for targeted mutation in mouse because we had previously achieved high level conversion of human ApoE genomic and cDNA targets in cultured cells and in transgenic mouse tissue (Tagalakis et al, 2001). The three common variants of human ApoE are ApoE2, ApoE3 and ApoE4. ApoE3 is the most frequent; the ApoE2 and ApoE4 alleles differ by virtue of single nucleotide polymorphisms that cause amino acid sequence changes. ApoE2 and ApoE4 are risk factors for atherosclerosis and Alzheimer's disease, respectively. We designed a C→T transition mutation that would simultaneously generate an RFLP and an R150C change in mouse ApoE protein (figure 1a), creating a SNP that is equivalent to the human ApoE2 SNP. A chimeraplast was synthesised to create this mutation (figure 1b) and was microinjected into the pronuclei of 1-cell mouse embryos.

For the production of transgenic mice, the volume of DNA injected into pronuclei is typically about $2x10^{-9}$ml, and the optimum concentration of DNA is around 2μg/ml; injection of DNA at concentrations above this significantly reduces the frequency of development of mice to term (Brinster et al, 1985). For a typical transgene of 10kb, this corresponds to about 350 molecules of the construct; the chimeraplast is 68nt long, so a $2x10^{-9}$ml injection would contain about 10^5 molecules. Double stranded DNA ends are recognised as a sign of chromosome damage; depending on the cell type and extent of the damage, the response to such damage may be cell cycle arrest, or apoptosis. To test whether chimeraplasts are toxic to preimplantation embryos we titrated the concentration of chimeraplast injected into pronuclei (0, 0.25, 2.5 and 25μg/ml), and embryos that survived the immediate trauma of injection were cultured for 4 days. Development to normal blastocyst was significantly reduced for embryos injected with buffer or with chimeraplast, but there was no significant difference between the chimeraplast and buffer injected embryos (figure 2). This shows that the major deleterious effects of injection are independent of the injected DNA, and are probably due to physical damage. For subsequent experiments we injected the DNA at 2.5μg/ml.

To test for mutagenic activity of the chimeraplast, we injected it into pronuclear mouse eggs, transferred them into oviducts of pseudopregnant foster mothers, and harvested embryos 12 days after transfer. A total of 662 eggs were injected, 420 of which developed to 2-cells and were transferred. 139 embryos were recovered at E12.5; DNA was extracted from each embryo, amplified and digested with HinP1I (figure 1c). None of the embryos gave the diagnostic 68bp fragment, and in other respects the restriction patterns were exactly as predicted (e.g. figure 3).

Discusssion

All of the vasectomised males used to generate the recipients were confirmed to be sterile by test matings, some of the embryo recipients were carrying very large numbers of foetuses (up to 17), and some recipients were not pregnant. In mice, embryos do not move from one uterine horn to the other. The embryo transfers were unilateral, and every foetus recovered was in the ipsilateral uterine horn; there was no sign of implantation in the contralateral uterine horns. For these reasons we are confident that all of the recovered foetuses were derived from the eggs that had been injected and transferred, and were not from accidental fertile matings.

Some recent publications (Albuquerque-Silva et al., 2001; van der Steege et al., 2001; Taubes, 2002) have highlighted the inconsistency of oligonucleotide-directed targeted mutation. Various possible reasons for this have been proposed, including inefficient delivery to the cell nucleus, degradation, toxicity, poor reagent quality and differential accessibility of different target genes. We circumvented many of these concerns: the ApoE gene was chosen because the human homologue has been successfully targeted; direct microinjection eliminates delivery problems as $>10^5$ molecules were injected per embryo; the chimeraplast gave a discrete band and showed no signs of degradation by gel electrophoresis (figure 4); and finally, the rate of development to blastocyst and to E12.5 of eggs microinjected with chimeraplast was incompatible with a toxicity effect.

