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A resource of vectors and ES cells for targeted deletion of microRNAs in mice

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Abstract

The 21-23 nucleotide single-stranded RNAs classified as microRNAs (miRNA) perform fundamental roles in a wide range of cellular and developmental processes. miRNAs regulate protein expression through sequence-specific base pairing with target messenger RNAs (mRNA) reducing both their stability and the process of protein translation[1, 2]. At least 30% of protein coding genes appear to be conserved targets for miRNAs[1]. In contrast to the protein coding genes[3, 4], no public resource of miRNA mouse mutant alleles exists. We have generated a library of highly germ-line transmissible C57BL/6N mouse mutant embryonic stem (ES) cells with targeted deletions for the majority of miRNA genes currently annotated within the miRBase registry[5]. These alleles have been designed to be highly adaptable research tools that can be efficiently altered to create reporter, conditional and other allelic variants. This ES cell resource can be searched electronically and is available from ES cell repositories for distribution to the scientific community[6].

The influence and importance of miRNAs as regulators of gene expression has led to their intensive study in many biological disciplines; ranging from cell cycle regulation[7], stem cell development and differentiation[8] to cardiac function [9, 10] neurobiology [11] and immunology[12]. The dysregulation of miRNA expression is frequently associated with cancer development [13] and the role of miRNAs in cellular proliferation and differentiation has raised opportunities for new strategies for therapeutic intervention in the disease process. A genome-wide miRNA knockout resource for the functional analysis of miRNAs currently only exists for the invertebrate *Caenorhabditis elegans*[14]. Much of our knowledge of miRNA function in mice comes from over-expression studies or the disruption of all miRNA by tissue restricted knockout of the processing enzyme Dicer[15-17]. A limited number of individual miRNA knockout mice have been described, which have provided information on areas such as B cell [1819] and regulatory T cell[20] function and cardiac development and function[9, 10]. The analysis of miRNA knockout alleles is complicated by the occurrence miRNA genes with related predicted specificities occurring at multiple loci[5] suggesting functional redundancy at the level of target specificity, although this is expected to be modulated by specific temporal and spatial expression patterns. Rigorous functional analysis of miRNA function will require the creation of compound mutants of miRNA family members for which a resource of knockout ES cells will be very valuable. We have

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Contributions H.M.P. planned and managed the project, constructed novel plasmid reagents, and performed high-throughput vector construction, ES cell targeting experiments, post targeting modifications, collated data and wrote the paper. H.Y. performed high-throughput vector construction planned and performed the ES cell targeting experiments, ES cell genotyping, and collated data. J.D.C. performed high-throughput vector construction, ES cell targeting and genotyping. F.C.L. performed ES cell targeting experiments. A.B. initiated and supervised the project and wrote the paper.

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designed the mirKO resource as a research toolbox of mutant alleles that can be used as reagents for studying miRNA function in a range of complementary approaches. We demonstrate how recombinase mediated cassette exchange (RMCE) can be used to efficiently modify each mutated miRNA locus, replacing the targeted allele with any desired sequence to generate reporter and conditional alleles, or a host of other possible allelic variants.

Results

mirKO targeting vectors production

The gene structures for most mammalian miRNAs are not well defined and the promoter regions have only been established in a few cases[21, 22]. However, miRNAs are generally transcribed from PolII promoters and the primary transcripts are 5′ 7-methyl guanylate capped and 3′-polyadenylated[21, 23]. miRNA genes can be intronic to coding or non-coding genes, within the exons of non-coding genes, or intergenic[24]. Since many miRNAs are encoded within the expanse of other loci, knockout alleles were designed to disrupt the activity of the miRNA without disturbing the function of the gene in which they are embedded.

For individual (singleton) miRNA genes targeting vectors were designed to generate small deletions (typically in the range 100 to 300bp). In cases where miRNA genes are in close proximity to one another (less that 300bp apart) the vectors were designed to delete clusters, to minimize the adventitious disruption of their neighbour(s). This general principle was adapted when it was considered that there was particular value in generating larger deletions, for instance clustered miRNA gene family members. In a small number of cases exons of coding or non-coding genes were disrupted by the targeting vectors.

