The NCI Mouse Repository
miRNA Resource

MicroRNAs (miRNAs) are small non-coding RNA molecules known to play an important role in fundamental cellular processes through negative post-transcriptional regulation of gene expression. In an effort to address the role that microRNAs play in human cancer, their use as diagnostic tools, and their potential function as new targets for therapeutic intervention in the treatment of cancer, the Division of Cancer Biology (DCB) of the National Cancer Institute (NCI) is supporting the generation of mouse embryonic stem cells (mESCs) harboring most known mouse miRNAs. In order to optimize novel information obtained on their role in cancer, 1520 genetically engineered mESC lines are being produced, harboring conditional microRNA transgenes. The NCI Mouse Repository will offer this new valuable resource to the cancer research community as it becomes available.

Drs. Gregory Hannon and Scott Lowe, at the Cold Spring Harbor Laboratory (CSHL), have utilized high-throughput technology to produce mES cells that conditionally express each known murine miRNA (1). The miR ESC lines, when used to generate genetically engineered mice, will produce a tet-inducible, reversible system to investigate the role of each miRNA in vivo, providing a powerful tool for the study of miRNA biology. Transgene expression for each ESC clone is controlled by either the tetracycline response element (TRE) or TRE-tight promoters, to accommodate a wide range of experimental designs. TRE-driven expression facilitates the study of microRNA in a variety of tissues, while TRE-tight promoter reduces leakiness and results in more restricted expression, suited for tissue-specificity, especially useful for study of miRNAs for which even the smallest level of expression may cause sterility or lethality (appendix 1). The following will be the categorical distribution of the miR ESC lines:

1. ***mES cell lines with mature microRNAs embedded in a miR30 precursor, designed to control the effect microRNA processing might have on overall expression***
   a) 508 mESC lines expressing the miR controlled by TRE-tight promoter (appendix 2).
   b) 300 mESC lines expressing the major miR controlled by TRE promoter.

2. ***mES cell lines with mature microRNA species in their endogenous context (referred to as: Primary-miRNAs)***
   a) 250 mESC lines expressing the miR controlled by TRE-tight promoter.
   b) 470 mESC lines expressing the miR controlled by TRE promoter.

The parental ES cell line used to produce all the microRNA-expressing clones is KH2 (C57BL/6 x 129/Sv). It contains an frt-homing cassette and reverse tet-transactivator that were previously targeted into its *ColA1* locus and *Rosa26* loci (2). The miR clones were generated by electroporation of pColTtGM Flp-in targeting vector into the ES cells (appendix 3, figure 3) containing either a TRE or a TRE-tight promoter and the fluorescent reporter gene GFP (green

fluorescent protein). Presence of the tetracycline responsive transactivator M2-rtTA in the parental KH2 ES cells, allows for promoter-driven miR expression in the presence of doxycycline (dox). All ES cell lines generated with this inducible system have been validated by flow cytometry for GFP expression. Additionally, many have also been authenticated for miRNA expression as determined by RT-qPCR. These results are noted on the description page for each of the miRNA-containing mESC lines. Selection of validation methods was determined based on the following parameters: **1)** In some cases the endogenous expression of the miRs was not detectable in ES cells in the absence of dox assessed by Taqman miR qPCR, while their expression was observed in the presence of dox indicating their induction. **2)** In several cases, the expression levels of miRs were beyond saturated amounts that could be assessed by Taqman miR-qPCR, therefore the expression levels of their primary transcript were measured by RT-qPCR, demonstrating the fold induction of the primary transcripts ranging from 4 to 55 for these miRs in miR-30 context. **3)** Induction of miR expression was unsuccessful in several clones even though the genomic sequences of the miRs for a select group among these were verified by PCR, possibly because the RNAi machinery could not efficiently process these mature miRs when placed into a miR-30 carrier. **4)** Validation for mESCs containing miRs larger than 22 bps was performed only by flow cytometry as miR-qPCR kits for these miRs were not readily available.

The first 508 genetically engineered miRNA mESC lines are available for distribution through the NCI Mouse Repository. Information regarding the progress of newly generated mES cells will be periodically updated on the NCI Mouse Repository web page. Sequencing data for each miRNA is listed in the oligonucleotide libraries (link to sequence data). Protocols utilized in the generation, care, manipulation, and use of the miR ESC clones are provided in the protocol section (appendix 4).

Orders for miR expressing clones may be submitted online. Please refer to http://mouserepository-staging.ncifcrf.gov/default.asp for more detailed instructions. Because the miR ES cell resource is limited, distribution to the scientific community will be prioritized according to the following organizational affiliation:

1) NCI-funded investigators
2) NIH-funded investigators
3) Government funded researchers, other than those funded by NCI or NIH
4) Investigators from non-profit organizations without government funding