The reason for our failure to obtain targeted conversions is not clear. We believe that it is not feasible to inject much more chimeraplast DNA without incurring significant postimplantation embryo losses. It has been proposed that chimeraplast activity is dependent on mismatch repair mechanisms. It is possible that preimplantation embryos lack the necessary mismatch repair activity; e.g. 1- to 4-cell stage rat embryos appear not to express Msh2 mRNA (Harrouk et al, 2000), but on the other hand, they may contain Msh2 protein of maternal origin. If not, it may be possible to stimulate chimeraplast-mediated mutagenesis by coinjection of Msh2 protein or components that may be lacking. Alternatively the use of a different chemistry may promote conversions in preimplantation embryos. There have been reports of targeted mutagenesis mediated by various types of single-strand oligonucleotides in yeast and/or cultured mammalian cells, including oligonucleotides that contain a triplex-forming region (Culver et al, 1999), those protected with phosphorothioates (PTO) (e.g. Kenner et al, 2002; Lu et al, 2003), locked nucleic acids (LNA) (Parekh-Olmedo et al, 2002) or including peptide nucleic acids (PNA) either conjugated with DNA oligonucleotides or as an adjuvant (Rogers et al, 2002). The PTO and LNA modifications probably function to stabilise the oligonucleotides by preventing exonuclease digestion; the triplex-forming oligonucleotides and PNA are thought to improve targeting by enhancing the interactions between the oligonucleotide and their targets. As yet the efficiencies obtained with single-strand oligonucleotides have not been high, but a number of factors have been reported to influence the efficiency of conversion. These include which strand is targeted with respect to the direction of transcription (Liu et al, 2002a; Pierce et al, 2003), transcriptional activity of the target (Liu et al, 2002; Igoucheva et al, 2003; Brachman & Kmiec, 2004), replication status of the target (Brachman & Kmiec, 2004) and single strandedness of the targeting oligonucleotide (Radecke et al, in press). Treatments that have been reported to increase the

efficiency of conversion include treatment of the cells with DNA damaging agents (Ferrara & Kmiec, 2004; Ferrara et al, 2004), modification of histone acetylation (and thereby chromatin structure; Parekh-Olmedo et al, 2003) and expression of normal or modified recombination proteins (Lio et al, 2002b, 2004). Because of the great attractiveness of gene repair for gene therapy, work is likely to continue in this direction, at least for somatic cells, and it is possible that this will result in improvements. Should significant progress be made in this direction, it may be worthwhile revisiting the possibility of oligonucleotide-mediated mutagenesis in preimplantation embryos. In this context it is obvious that any modified treatment regime must be compatible with normal development to term, as well as efficient mutagenesis of most or all targets. A further important consideration for any targeted mutagenesis programme is that second site mutations caused by the treatment should be kept to a minimum. While it may be possible to breed out unlinked second site mutations, it is likely that treatments such as the deliberate induction of DNA damage would give rise to sufficient random mutations to confound the analysis of phenotype.

Conclusions

Whatever the reason for the apparent lack of chimeraplast mutagenic activity in mouse embryos, at present this technology is not suitable for large scale targeted mutagenesis programmes. Clearer understanding of the mechanisms of oligonucleotide-mediated targeted mutagenesis and the development of methods to increase the efficiency may, in the future, make this approach feasible.

Acknowledgements

This work was funded by a Sir Henry Wellcome Commemorative Award from the Wellcome Trust (067727). Correspondence and requests for materials should be addressed to Paul Simons (simons@rfc.ucl.ac.uk).

References

Albuquerque-Silva J, Vassart G, Lavinha J, Abramowicz MJ. 2001 Chimeraplasty validation. Nat Biotechnol 19: 1011.

229 Alexeev V, Igoucheva O, Domashenko A, Cotsarelis G, Yoon K. 2000 Localized in vivo
230 genotypic and phenotypic correction of the albino mutation in skin by RNA-DNA
231 oligonucleotide. Nat Biotechnol. 18:43-7

232 Alexeev V, Yoon K. 1998 Stable and inheritable changes in genotype and phenotype of
233 albino melanocytes induced by an RNA-DNA oligonucleotide. Nat Biotechnol. 16:1343-6.

234 Bandyopadhyay P, Ma X, Linehan-Stieers C, Kren BT, Steer CJ. 1999 Nucleotide exchange
235 in genomic DNA of rat hepatocytes using RNA/DNA oligonucleotides. Targeted delivery of
236 liposomes and polyethyleneimine to the asialoglycoprotein receptor. J Biol Chem.
237 274:10163-72.

238 Brachman EE, Kmiec EB 2004. DNA replication and transcription direct a DNA strand bias
239 in the process of targeted gene repair in mammalian cells. J Cell Sci. 117:3867-74.

240 Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD. 1985 Factors affecting the
241 efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc Natl Acad Sci
242 U S A. 82:4438-42.