Targeting vectors were designed with slightly asymmetric homology arms of 3kb and 5kb to support robust long-range PCR genotyping for correct targeting. The targeting vectors incorporate site-specific recombinase sites flanking the selection cassette to facilitate removal of the cassette leaving just a small deletion and/or enabling further modification of the locus. We have attempted to make targeting vectors for all of the genes in the miRBase registry[5] of miRNAs except for those on Chromosome 2 between NCBIm37 coordinates 10,386,914 and 10,439,906 bp, as this region contained several families of repetitive miRNAs genes of low sequence complexity. Excluding this region, the 513 other miRNA genes in miRBase (release 14) corresponded to 455 individual targeting vector designs. Of these 9 have not yet been attempted and a further 8 failed at the automated vector design stage leaving 438 possible vector designs which correspond to 494 (97%) of the miRNA genes on our target list.

Targeting vectors were constructed using three cycles of recombineering in *E.coli* as outlined in Fig 1. (see Supplementary Figures 1a and 1b for novel plasmid reagents)[25]. This process yielded vectors for 428 (98 %) of the available designs, covering 476 miRNA genes (Supplementary Table 1). For each design two targeting vectors were produced with either a Neomycin or a Puromycin-delta thymidine kinase fusion gene (*puroΔtk*) [26] for positive or positive-negative selection in ES cells, respectively. The availability of targeting vectors with two different selection markers supports the generation of homozygous mutant ES cells for each miRNA by serial targeting in ES cells. Both versions possessed selection cassettes flanked by loxP sites and also the Flp recombinase recognition sites FRT and the mutant F3 in reverse orientation[27].

mirKO targeted ES cell production

ES cells from the C57BL/6N mouse strain were used for generating the resource, enabling the mutant alleles to be produced in a defined genetic background and thus avoiding the complication of assessing mouse phenotypes in an out-bred population [28]. Gene targeting was initially piloted in the wild type JM8.F6 ES cell clone and the isogenic derivative JM8.A3[28] was used subsequently to generate the bulk of the resource. The JM8.A3 cell line enables chimaeric mice to be scored by the presence of agouti coat colour using host embryos from the C57BL6 strain while germ-line transmission can be scored in crosses with C57BL/6N females by the presence of the agouti coat colour in their offspring.

The miRNA targeting vectors with the *puroΔtk* cassette were used to generate the resource, and representative examples for the gene targeting of the *mir-290~295* cluster, *mir-21* and *mir-106a~363* cluster are depicted in Figs. 2, 3 and 4 respectively. In general, sets of 32 puromycin resistant clones were screened by long-range PCR across the short arm of the vector to identify targeted clones. Overall, 70% of targeting vectors yielded at least one targeted clone from the first set of 32 clones analysed an overall average targeting frequency of 25%. The targeting frequencies are summarized in Supplementary Fig. 2 and ranged from 92% to below 1%. In cases where targeting failed, or generated less than 3 positive clones, additional rounds of targeting were undertaken and successive sets of clones were screened, which increased the overall vector success rate to 81%. Overall, we have successfully generated targeted deletions for 392, or 79%, of the intended 495 miRNA genes, either as singletons or as clusters, after excluding candidate miRNAs that were removed from later versions of miRBase (Supplementary Table 1).

Up to 6 independent targeted ES cell clones for each allele were re-assessed by long range PCR across both homology arms and archived in replicate copies. The presence of products migrating at the expected size on standard 0.8% agarose gels for both PCR reactions was taken as high confidence that the targeting was successful and that the correct clones had been expanded (Figs. 2b, 3b and 4b for examples) . In a few cases PCR genotyping of the long homology arm was not successful for all clones corresponding to one allele. Analysis of 3 sets of failures by Southern blotting revealed that in most cases these were correctly targeted false negatives resulting from failure in long-range PCR (data not shown).

The sets of recombinase recognition sites flanking the selection cassette in the targeted loci supports directed manipulation of each allele while the *puroΔtk* positive-negative selection cassette makes these events selectable in Gancyclovir or FIAU [26]. The *puroΔtk* selection cassette flanked by loxP sites in direct orientation can be efficiently removed by expression of Cre followed by FIAU selection (Figs. 2 and 3 Supplementary Fig. 3) leaving a marker-free locus with a small deletion.

The utility of the resource for the establishing alleles in mice was assessed by injection of targeted clones into C57BL/6J-*Tyr*$^{c\text{-}Brd}$ blastocycts and test breeding of the resultant chimaeric mice (Table 1). Half of targeted clones generated from the JM8.F6 parental line gave germ-line transmission of the targeted allele (n=4) while for the JM8.A3 ES cell clones used for the majority of the resource ES cell clones 71% of injected clones (n=24), or 91% of those clones that gave male chimaeras (n=19) yielded germline transmission.

mirKO alleles are efficient substrates for RMCE

The FRT and F3 sites flanking the *puroΔtk* cassette facilitate the technique of recombinase mediated cassette exchange (RMCE)[27]. Co-transfection of a codon optimized FLPo [29] expression vector with a suitable exchange plasmid carrying a *PGK-neo-bpA* expression cassette catalyzed 100% efficient cassette exchange in G418 plus FIAU selected ES cell clones at all loci tested (Table 2; Supplementary Table 2; Supplementary Fig. 4). Selection

in G418 alone also yielded successful cassette exchange events, but at a lower frequency (Supplementary Table 2). The efficiency of RMCE allows the *puroΔtk* alleles in the heterozygous ES cell resource to be rapidly converted into alternative allelic variants (Fig. 2, 3 and 4; Supplementary Fig. 4).