Appendix 1

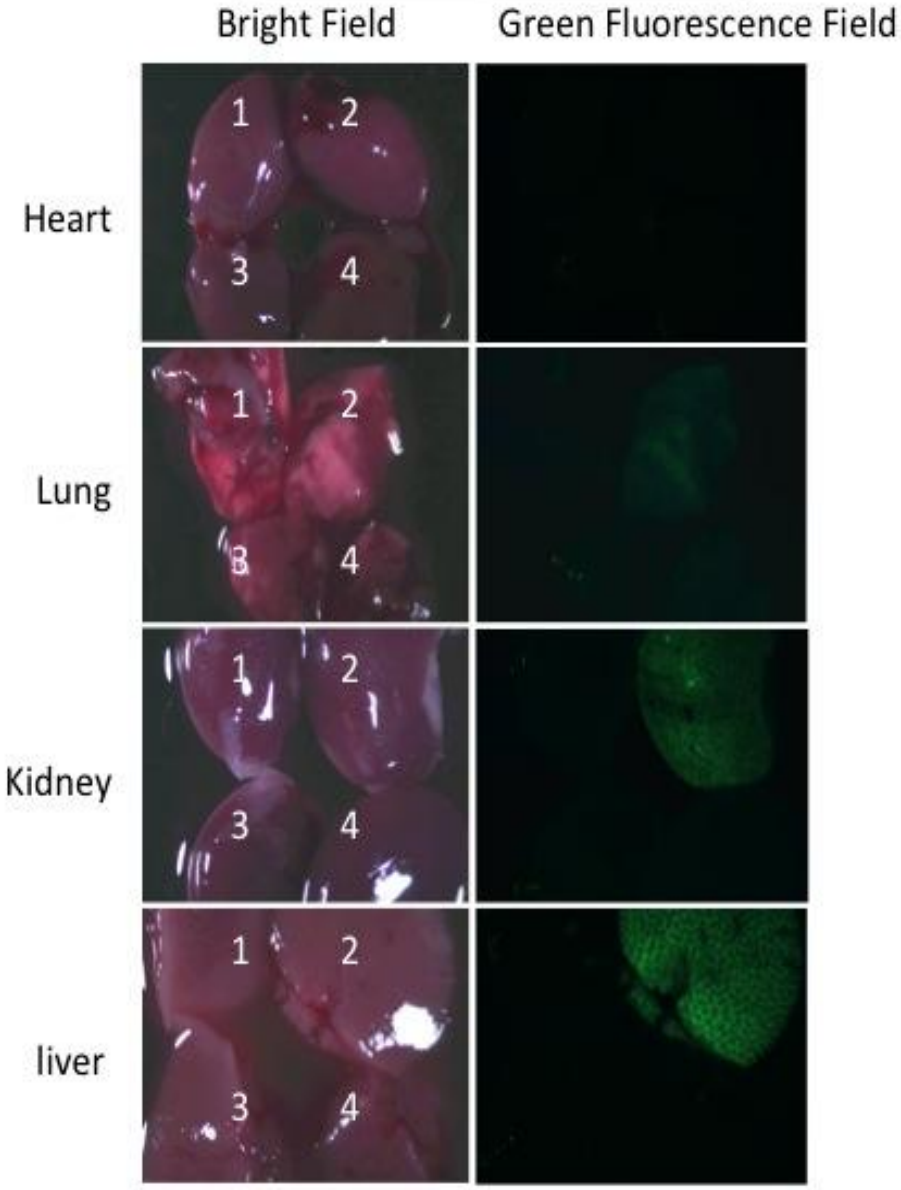
Comparison of tissue specificity of GFP expression in TRE-tight- let-7c and TRE-let-7c genetically engineered mice

A comparison of the expression pattern of GFP in various organs from TRE-tight-versus TRE-let-7c mice is provided (see Figure 1). Consistent with previous reports (1, 3), significant variation in the level of GFP induction between different organs from TRE-mice (heterozygous let-7c/heterozygous rtTA2) was observed. Imaging analysis showed that GFP fluorescence was readily detected in the thymus, skin, small intestine, and colon (not shown) from TRE-let-7c as well as TRE-tight-let-7c when doxycycline (dox) in drinking water was given to mice for four days. However, GFP positivity was only observed in lung, kidney and liver from TRE-let-7c mice with the presence of a single R26-rtTA transgenic allele (labeled as hetero-rtTA2; see #2 organs in Figure 1), but not from TRE-tight-let-7c mice (see #3 organs in Figure 1). Previously, the presence of two R26-rtTA transgenic alleles increased the dox-dependent expression of GFP-shRNAs relative to a single allele in TRE-mice was reported (1). However, no GFP positivity was observed in kidney, lung and liver from TRE-tight-let-7c mice even in the presence of two R26-rtTA alleles (labeled as homo-rtTA; see #4 organs in Figure 1).