243 Chen Y, Yee D, Dains K, Chatterjee A, Cavalcoli J, Schneider E, Om J, Woychik RP,
244 Magnuson T. 2000 Genotype-based screen for ENU-induced mutations in mouse embryonic
245 stem cells. Nat Genet. 24:314-7.

246 Cole-Strauss A, Yoon K, Xiang Y, Byrne BC, Rice MC, Gryn J, Holloman WK, Kmiec EB.
247 1996 Correction of the mutation responsible for sickle cell anemia by an RNA-DNA
248 oligonucleotide. Science. 273:1386-9.

249 Culver KW, Hsieh WT, Huyen Y, Chen V, Liu J, Khripine Y, Khorlin A. 1999 Correction of
250 chromosomal point mutations in human cells with bifunctional oligonucleotides. Nat
251 Biotechnol. 17:989-93.

252 Eggan K, Rode A, Jentsch I, Samuel C, Hennek T, Tintrup H, Zevnik B, Erwin J, Loring J,
253 Jackson-Grusby L, Speicher MR, Kuehn R, Jaenisch R. 2002 Male and female mice derived
254 from the same embryonic stem cell clone by tetraploid embryo complementation. Nat
255 Biotechnol. 20:455-9.

Ferrara L, Kmiec EB. 2004 Camptothecin enhances the frequency of oligonucleotide-directed gene repair in mammalian cells by inducing DNA damage and activating homologous recombination. Nucleic Acids Res. 32:5239-48

Ferrara L, Parekh-Olmedo H, Kmiec EB. 2004 Enhanced oligonucleotide-directed gene targeting in mammalian cells following treatment with DNA damaging agents. Exp Cell Res 300:170-9.

Harrouk W, Codrington A, Vinson R, Robaire B, Hales BF. 2000 Paternal exposure to cyclophosphamide induces DNA damage and alters the expression of DNA repair genes in the rat preimplantation embryo. Mutat Res. 461:229-41.

Hogan B., Beddington, R., Costantini, F, Lacy E (1994) Manipulating the Mouse Embryo: A laboratory manual (2nd edition) Cold Spring Harbor Press.

Hrabe de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, et al 2000 Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet. 25:444-7.

Igoucheva O, Alexeev V, Pryce M, Yoon K. 2003 Transcription affects formation and processing of intermediates in oligonucleotide-mediated gene alteration. Nucleic Acids Res. 31:2659-70.

Kenner O, Kneisel A, Klingler J, Bartelt B, Speit G, Vogel W, Kaufmann D. 2002 Targeted gene correction of hprt mutations by 45 base single-stranded oligonucleotides. Biochem Biophys Res Commun. 299:787-92.

Kren BT, Bandyopadhyay P, Steer CJ 1998 In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. Nat Med. 4:285-90.

Kren BT, Cole-Strauss A, Kmiec EB, Steer CJ. 1997 Targeted nucleotide exchange in the alkaline phosphatase gene of HuH-7 cells mediated by a chimeric RNA/DNA oligonucleotide. Hepatology. 25:1462-8.

Kren BT, Parashar B, Bandyopadhyay P, Chowdhury NR, Chowdhury JR, Steer CJ. 1999 Correction of the UDP-glucuronosyltransferase gene defect in the Gunn rat model of Crigler-Najjar syndrome type I with a chimeric oligonucleotide. Proc Natl Acad Sci U S A. 96:10349-54.

284 Liu L, Cheng S, van Brabant AJ, Kmiec EB. 2002a Rad51p and Rad54p, but not Rad52p,
285 elevate gene repair in Saccharomyces cerevisiae directed by modified single-stranded
286 oligonucleotide vectors. Nucleic Acids Res. 30:2742-50.

287 Liu L, Maguire KK, Kmiec EB. 2004 Genetic re-engineering of Saccharomyces cerevisiae
288 RAD51 leads to a significant increase in the frequency of gene repair in vivo. Nucleic Acids
289 Res. 32:2093-101.

290 Liu L, Rice MC, Drury M, Cheng S, Gamper H, Kmiec EB. 2002b Strand bias in targeted
291 gene repair is influenced by transcriptional activity. Mol Cell Biol. 22:3852-63

292 Lu IL, Lin CY, Lin SB, Chen ST, Yeh LY, Yang FY, Au LC. 2003 Correction/mutation of
293 acid alpha-D-glucosidase gene by modified single-stranded oligonucleotides: in vitro and in
294 vivo studies. Gene Ther 10:1910-6.