Reporter alleles generated by RMCE

We demonstrate the ability to manipulate mirKO mutants by the introduction of the fluorescent reporter gene Td-tomato at two miRNA loci, the *mir-290~295* cluster and *mir-21*, that have very different expression patterns upon ES cell differentiation (Fig. 2 and 3; Supplementary Fig. 1c). To facilitate authentic expression from the endogenous miRNA promoter the *PGK-neo-bpA* selection cassette was removed by recombination between the flanking Rox sites mediated by Dre recombinase expression by transient transfection [30, 31]. The miR-290~295 cluster miRNAs are highly represented in ES cells[32, 33] being expressed intergenically from a defined promoter[22] and function in the maintenance of ES cell proliferation and pluripotency[7, 8]. Td-tomato at the *mir-290~295* cluster showed high expression in undifferentiated ES cells that subsided upon differentiation consistent with the reported expression pattern of the cluster (Fig. 2). *mir-21* is located within the 3′UTR of the *Tmem49* gene, but is expressed from a promoter located between *Tmem49* exons 11 and 12[21]. Td-tomato expression at the *mir-21* locus was not detectable in living undifferentiated ES cells or embryoid bodies formed during the first few days of the differentiation process. However, for later stage differentiating embryoid bodies (about 10d and later) red fluorescence appeared in some cells expressing Td-tomato (Fig. 3). There was no fluorescence within control *mir-21*^{PuΔtk} cells not expressing Td-tomato. This is consistent with the previously reported low representation of miR-21 in undifferentiated ES cells[22, 33] and the greatly increased presence upon differentiation *in utero*[33].

Conditional alleles generated by RMCE

To demonstrate the conversion of the *puroΔtk* allele to a conditional allele we have chosen the X linked *mir-106a~363* cluster (Fig. 4). The genomic sequence of the cluster was re-introduced by RMCE in place of the *puroΔtk* alleles such that it is flanked by loxP sites. The conditional allele was finalized by Dre deletion of the PGK neo cassette. The status of the *mir-106a~363* cluster at each step of the conversion process was assessed by PCR with flanking primers. The expression levels relative to parental JM8.A3 ES cells for each mature miRNA within the cluster for the allelic variant ES cell clones was determined by comparative quantitative PCR. The expression of miR-18b, miR-20b and miR-363 where all greatly reduced or undetectable in the null ES cell clones, but were expressed in the conditional ES cell clone. There was high expression of miR-19b and miR-92a in *mir-106a~363* cluster null ES cell clones (data not shown) but this is explained by the presence as orthologues of these miRNAs expressed from the miR-17~92a-1 cluster on chromosome 14 which are also detected by the TaqMan assays used. The reason for the detection of high levels of miR-106a is unresolved as the TaqMan assay was described as being specific for this miRNA.

Accessing and utilizing the mirKO resource

To inform researchers of the availability and technical details of the mirKO reagents this information has been entered onto the International Knockout Mouse Consortium (IKMC) web portal (www.knockoutmouse.org/martsearch) which is editable and will be modified as the resource develops[6]. Contingent upon successful expansion and genotyping quality control by long range PCR targeted ES cell clones are being transferred to repositories for distribution. Within the IKMC web portal links are provided to order clones form either of the two participating repositories MMRRC (www.mmrrc.org) at the University of California

Davis, USA, and EuMMCR at the Helmholtz Zentrum München (www.eummcr.org), Munich, Germany.

The IKMC web portal has been described in detail elsewhere [6] so that the following is a brief description relating to the mirKO reagents. The mirKO reagents on the IKMC web portal can be searched using the MGI number, Ensembl gene identifier or the miRBase registry designation. Viewable at this website is an overview of resources for the particular project and their availability. A link through a mirKO project number provides a summary diagram of individual targeting vectors. Each vector has a unique design ID that links through to the high throughput gene targeting design for the particular miRNA knockout which provides information on the oligonucleotides homology arms used for recombineering with associated genomic coordinates (NCBIM37 assembly). Each targeting vector has been given a name with a prefix (mirKO_TV_PuDtk) followed by a unique address within the stock glycerol plates. Each targeting vector has a linked annotated sequence file deposited within the IKMC database. The targeting vectors will be available to order from repositories so as to enable the initiation of new targeting experiments.