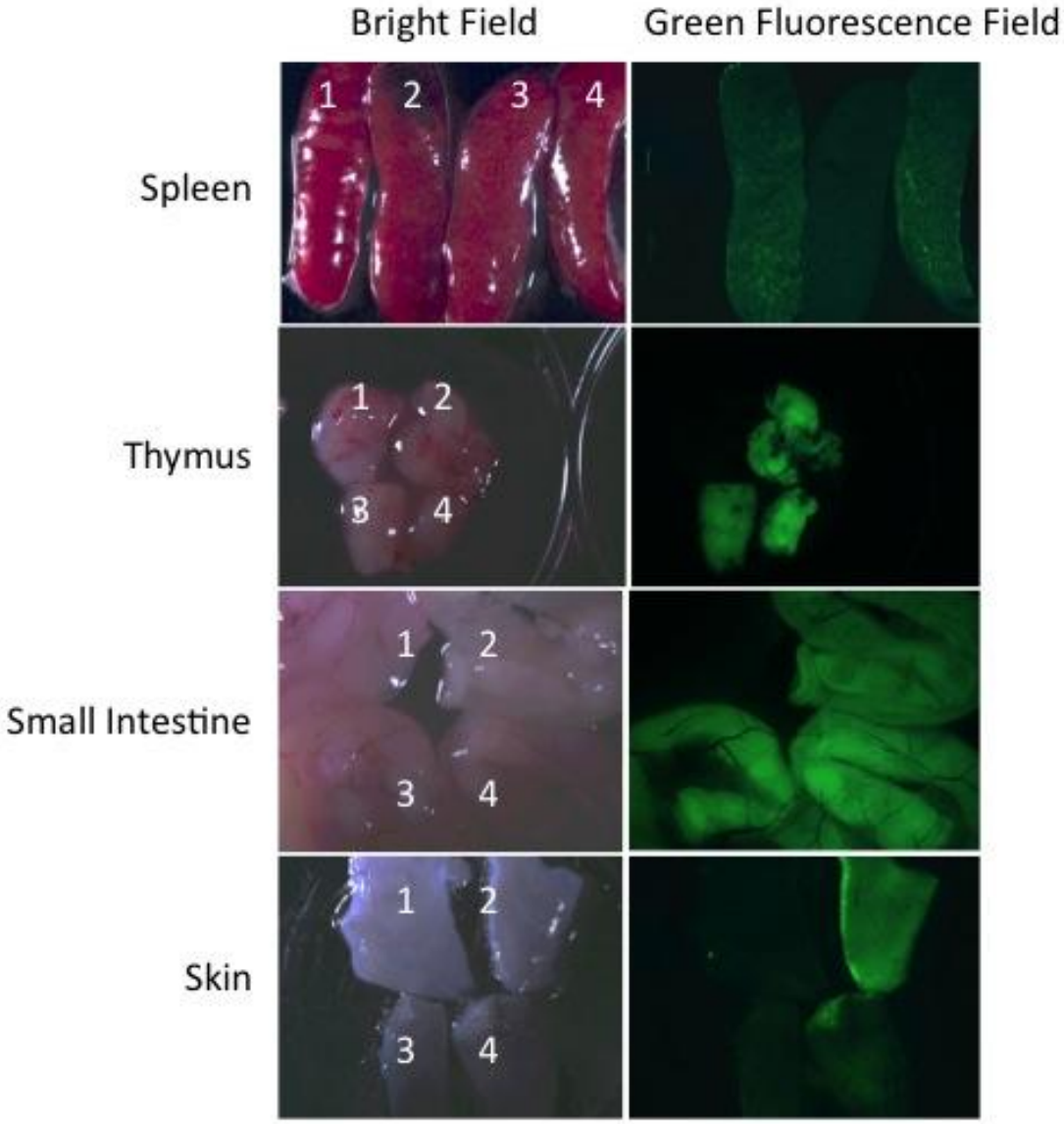
Finally, rtTA-dependent GFP induction in spleen from TRE-tight let-7c mice was observed (Figure 1). The level of GFP fluorescence with two R26-rtTA alleles in TRE-tight-let-7c mice was comparable to the level with one R26-rtTA allele in TRE-let-7c mice (Figure 1, #2 vs. #4 organs). These results suggested that TRE-tight promoter-driven GFP induction was more tissue-restricted than TRE promoter-driven GFP induction. In homozygous ROSA mouse strains with other shRNAs driven by TRE-tight promoter, fluorescence was observed in more widespread tissues including the pancreas, testis, and uterus, which could be comparable to the levels seen in mice having heterozygous ROSA with TRE promoter (data not shown). In the bone marrow, 60-80 % cells were GFP positive, though fluorescence in the liver, kidney, lung, and heart from TRE-tight was below the sensitivity threshold of the imaging assay. Speckled GFP positive cells were detected by immunohistochemistry in the lung, kidney and liver (data not shown). The increased expression of let-7c in the intestine, spleen and thymus from TRE-tight-let-7c mice was confirmed using small RNA northern analysis. In contrast to the restricted tissue distribution of GFP fluorescence in adult tissues, much stronger GFP induction was observed in most of the tissues including liver and brain in embryos at E15.5 after dox was given to the mother for 4 days.