295 Munroe RJ, Bergstrom RA, Zheng QY, Libby B, Smith R, John SW, Schimenti KJ,
296 Browning VL, Schimenti JC. 2000 Mouse mutants from chemically mutagenized embryonic
297 stem cells. Nat Genet. 24:318-21.

298 Nadeau JH, Balling R, Barsh G, Beier D, Brown SD, Bucan M, et al 2001 Functional
299 annotation of mouse genome sequences. Science 291,1251-1255.

300 Nolan PM, Peters J, Strivens M, Rogers D, Hagan J, Spurr N, Gray IC, et al 2000 A
301 systematic, genome-wide, phenotype-driven mutagenesis programme for gene function
302 studies in the mouse. Nat Genet. 25:440-3.

303 Papaioannou VE, Fox JG. 1993 Efficacy of tribromoethanol anesthesia in mice. Lab Anim
304 Sci. 43:189-92.

305 Parekh-Olmedo H, Drury M, Kmiec EB. 2002 Targeted nucleotide exchange in
306 Saccharomyces cerevisiae directed by short oligonucleotides containing locked nucleic acids.
307 Chem Biol. 9:1073-84.

308 Parekh-Olmedo H, Engstrom JU, Kmiec EB. 2003 The effect of hydroxyurea and trichostatin
309 A on targeted nucleotide exchange in yeast and Mammalian cells. Ann N Y Acad Sci
310 1002:43-55.

311 Pierce EA, Liu Q, Igoucheva O, Omarrudin R, Ma H, Diamond SL, Yoon K. 2003
312 Oligonucleotide-directed single-base DNA alterations in mouse embryonic stem cells. Gene
313 Ther. 10:24-33.

314 Radecke F, Radecke S, Schwarz K. 2004 Unmodified oligodeoxynucleotides require single-
315 strandedness to induce targeted repair of a chromosomal EGFP gene. J Gene Med. in press

316 Rogers FA, Vasquez KM, Egholm M, Glazer PM 2002. Site-directed recombination via
317 bifunctional PNA-DNA conjugates.Proc Natl Acad Sci U S A.99:16695-700.

318 Seibler J, Zevnik B, Kuter-Luks B, Andreas S, Kern H, Hennek T, Rode A, Heimann C,
319 Faust N, Kauselmann G, Schoor M, Jaenisch R, Rajewsky K, Kuhn R, Schwenk F. 2003
320 Rapid generation of inducible mouse mutants. Nucleic Acids Res. 31:e12.

321 Tagalakis AD, Graham IR, Riddell, DR, Dickson JG & Owen JS 2001 Gene Correction of
322 the Apolipoprotein (Apo) E2 Phenotype to Wild-Type ApoE3 by In Situ Chimeraplasty. J.
323 Biol. Chem 276:13226-13230.

324 Taubes G. 2002 The strange case of chimeraplasty. Science 298: 2116- 2120.

325 Valenzuela DM, Murphy AJ, Frendewey D, Gale NW, Economides AN, Auerbach W,
326 Poueymirou WT, Adams NC, Rojas J, Yasenchak J, Chernomorsky R, Boucher M, Elsasser
327 AL, Esau L, Zheng J, Griffiths JA, Wang X, Su H, Xue Y, Dominguez MG, Noguera I,
328 Torres R, Macdonald LE, Stewart AF, DeChiara TM, Yancopoulos GD. 2003 High-
329 throughput engineering of the mouse genome coupled with high-resolution expression
330 analysis. Nat Biotechnol. 21:652-9.

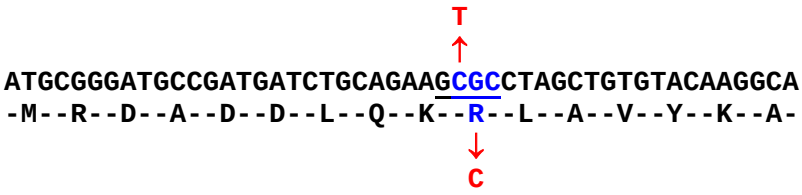
331 van der Steege G, Schuilenga-Hut PH, Buys CH, Scheffer H, Pas HH, Jonkman MF. 2001
332 Persistent failures in gene repair. Nat Biotechnol. 19:305-6

333 Wiles MV, Vauti F, Otte J, Fuchtbauer EM, Ruiz P, Fuchtbauer A, Arnold HH, Lehrach H,
334 Metz T, von Melchner H, Wurst W 2000 Establishment of a gene-trap sequence tag library to
335 generate mutant mice from embryonic stem cells. Nat Genet 24:13-4