For each miRNA project all available targeted ES cell clones are listed in the form mirKO_ES_PuDtk_ID, where the ID number describes the matrix plate number and address where the clone is stored. The parental ES cell line (JM8.F6 or JM8.A3) is listed against each ES cell clone. There is a linked annotated sequence file comprising the selection cassette that replaced the mRNA and genomic flanking sequence. The binding sites of the long-range PCR primers used for genotype verification at both ends of the targeting event are indicated. Within the QC data the status of the long-range genotyping PCR is indicated.

Following receipt of targeted ES cell clones from repositories we recommend that they be expanded on feeder cells under antibiotic selection and frozen into multiple vials for future use in addition to making genomic DNA[28]. It is important that the targeting status is independently verified by the recipient prior to use. As a minimum this should be by successful repetition of the long range PCR at both ends of the targeting event. In cases where the production and distribution centres have not been successful in performing the PCR at the long-arm we strongly recommend that a Southern blot strategy be configured by the recipient to verify correct targeting before use. Although we do not believe it is essential where long-range PCR results are clear, and indeed it was impractical for us to develop at large-scale during knockout production, a Southern strategy will be a useful genotyping protocol to have available for all mirKO projects. Following genotype verification where a simple miRNA deletion is required and the *puroΔtk* selection cassette is removed by Cre recombinase transfection in tissue culture then an additional short-range PCR should be developed by the recipient. This is most easily performed by PCR across the deletion although alternative approaches may be required where the mutant and wild type PCR products are very similar in size (indistinguishable) or very different in size (the longer PCR product may be inefficiently amplified) (Supplementary Fig. 3).

Discussion

We established a mouse miRNA knockout resource in ES cells for the majority of the miRNAs currently in the miRBase registry. These miRNA alleles were generated in C57BL/6N ES cells, which has been adopted as the standard genetic background for knockouts. Most of the targeted alleles were made using the JM8.A3 ES cell clone which enables visual identification of chimaerism and germ line transmission by agouti coat colour in the widely used C57BL/6 strain. In practical terms, this allows an inbred genetic background to be maintained while still using coat colour markers, reducing mouse usage and genotyping[28]. The germline transmission efficiency of the clones from the resource are similar to those

reported previously[28]. The knockouts are configured as simple deletions of the miRNA but the design enables efficient post targeting modification to be accomplished by RMCE allowing a multitude of alternative alleles to be efficiently produced. We have demonstrated the conversion of *puroΔtk* targeted alleles to reporter and conditional alleles. Other examples of possible applications might include the variation of miRNA copy number and mutating individual miRNAs within clusters. Clones are available in public repositories and details of the targeted ES cell clones and alleles have been entered onto a publically viewable database of the International Mouse Knockout Consortium (http://www.knockoutmouse.org/martsearch)[6].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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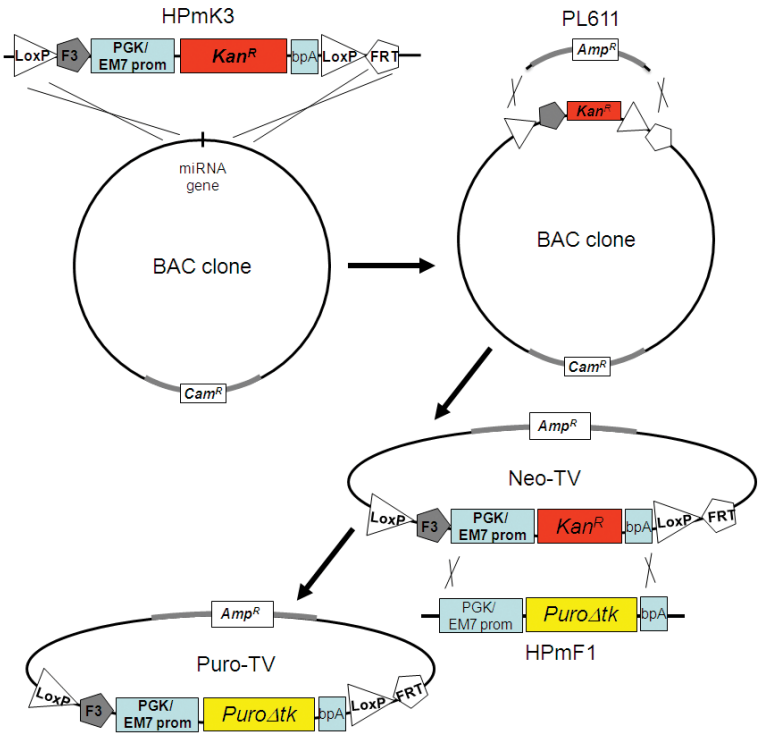