Figure 1. Comparison of GFP expression in various organs from TRE vs TREtight mice

1) TREtight No let-7c Hetero-rtTA2	**2) TRE** Hetero-let-7c Hetero-rtTA2
3) TREtight Hetero-let-7c Hetero-rtTA2	**4) TREtight** Hetero-let-7c Homo-rtTA2



1) TREtight No let-7c Hetero-rtTA2	**2) TRE** Hetero-let-7c Hetero-rtTA2
3) TREtight Hetero-let-7c Hetero-rtTA2	**4) TREtight** Hetero-let-7c Homo-rtTA2



Appendix 2

List of 508 mESC lines expressing the miR controlled by TRE-tight promoter in the miR30 context

let-7a	let-7a-st	let-7b	let-7c	let-7c-1-st
let-7d	let-7d-st	let-7e	let-7f	let-7g
let-7g-st	let-7i	miR-1	miR-100	miR-101a
miR-101a-st	miR-101b	miR-103	miR-106a	miR-106b
miR-107	miR-10a	miR-10a-st	miR-10b	miR-10b-st
miR-1186	miR-1188	miR-1191	miR-1192	miR-1193
miR-1194	miR-1197	miR-1198	miR-122	miR-1224
miR-124	miR-124-st	miR-125a-3p	miR-125a-5p	miR-126-3p
miR-126-5p	miR-127	miR-127-st	miR-128	miR-129-3p
miR-129-5p	miR-1-2-as	miR-1306	miR-130a	miR-130b
miR-132	miR-133a	miR-133a-st	miR-134	miR-135a-st
miR-135b	miR-136	miR-136-st	miR-137	miR-138
miR-139-3p	miR-140-st	miR-141	miR-141-st	miR-142-3p
miR-142-5p	miR-143	miR-144	miR-145-st	miR-147
miR-148a-st	miR-148b	miR-150	miR-150-st	miR-151-3p
miR-151-5p	miR-152	miR-153	miR-154	miR-154-st
miR-155	miR-15a	miR-15b	miR-15b-st	miR-16
miR-16-st	miR-17	miR-17-st	miR-181a	miR-181a-1-st
miR-181a-2-st	miR-181b	miR-181c	miR-181d	miR-182
miR-1839-5p	miR-183-st	miR-184	miR-185	miR-186-st
miR-187	miR-188-3p	miR-188-5p	miR-1892	miR-1894-3p
miR-1895	miR-1896	miR-1897-3p	miR-1897-5p	miR-1898
miR-18b	miR-190	miR-1902	miR-1903	miR-1904
miR-1906	miR-1907	miR-190b	miR-191	miR-191-st
miR-192	miR-1927	miR-1929	miR-193	miR-1930
miR-1932	miR-1933-3p	miR-1933-5p	miR-1935	miR-1936
miR-1938	miR-1939	miR-193-st	miR-194	miR-1940
miR-1941-5p	miR-1943	miR-1945	miR-1946b	miR-1947
miR-1948	miR-1949	miR-195	miR-1950	miR-1951
miR-1953	miR-1954	miR-1956	miR-1957	miR-1958
miR-1959	miR-1960	miR-1961	miR-1962	miR-1963
miR-1966	miR-1967	miR-1968	miR-1969	miR-196a
miR-196a-st	miR-196b	miR-1970	miR-1971	miR-1981
miR-1982.1	miR-1982.2	miR-1982-st	miR-1983	miR-199a-5p
miR-199b-st	miR-19a	miR-19b	miR-200a	miR-200a-st
mir-200b	miR-200b-st	miR-200c	miR-201	miR-202-3p

miR-202-5p	miR-203	miR-203-st	miR-204	miR-206
miR-208a	miR-208b	miR-20a	miR-20a-st	miR-20b
miR-20b-st	miR-21	miR-214	miR-214-st	miR-215
miR-216a	miR-217	miR-218	miR-219	miR-220
miR-21-st	miR-221	miR-222	miR-224	miR-22-st
miR-23a	miR-23b	miR-24	miR-24-1-st	miR-24-2-st
miR-25	miR-26a	miR-26b	miR-26b-st	miR-27a
miR-27a-st	miR-27b	miR-27b-st	miR-28	miR-28-st
miR-290-3p	miR-290-5p	miR-291a-3p	miR-291b-3p	miR-292-3p
miR-292-5p	miR-293-st	miR-294	miR-294-st	miR-295
miR-296-5p	miR-297a	miR-297b-3p	miR-297b-5p	miR-297c
miR-298	miR-299	miR-299-st	miR-29a	miR-29a-st
miR-29b	miR-29b-st	miR-29c	miR-29c-st	miR-300
miR-300-st	miR-301a	miR-301b	miR-302a	miR-302b
miR-302b-st	miR-302c	miR-302c-st	miR-302d	miR-30a
miR-30a-st	miR-30b-st	miR-30c	miR-30c-1-st	miR-30c-2-st
miR-30e-st	miR-31	miR-31-st	miR-32	miR-320
miR-322	miR-322-st	miR-323-5p	miR-324-5p	miR-325
miR-325-st	miR-327	miR-329	miR-33	miR-330
miR-330-st	miR-331-5p	miR-335-3p	miR-335-5p	miR-337-3p
miR-337-5p	miR-338-3p	miR-338-5p	miR-33-st	miR-340-3p
miR-340-5p	miR-341	miR-342-5p	miR-343	miR-344
miR-345-3p	miR-34a	miR-34b-3p	miR-34b-5p	miR-350
miR-351	miR-361	miR-362-5p	miR-363	miR-365
miR-367	miR-369-3p	miR-369-5p	miR-370	miR-374
miR-374-st	miR-375	miR-376a	miR-376a-st	miR-376b
miR-376b-st	miR-376c	miR-376c-st	miR-377	miR-378
miR-378-st	miR-379	miR-380-3p	miR-380-5p	miR-381
miR-382	miR-382-st	miR-383	miR-384-3p	miR-384-5p
miR-410	miR-411	miR-411-st	miR-421	miR-423-5p
miR-429	miR-431-st	miR-433	miR-433-st	miR-434-5p
miR-448	miR-449a	miR-449b	miR-449c	miR-450a-3p
miR-450a-5p	miR-450b-3p	miR-450b-5p	miR-451	miR-452
miR-453	miR-463	miR-464	miR-465a-3p	miR-465a-5p
miR-465b-5p	miR-465c-5p	miR-466a-3p	miR-466a-5p	miR-466b-3p
miR-466b-5p	miR-466d-3p	miR-466d-5p	miR-466e-5p	miR-466f
miR-466f-5p	miR-466h	miR-466j	miR-466l	miR-467a
miR-467a-st	miR-467b	miR-467c	miR-467d	miR-467e
miR-467e-st	miR-467h	miR-468	miR-469	miR-471
miR-483	miR-487b	miR-488	miR-488-st	miR-489

