Generation of a murine ES cell system deficient in microRNA processing for the identification of microRNA targets

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. No part of this thesis has been submitted for any other qualification.

This thesis is within the 300-page limit laid down by the Biology Degree Committee.

Matthew P Davis

Abstract

MicroRNAs (miRNAs) are 21-22nt RNA molecules that regulate mRNAs, generally by triggering their degradation or blocking their translation. This effect is mediated via direct binding of the miRNA to its mRNA target at sites of partial complementarity. The number of miRNAs annotated in miRBase has grown rapidly in the last decade. There are now 695 human miRNAs and 488 mouse miRNAs. The significance of miRNA mediated post-transcriptional regulation has led to rapid advances in our understanding of miRNA expression, biogenesis and functional mechanism. However, with miRNAs predicted to regulate up to 60% of the human genome, there is a necessity for the development of methods to identify miRNA target sets on a large scale. It is increasingly evident that miRNAs can be functional components of large regulatory networks. The complexity of these associations is compounded by the ability of multiple miRNAs to regulate the same target mRNA simultaneously. It is also understood that miRNAs with a high degree of sequence similarity at their 5' end may be functionally redundant; this makes the analysis of target associations more challenging.

To address these problems I have developed a system in mouse embryonic stem (ES) cells to simply and rapidly derive gene lists enriched for miRNA targets. DGCR8 is a doublestranded RNA binding protein essential for the first cleavage of miRNA primary transcripts in the canonical miRNA processing pathway and is required for the maturation of these miRNAs. I have disrupted miRNA processing by the targeted insertion of a gene trap cassette into the second allele of *Dgcr8* in cell lines that already bear a gene trap within their first allele. This led to a broad reduction of miRNA processing in these cells and a depletion of mature miRNAs. As a

consequence of the disruption of this locus I was able to identify a number of miRNAs that appear to be processed in DGCR8 independent manner.

I proceeded to transfect these cells with ES-cell-expressed miRNA mimics. I used microarrays to identify transcripts that are down regulated as a consequence of the miRNA reintroduction. By comparing transcripts that had been up regulated upon the depletion of *Dgcr8* to this set I was able to create miRNA target lists for mmu-miR-25 and mmu-miR-291a-3p. These lists should be enriched for functionally relevant, co-expressed targets, moderated for miRNA mimic over expression and to a large extent devoid of interference from target saturation and combinatorial regulation. The system should also not be susceptible to problems associated with functional redundancy. In total I identified 25 target candidates for miR-291a-3p and 40 candidates for miR-25. Amongst these genes are a number of oncogenes and tumour suppressor genes in addition to genes involved in cell cycle regulation and extra-cellular signal transduction.

In conclusion it appears that miRNAs play a fundamental role in the regulation of the ES cell transcriptome and as such are deserving of considerable future research. It is my belief that the method presented in this thesis could contribute significantly to this effort by providing substantial and experimentally derived miRNA candidate target lists upon which to base future hypotheses.

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Abbreviations

ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BLAST	Basic Logical Alignment Search Tool
BMP	Bone morphogenic protein
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP-Sequencing
CRI	Cambridge Research Institute
DAS	Distributed annotation system
DDW	Double distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's PBS
dsRNA	Double stranded RNA
EB	Embryoid body
EDTA	Ethylene-diamine-tetra-acetic acid
EGFP	Enhanced green fluorescent protein
ES cell	Embryonic stem cell
EST	Expressed sequence tag
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
GFP	Green fluorescent protein
GO	Gene ontology
GSK3	Glycogen synthase Kinase 3
IGTC	International Gene Trap Consortium
Indels	Insertion deletions
iPS cell	Induced pluripotent stem cell
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
IQR	Inter-quartile range
IRES	Internal ribosome entry site

kb	Kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria-Bertani
LFC	Log fold change
LIF	Leukemia inhibitory factor
LNA	Locked Nucleic Acid
MAPK	Mitogen-activated protein kinase
MEFs	Mouse embryonic fibroblasts
mRNA	Messenger RNA
MFI	Median fluorescence intensity
miRNP	microRNA-associated ribonucleoprotein complex
miRNAs	microRNAs
ncRNAs	non-coding RNAs
nt	Nucleotides
ORF	Open reading frame
P-body	Processing body
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PITA	Probability of interaction by target accessibility
pol	RNA polymerase
polyA	polyadenylation
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
pSILAC	Pulsed stable isotope labeling with amino acids in cell culture
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RA	Retinoic acid
RACE	Rapid amplification of complementary DNA ends
RNA	Ribonucleic Acid
RNAi	RNA interference
RNP	Ribonucleoprotein complex

RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPE	Streptavidin R-phycoerythrin
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
SSC	Saline sodium citrate
ssRNA	Singe stranded RNA
TBE	Tris-Borate EDTA
TF	Transcription Factor
TK promoter	Thymidine kinase promoter
T_m	Melting temperature
tRNA	Transfer RNA
UTR	Untranslated region