Fig. 1. Schematic illustration of targeting vector construction

The Kanamycin resistance recombineering cassette was PCR amplified from the pHPmK3 vector and recombineered into BACs in *E.coli* deleting the miRNA gene. Retrieval of the homology arms and kanamycin selection cassette was achieved by recombineering a PCR amplified pPL611 vector backbone to create a *PGK-Neo* targeting vector (Neo-TV). The kanamycin/neomycin selection cassette of the targeting vector was switched to *PuroΔtk* by using a *PGK-EM7-PuroΔtk-bpA* restriction fragment for recombineering to generate the puromycin version of the targeting vector (Puro-TV).

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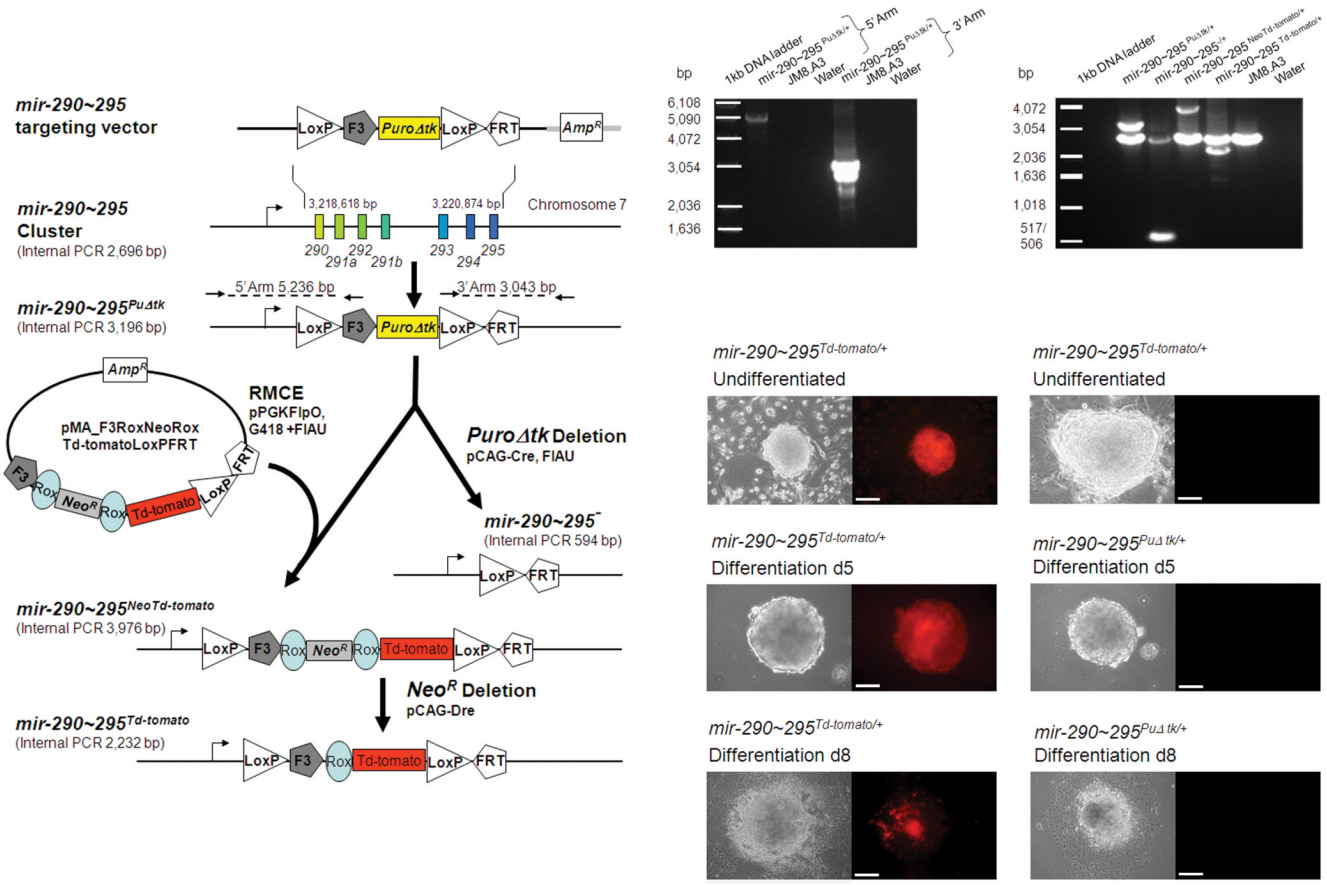