miR-490	miR-493	miR-494	miR-495	miR-496
miR-497	miR-499	miR-500	miR-501-3p	miR-503
miR-503-st	miR-504	miR-505	miR-509-3p	miR-509-5p
miR-532-5p	miR-539	miR-540-3p	miR-541	miR-542-3p
miR-542-5p	miR-543	miR-544	miR-546	miR-547
miR-568	miR-590-3p	miR-590-5p	miR-592	miR-598
miR-652	miR-653	miR-654-5p	miR-665	miR-665-5p
miR-666-st	miR-669a	miR-669b	miR-669c	miR-669d
miR-669e	miR-669f	miR-669g	miR-669h-3p	miR-669h-5p
miR-669i	miR-669j	miR-669k	miR-669l	miR-669m
miR-669o	miR-670	miR-671-5p	miR-672	miR-673-3p
miR-673-5p	miR-674	miR-676	miR-676-st	miR-677
miR-678	miR-679	miR-680	miR-682	miR-684
miR-685	miR-686	miR-687	miR-691	miR-693
miR-694	miR-695	miR-697	miR-701	miR-703
miR-704	miR-705	miR-706	miR-708	miR-708-st
miR-709	miR-710	miR-711	miR-717	miR-719
miR-721	miR-741	miR-742	miR-742-st	miR-743a
miR-743b-3p	miR-743b-5p	miR-744	miR-759	miR-760
miR-763	miR-764-3p	miR-770-3p	miR-7a	miR-7b
miR-802	miR-804	miR-805	miR-871	miR-872
miR-873	miR-875-3p	miR-875-5p	miR-876-3p	miR-876-5p
miR-877	miR-878-5p	miR-879	miR-879-st	miR-880
miR-881	miR-881-st	miR-882	miR-883a-3p	miR-883a-5p
miR-883b-3p	miR-883b-5p	miR-93	miR-96	miR-98
miR-99a	miR-99b-st	miR-9-st		

Appendix 3

MicroRNA ES cell resource construction

The miRNA ES cell resource was generated in the following four phases, carried out concurrently, to achieve the goal of generating all 1520 miRNA-harboring mESC:

1- Construction of the recipient pColTtGM Flp-in Targeting vector
2- Design of the libraries of oligonucleotides of mature miRNAs (embedded in miR30 precursor) and the library of individual pri-miRs
3- Production of distinct libraries of pColTtg targeting vectors harboring miRNAs
   a. Production of the library of targeting vectors carrying miR-30-based miRNAs
   b. Production of library of targeting vectors expressing Pri-miRs
4- Production of mESC lines harboring miRNAs and validation by flow cytometry and RT-qPCR

1) Construction of the recipient pColTtGM Flp-in targeting vectors:

The frt-mediated targeting strategy was applied to generate ES cells expressing regulatable miRNA. KH2 ES cells were used in which an frt-homing cassette and reverse tet-transactivator were previously targeted into *ColA1* locus and *Rosa26* loci (2). Expression of FLPe Recombinase mediates recombination between the frt site on pColTGM vector and those within the homing cassette at the *ColA1* locus (see figure 3).

It has been shown that miR30-based shRNAs can be inducibly expressed in a tissue specific and reversible manner (3). The miR30 cassette serves as the backbone to receive predicted 22nt shRNA, included in the Hannon-Elledge genome library. Therefore, the pBS31 vector was modified by the insertion of miR-30 based cassette downstream of GFP to generate the recipient targeting vector (called pColTtGM, see Figure1A). Additionally, the TRE promoter was replaced with a TRE-tight version to reduce leaky expression of either shRNAs/miRNAs. The miR-30 based cassette contains convenient and unique XhoI and EcoRI sites for rapid single-step cloning of miRNA expression targeting vectors to generate the library. Moreover, for pri-miRNA expression, the miR30 cassette was replaced with a linker, having multiple cloning sites, to generate the new targeting vector (called pColTtG-linker, see figure 1B) for construction of the library of targeting vectors expressing pri-miRs. Sequences for all recipient vectors were verified using 5 different sets of sequencing primers.