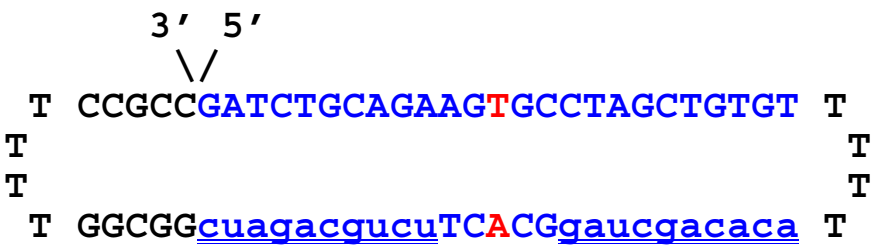
336 Zambrowicz BP, Friedrich GA, Buxton EC, Lilleberg SL, Person C, Sands AT. 1998
337 Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells.
338 Nature. 392:608-11

Figure 1

A



B



C

ccgacatggaggatctacgcaaccgactcgggcagtaccgcaacgaggtgcac
accatgctgggccagagcacagaggagatacgggcgcggctctccacacacct
gcgcaagatgcgcaagcgcttgatgcgggatgccgatgatctgcagaagcgcc
tagctgtgtacaaggcaggggcacgcgagggcgccgagcgcggtgtgagtgcc
atccgtgagcgcctggggcctctggtggagcaaggtcgc

A: **Design of the mutation.**

The wild-type DNA and peptide sequence are shown. The target codon is shown in blue, and the targeted DNA sequence alteration is indicated in red; similarly the wild-type and mutant amino acids are shown. To test for successful mutation, we made use of the HinP1I restriction site (underlined) that overlaps the target codon; the wild-type sequence possesses a restriction site; the mutation was designed to destroy this site.

B: **Structure of the chimeraplast.** The sequence that is homologous to the mouse ApoE target is shown in blue, with the bases mismatched with the target (and corresponding to the intended sequence change) shown in red; DNA residues are shown in UPPERCASE, 2'O-Me-RNA residues are shown in lowercase; the GC clamp and T residues of the hairpin loops are shown in black.

C: **PCR-RFLP analysis.**

The complete sequence of the wild-type PCR product is shown, with the targets of the PCR primers underlined, HinP1I sites are double underlined, and the target nucleotide is shown in red. The diagnostic restriction site is flanked by sites 33bp upstream and 35bp downstream. The wild-type allele is predicted to give fragments of 87, 35, 33, 30, 24, 20, 9, 7 and 6bp in length; mutation would cause loss of the 35 and 33bp fragments and a new 68bp fragment.