Fig. 2. Targeting and reporter modification of the *mir-290~295* cluster

a, Targeting of the intergenic miRNA cluster *mir-290~295* replaces 2256bp of genomic sequence with the *PGK-PuroΔtk* cassette. The PGK-*PuroΔtk* deletion was by Cre recombinase transfection and FIAU selection. Alternatively RMCE by FLPo recombinase transfection was used to integrate a neomycin resistance (*Neo*^{R}) plasmid (e.g. pMA_F3RoxNeoRoxTd-tomatoLoxPFRT). The *PGK-Neo*^{R} deletion was by Dre recombinase transfection. The predicted sizes of PCR products generated by PCR across the mir-290~295 cluster or modified alleles are indicated in brackets. **b**, Products for long-range PCR between primers specific for the selection cassette and the region external to the 5′ and 3′ homologous arms of the targeting vector. **c**, Products for PCR between primers specific for flanking sequences of *mir-290~295* locus and the allelic variants. **d**, Bright field and corresponding fluorescent images of the same *mir-290~295*$^{Td\text{-}tomato/+}$ ES cell colony (top left), *mir-290~295*$^{PuΔtk/+}$ control ES cell colony (top right), embryoid bodies at d5 and d8 into the differentiation regime for *mir-290~295*$^{Td\text{-}tomato/+}$ (middle and bottom left) and *mir-290~295*$^{PuΔtk/+}$ (middle and bottom right). Scale bars are 100μm.

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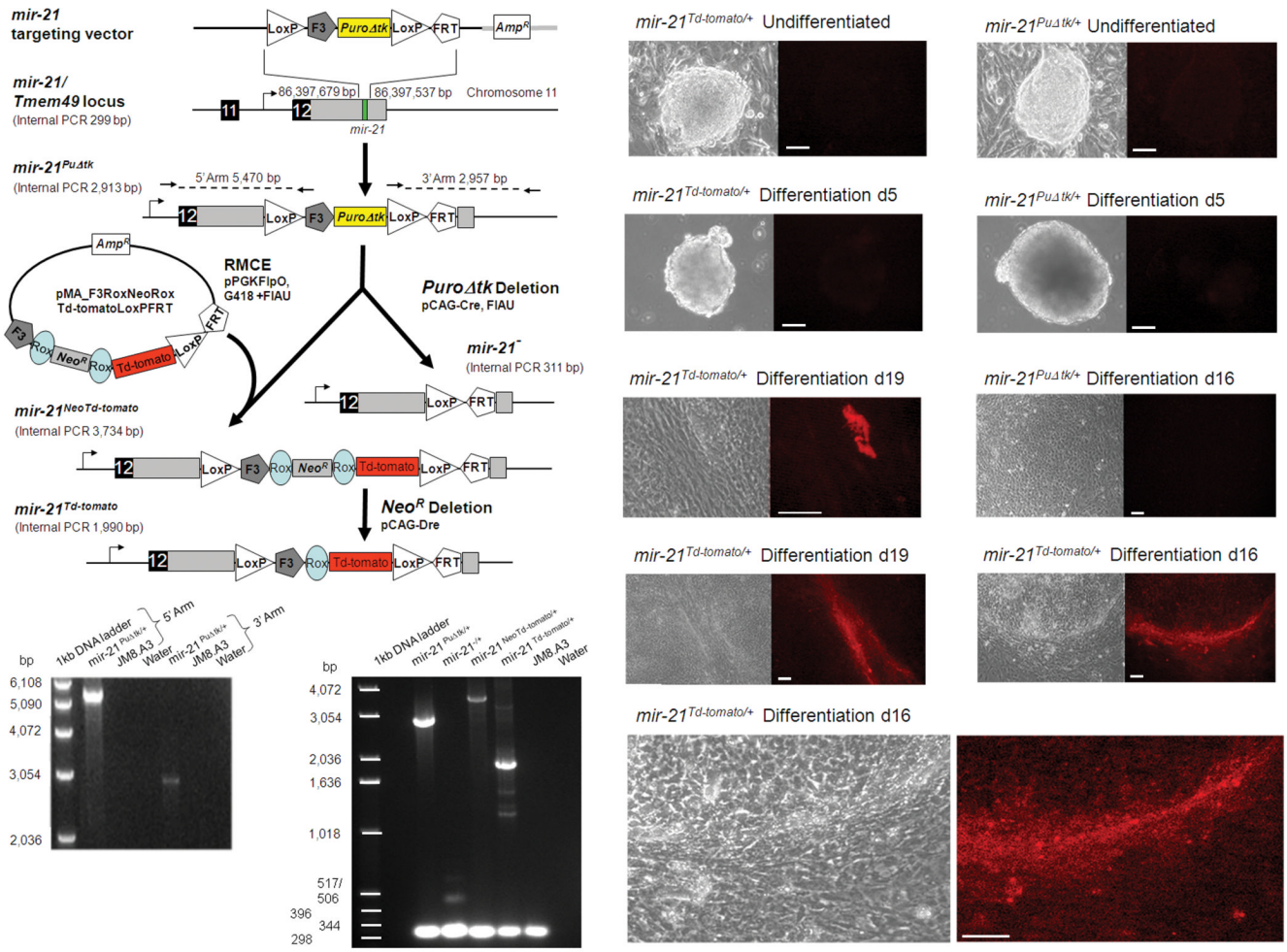