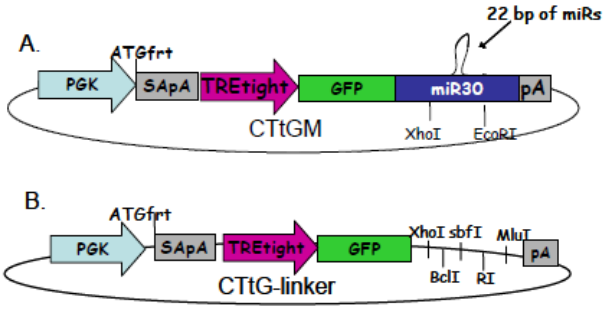


Figure 1. Schematic diagram of recipient Flp-in targeting vectors. A) CTtGM as a recipient vector to construct Flp-in targeting vectors carrying miRs in a miR30 carrier. B) CTtG-linker recipient vector to construct Flp-in targeting vectors harboring Pri-miRNAs.

2) Design the libraries of oligonucleotides of mature miRNAs (embedded in miR30 precursor) and the library of individual pri-miRs:

CSHL has generated populations of 96-mers of 680 miR-30 based mature miRNAs which contain exact complementary to the known mature miRNA sequences on a surface chip (Agilant Technology,CA). These oligonucleotides (there will be a link on the website to the library of oligonucleotides) were used as templates to produce PCR products for the construction of the libraries of miRNA-express-targeting vectors. Sequences of PCR primers for the amplification of the library of oligonucleotides follow:

a) miRXho-F; 5' -tacaatactcgagaaggtatattgctgttgacagtgagcg-3'
b) miREco-R; 5' -acttagaagaattccgaggcagtaggca-3

In addition, two sets of 487 PCR primers were designed by Open Biosystems for the amplification of pri-miRNA transcripts. Each primer contains a combination of XhoI, SalI, or BclI and EcoRI or MfeI or MluI in the 5' and 3' end respectively (there will be a link on the website to the list of primers). The mouse genomic DNA was used as a template for PCR to amplify pri-miRs. Predicted 431 pri-miRNA-PCR products were amplified and resolved on an agarose gel, stained with ethidium bromide and photographed (See Figure 2). The mouse genomic DNA was used as a template for PCR, for the amplification of pri-miRs. Predicted pri-miRNA- PCR products, ranging from 200 bps to 1500 bps, were resolved on the agarose gel, stained with ethidium bromide, and photographed (See Figure 2).

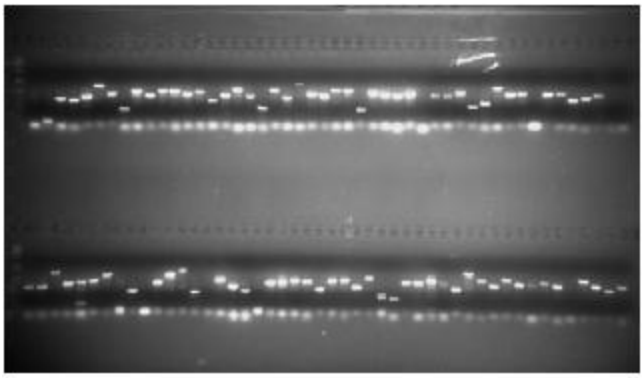


Figure 2. The representative photographic image of 98 PCR-products of primary miRNAs amplified using mouse genomic DNA and primers corresponding to the 5' and 3' ends of predicted pri-miRNA sequences.

3) Production of distinct libraries of pColTtG targeting vectors harboring miRNAs:

a. Production of the library of targeting vectors carrying miR-30-based miRNAs

The PCR products (110 bps) of miR-30-based miRNAs were amplified using oligonucleotides from Agilant chips, digested with XhoI and EcoRI, and ligated into the same sites within pColTtGM vector. Correct clones were identified by sample sequencing of the pool. Forty five hundred colonies on the plates were sequence analyzed at the Cold Spring Harbor Lab Sequencing Facility. Sequencing results showed that 375 miRNAs out of a total of 680 miRNAs (55%) were successfully cloned into targeting vector, pColTtGM. The miniprep-DNAs were stored in 96-well plates. Individual targeting vectors would be re-prepped by Qiagen Maxi prep (Qiagen, CA) and sequence verified again prior to transfection into ES cells.

b. ***Production of library of targeting vectors expressing Pri-miRs***

Due to various sizes and different availability of cloning sites of each PCR products, PCR products were pooled by three sets of sizes (200-500, 500-1000, and 100-1500 bps) and four sets of cloning sites (Xho or BclI as a 5 site, and EcoRI or MluI as a 3' site).