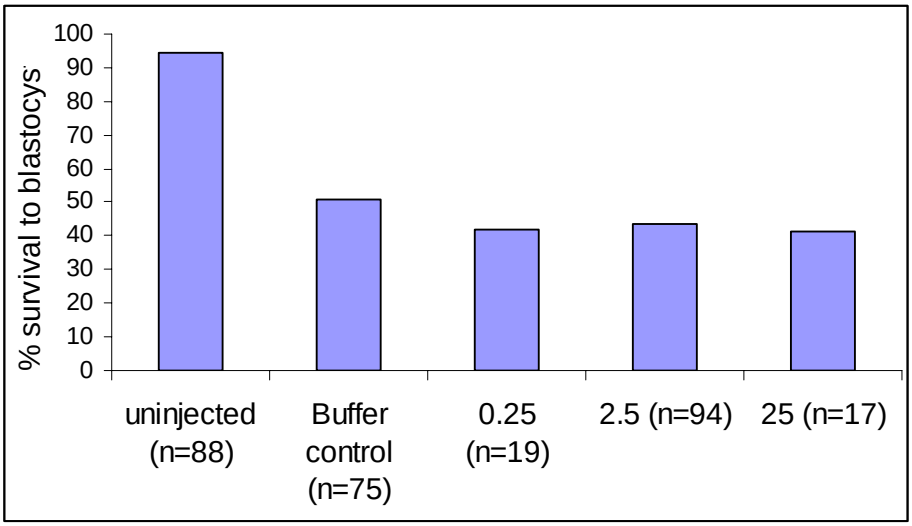


Figure 2 **Survival of embryos following injection of the chimeraplast.**

Pronuclear mouse embryos were injected with chimeraplast at 0.25, 2.5 or 25 ng/μl, or with buffer alone; the embryos were cultured overnight. The following day, the lysed embryos were discarded, and the remaining 2-cell embryos were transferred to fresh medium (only 1/294 embryos had failed to divide). Control uninjected 2-cell embryos were also transferred to fresh medium. Following a further 3 days in culture, the normal blastocysts were counted. Chi-squared analysis shows that injections of buffer or 2.5μg/ml chimeraplast increased the frequency of abnormal development significantly ($P<0.001$; $\chi^2=40.336$, 1d.f. and $P<0.001$; $\chi^2=53.811$, 1d.f., respectively); the rates of normal development of chimeraplast-injected and control buffer-injected eggs were indistinguishable (for 2.5μg/ml chimeraplast, $P=0.361$; $\chi^2=0.833$, 1 d.f.)

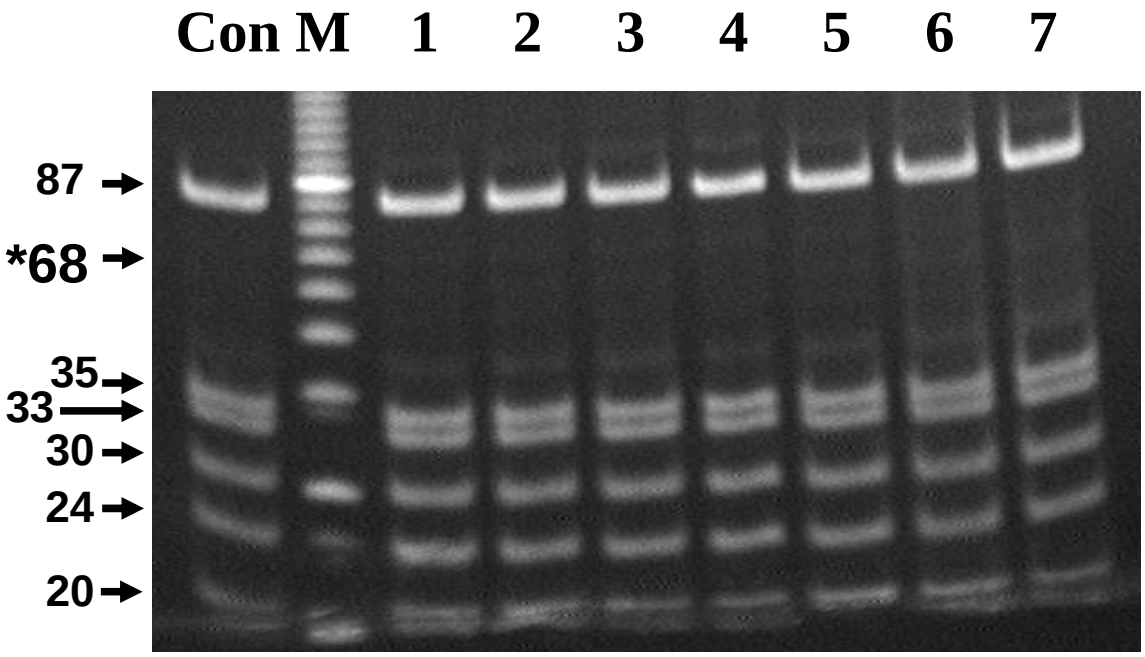


Figure 3 **Representative screening results.**

ApoE PCR products from chimeraplast-injected embryos recovered at mid-gestation (E12.5) were digested with HinP1I to screen for the introduction of the C→T mutation. Lanes 1-6 show representative patterns from such embryos. The mutant allele would give a new 68bp fragment (*), with the 35 and 33bp fragments being depleted. We obtained no evidence for successful mutation in any of the 139 embryos analysed. The lane labelled Con shows the pattern obtained from an untreated wild-type mouse; lane M contains molecular weight standards (Invitrogen 10bp ladder).

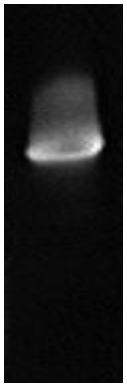


Figure 4 **Intactness of the chimeraplast.**

The chimeraplast was run on a 15% polyacrylamide gel and stained with ethidium bromide. The bulk of the material migrated in a discrete band; the higher molecular weight smear is typical of chimeraplasts and is probably caused by intermolecular base pairing.