Fig. 3. Targeting and reporter modification of the *mir-21 gene*

a, The *mir-21* gene was removed as a 191bp deletion using the *PuroΔtk* cassette which could then be a substrate for either deletion or RMCE using a reporter construct. The predicted sizes of PCR products generated by PCR across the *mir-21* locus or modified alleles are indicated in brackets. **b**, Products for long-range PCR between primers specific for the selection cassette and the area external to the 5′ and 3′ homologous arms of the targeting vector. **c**, Products for PCR across the targeted and modified mir-21 locus. Note that the *mir-21*$^{-}$ allele yields a PCR product which migrates as a doublet with the wild type allele/JM8.A3 PCR product. **d**, Bright field and fluorescent images of the same *mir-21*$^{Td\text{-}tomato/+}$ undifferentiated ES cell colonies (top left) *mir-21*$^{Pu\Delta tk/+}$ control ES cell colonies (top right), embryoid bodies at d5 and d16 and d19 of differentiation for *mir-21*$^{Td\text{-}tomato/+}$ and *mir-21*$^{Pu\Delta tk/+}$ as indicated. The bottom image shows a close-up of a d16 differentiation *mir-21*$^{Td\text{-}tomato/+}$ embryoid body. Scale bars are 100 μm.

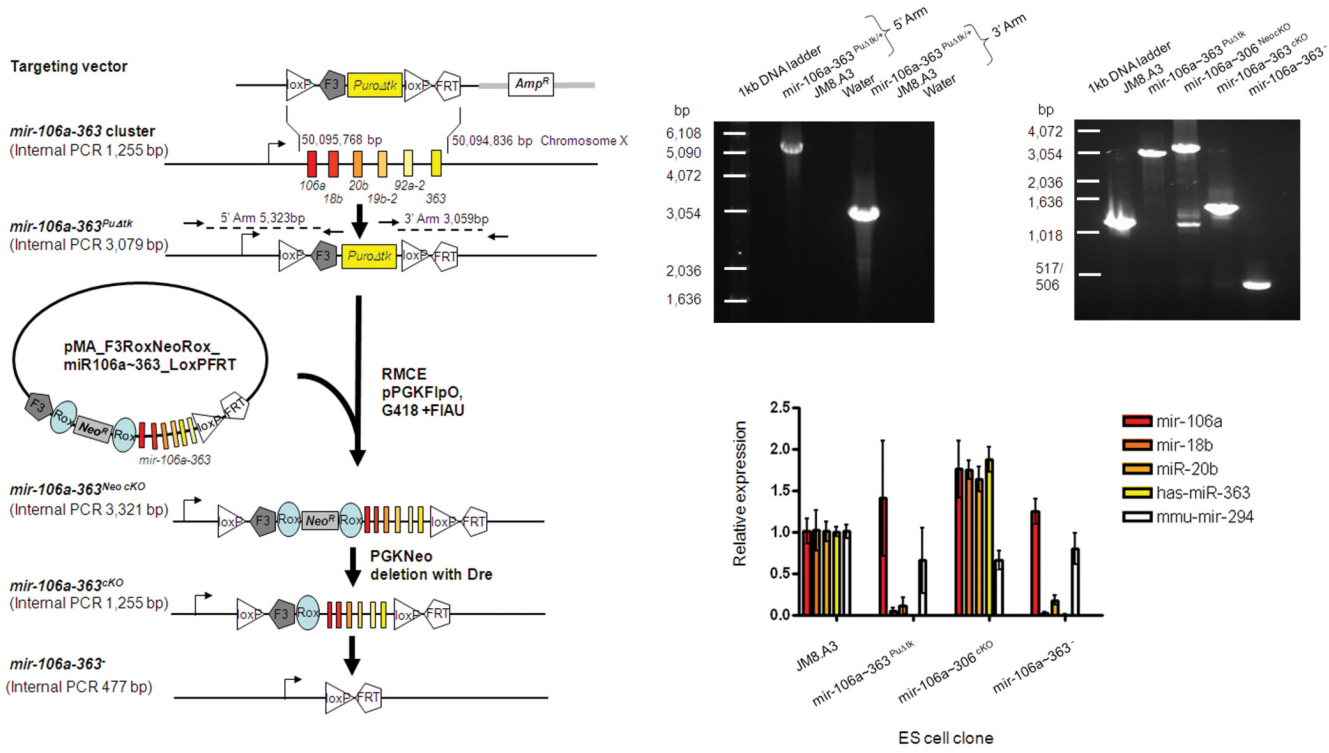

Fig. 4. Targeting and conditional modification of the *mir-106a~363* cluster

a. The *mir-106a~363* cluster was removed as a 932bp deletion using the *PuroΔtk* cassette. The predicted sizes of PCR products generated by PCR (internal to the targeting vector) across the *mir-106a-363* cluster or modified alleles are indicated in brackets. An RMCE cassette containing a cloned genomic sequence for the wildtype *mir-106a~363* cluster (pMA_F3RoxNeoRox_miR106a~363_LoxPFRT) was inserted into the targeted locus by co-transfected with *PGK-FlpO* followed by FIAU and G418 selection. The *PGK-Neo*^{R} deletion was by Dre recombinase transfection. Cre deletion successfully deleted the conditional allele, although the *mir-106a~363*$^{-}$ clone analysed in this figure was derived by Cre deletion of the *mir-106a~363*^{PuΔtk} clone. **b**, Long-range PCR between primers specific for the selection cassette and the region external to the 5′ and 3′ homologous arms of the targeting vector. **c**, PCR between primers (internal to the targeting vector) specific for flanking sequences of *mir-106a~363* locus and the allelic variants. **d**, Comparative quantitative PCR for miRNAs within the *mir-106a~363* cluster and miR-294 as a control. The TaqMan MicroRNA assays used are color coded. n=3; error bars show SD.

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Table 1

Chimaerism and germline transmission rates

	Microinjections			Male Chimaeras		Test breeding		
Parental cell line	**Clones injected**	**Clones at birth**	**Percentage of injected**	**Clones at weaning**	**Percentage of injected**	**Clones set-up**	**Clones with GLT**	**Percentage of injected**
JM8.F6	4	3	75%	2	50%	2	2	50%
JM8.A3	24	23	96%	19	79%	19	17	71%

GLT, germline transmission.

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Table 2

Efficiency of RMCE at different chromosomal loci

Targeted miRNA gene(s)	Chromosome: Location (bp) (NCBIM37)	RMCE construct	% RMCE (analysed colonies)
mir-29b-2, mir-29c	1: 196,863,234 - 196,863,828	pHPmK3	100 (n=20)
mir-124-3	2: 180,628,745 - 180,628,812	pHPmK3	100 (n=23)
mir-124-2	3: 17,695,662 - 17,695,770	pHPmK3	100 (n=24)
mir-9-1	3: 88,019,520 - 88,019,608	pHPmK3	100 (n=24)
mir-29a, mir-29b-1	6: 31,012,660 - 31,013,093	pHPmK3	100 (n=22)
mir-128a	6: 130,098,938 - 130,099,007	pHPmK3	100 (n=24)
mir-290~295	7: 3,218,627 - 3,220,842	pHPmK3	100 (n=24)
mir-290~295	7: 3,218,627 - 3,220,842	pMA_ F3RoxNeoRoxloxPFRT	100 (n=87)
mir-210	7: 14807283 - 148407392	pHPmK3	100 (n=43)
mir-21	11: 86397569 - 86397660	pMA_ F3RoxNeoRoxloxPFRT	100 (n=71)

Derivatives of the RMCE construct pMA_FRTRoxNeoRoxloxPF3 carrying different inserted cargos were used.