4) Production of ES cell lines harboring miRNAs and validation by flow cytometry and RT-qPCR:

Established FRT mediated gene targeting protocols were used (2) with modifications (Figure 3). Briefly described, FLPe recombinase (2.5 µg) and Flp-in targeting vectors (5 µg) were transfected into KH2 cells using a nucleofector 96-well shuttle system (Lonza, Germany). Expression of *FLPe* mediates recombination between frt sites at the *ColA1* locus in KH2 cells

and those in the targeting vector. Since correct integration of targeting vector at *ColA1* locus confers hyromycin resistance and neomycin sensitivity, positive clones were selected with hygromycin (140 µg/ml). Four different single clones per miRNAs were picked about 11-13 days post-electroporation. Correct single-site integration was confirmed by Southern Blotting (results not shown). Cells were then plated into 24-well plates and grown to confluence. They were then expanded to 6-cm plates and used to make frozen stocks. A subpopulation of these cells was re-plated onto a 24-well plate and used for flow cytometric analysis and RNA purification. Since KH2 cells express rtTA (reverse tet-transactivator), transgene expression can be induced in the ES cells by addition of doxycycline and assessed by flow cytometric analysis and RT-qPCR respectively. Forty-eight hours post-doxycycline treatment, cells were either lysed into Trizol solution for RNA purification, or trypsinized to measure GFP levels using Guava flow cytometer (Millipore, CA). RT-qPCRs were performed using Taqman-microRNA assay kit (Applied Biosystems, ID # 001141).

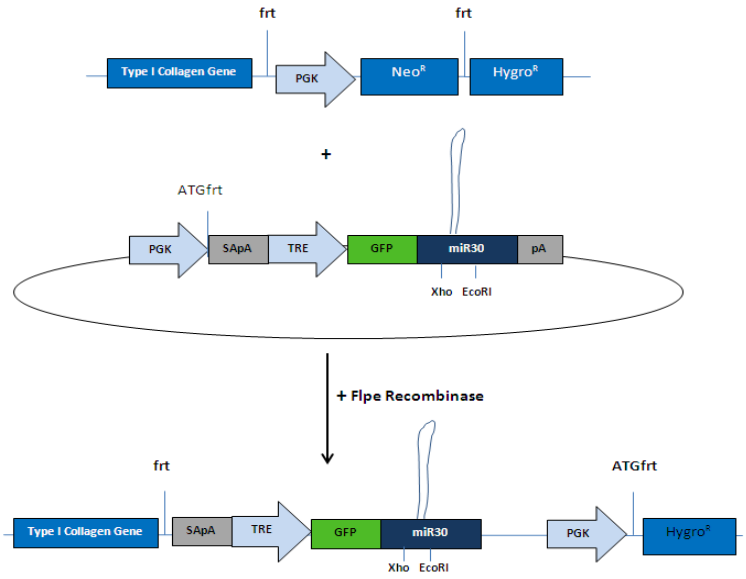


Figure 3. (a) Strategy for Flpe-mediated recombination at the *ColA1* locus in KH2 mES cells. Previously engineered KH2 cells contain, at independent loci, *Rosa26*-M2rtTA and the "FRT homing cassette" consisting of *PGK*-Neo^R and a nonfunctional hygromycin resistance cassette with no ATG site or promoter Co-electroporation of the ColFLp-TGM and pCAGGs-Flpe Recombinase vectors promotes inter- and intra-chromosomal recombination at the *ColA1* locus downstream of the Type I Collagen gene, resulting in excision of *PGK*-Neo^R and integration of the ColFLp-TGM. Correct integration restores hygromycin resistance.

Appendix 4

ES Cell Culture and Maintenance

The following list of supplies and reagents are needed:

Item	Vendor	Catalog No.
KNOCKOUT DMEM	Invitrogen	10829-018
0.25% Trypsin-EDTA	Invitrogen	25200-056
β-mercaptoethanol	Sigma	M7154
FBS (ES cell qualified)	Stem Cell Technologies	6952
Gelatin	Millipore	G-2500
L-Glutamine, 200mM	Invitrogen	25030-081
LIF(ESGRO)	Millipore	ESG1107
Penicillin G	ICN	194537
Streptomycin sulfate	Invitrogen	11860-038
D-PBS	Invitrogen	10010-023

Media Preparation

M15 Growth Media:

500 ml of media KNOCKOUT DMEM
90 ml of ES Cell-Tested FBS
6 ml of ***GPS***
6 ml ***BME***
60 µl LIF

GPS solution (Glutamine/Penicillin/Streptomycin)

Weigh 500 mg of Penicillin
Weigh 300 mg of Streptomycin
Dissolve the powders to 100 ml of 100 X Glutamine solution
Filter through 0.2 UM filter unit
Aliquot them into 6 ml each
Place them in -20˚ C freezer

100 X β-mercaptoethanol(BME)

Add 75 µl of BME to the 500 ml of PBS
Filter BME-PBS and aliquot 50 ml each

MEF Feeder Media:

500 ml Knock out DMEM
60 ml ES cell-tested FBS
6 ml ***GPS***

Freezing Media (10% DMSO):

6 ml **M15** (see above)
1 ml DMSO
3 ml ES cell-tested FBS
May be kept at 4 ℃ for up to one week

Preparation of feeder plates

- Add the appropriate volume of 0.1% gelatin solution to the plates and leave at room temperature for 30-60 minutes (See below: Table 1)
- Aspirate the gelatin solution
- Immediately, add appropriate volume and concentration of mitotically inactive MEF feeder cells (treated with mitomycin C, or irradiated) to the plates (See below: Table 1)

Table 1.

Size of TC Plate	Volume of Gelatin	No. of Feeder Cells per plate	Volume of Media per Plate
24-Well	0.5 ml	$1x10^5$	1.0ml
6-Well	1.5ml	$5x10^5$	3.0ml
60mm	2.0ml	$7x10^5$	5.0ml
100mm	3.0ml	$1x10^6$	10.0ml

Thawing the vial

Each vial contains a minimum of $2x10^6$ ES cells and may be thawed on a 60mm feeder plate

- Thaw the vial very quickly in a water bath at 37 ˚ C (1-2 minutes)
- Wipe the outside of the vial with 70% ethanol
- Immediately transfer the content into a 15 ml centrifuge tube containing 9 ml warm culture media (M15)
- Spin cells at 1000 rpm for 5 minutes using a table-top centrifuge
- Aspirate the supernatant and re-suspend the cells in 5 ml of warm culture media (M15)
- Transfer the entire volume to a 60 mm feeder plate, and incubate at 37 ˚ C, 5% CO_2
- Feed daily until 70%-80% confluent

Note: ***ES cells should be passaged every 2-3 days and media should be changed prior to turning yellow***

Passaging ES cells

ES cells may be split 1:3 to 1:10

- Aspirate the media
- Rinse the cells with D-PBS
- Add 0.6 ml of 0.25% trypsin to the 60 mm plate and incubate for 5 min (37 ˚ C, 5% CO_2)
- Add 4 ml of warm media (M15) and pipet up and down 3-4 times to separate cells
- Transfer cells to a 15 ml centrifuge tube and spin at 1000 rpm for 5 minutes
- Aspirate the supernatant and re-suspend the cells in 5 ml of warm media (M15)
- Determine cell number (using a counting chamber: Hemocytometer, makler chamber, microcell,…)
- Seed ES cells as follow:

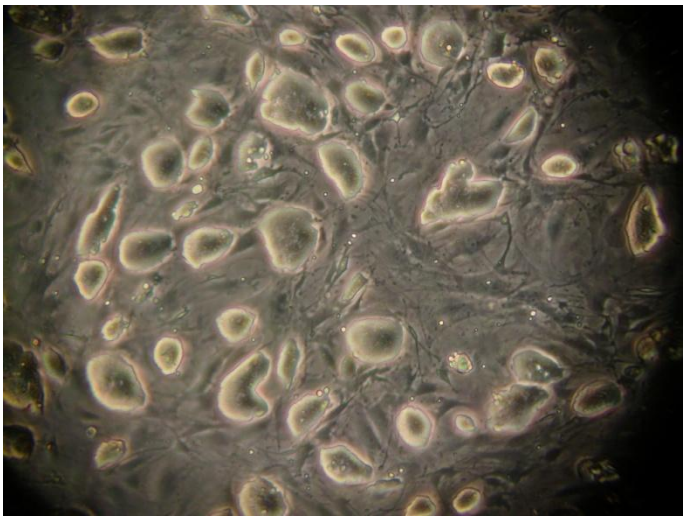
  $2.0x10^6$ in a 60 mm feeder plate or $5.0x10^6$ in a 100 mm feeder plate

Preparing ES cells for microinjection

- Aspirate the media from a semi-confluent 60 mm plate
- Rinse the cells with D-PBS
- Add 0.6 ml of 0.25% trypsin to the 60 mm plate and incubate for 5 min (37 ˚ C, 5% CO2)
- Add 4 ml of warm media (M15) and pipet up and down 3-4 times to separate cells
- Transfer cells to a 15 ml centrifuge tube and spin at 1000 rpm for 5 minutes
- Aspirate the supernatant and re-suspend the pellet in 5 ml of warm media (M15), leave on ice
- ES cells may be kept on ice until microinjection is complete (2-4 hours)

ES cell culture after 24 hours, at approximately 40% confluence:

Note: ES cell colonies with bright edges and smooth three-dimensional appearance



Freezing ES cells

- Aspirate the media from a semi-confluent 60 mm plate (when cells are approximately 80% confluent)
- Rinse the cells with D-PBS
- Add 0.6 ml of 0.25% trypsin to the 60 mm plate and incubate for 5 min (37 ˚ C, 5% CO2)
- Add 4 ml of warm media (M15) and pipet up and down 3-4 times to separate cells
- Transfer cells to a 15ml centrifuge tube and spin at 1000 rpm for 5 minutes
- Aspirate the supernatant and re-suspend the cells in appropriate volume of ice-cold freezing media (generally 1ml of freezing media per 60 mm plate)
- Aliquot 1/3 of total volume into each pre-labeled cryovial (0.33 ml per vial)
- Place the cryovials into an isopropanol freezing container (also called blue-frosty box) and immediately transfer the container to a -80 ˚C freezer (keep at -80 ˚C overnight)
- The next day, transfer the cryovials to Liquid nitrogen tank for long term storage

Appendix 5

References

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