How to measure miRNA expression
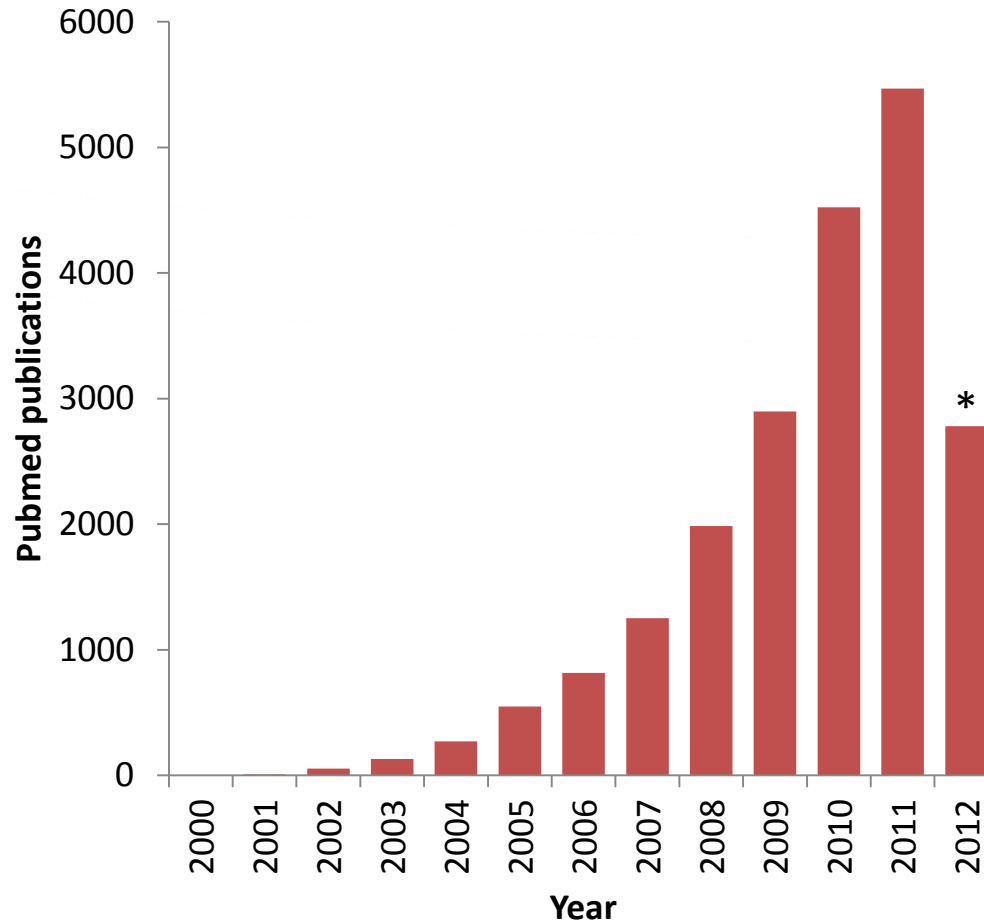
Matt Barter

Institute of Cellular Medicine

Expansion of microRNA research

'Micro-managers of gene expression'

Talk plan

1. microRNAs

- Biogenesis
- Action
- Targetting

2. microRNA function

- Role of microRNAs in homeostasis, cell processes and disease
- Serum biomarkers
- Therapeutic microRNA inhibition

3. Measuring microRNA expression

- Extraction
- Assays and profiling
- Analysis

4. Experimental follow up

1. What are microRNAs?

MicroRNAs

- ~22nt long post-transcriptional regulators of gene expression

- Small, single stranded non-coding RNAs

- Evolutionary ancient component of genetic regulation

- Current estimates ~1000 in humans
  - miRBase – repository of sequence and nomenclature

- microRNA families – similar sequence

- Clusters and multiple copies in genome
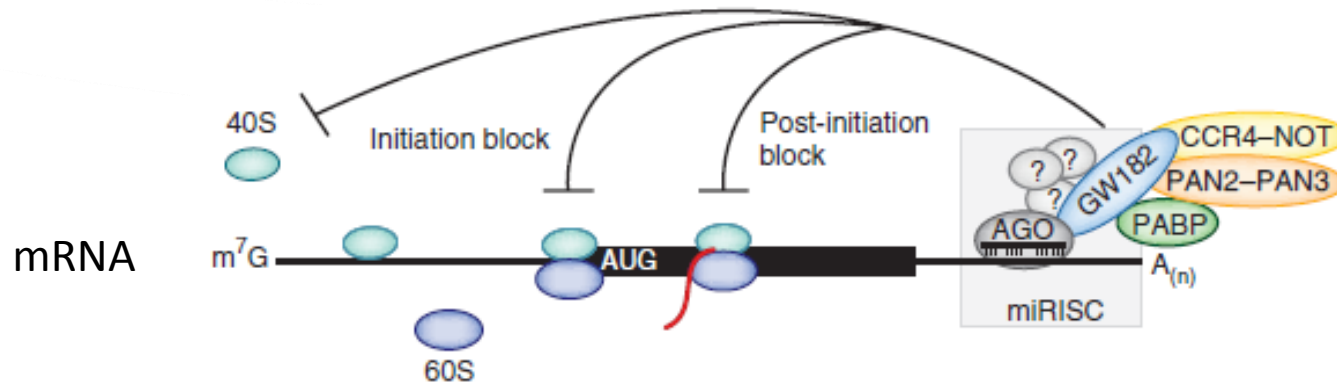
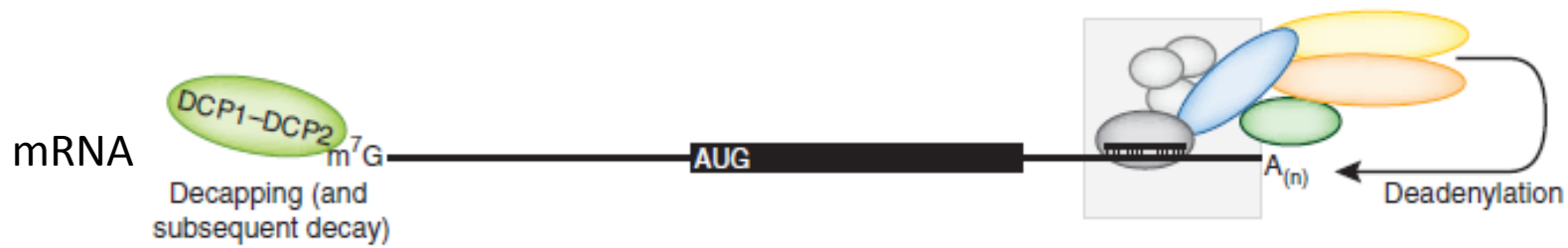- Own promoter or intronic

microRNA processing
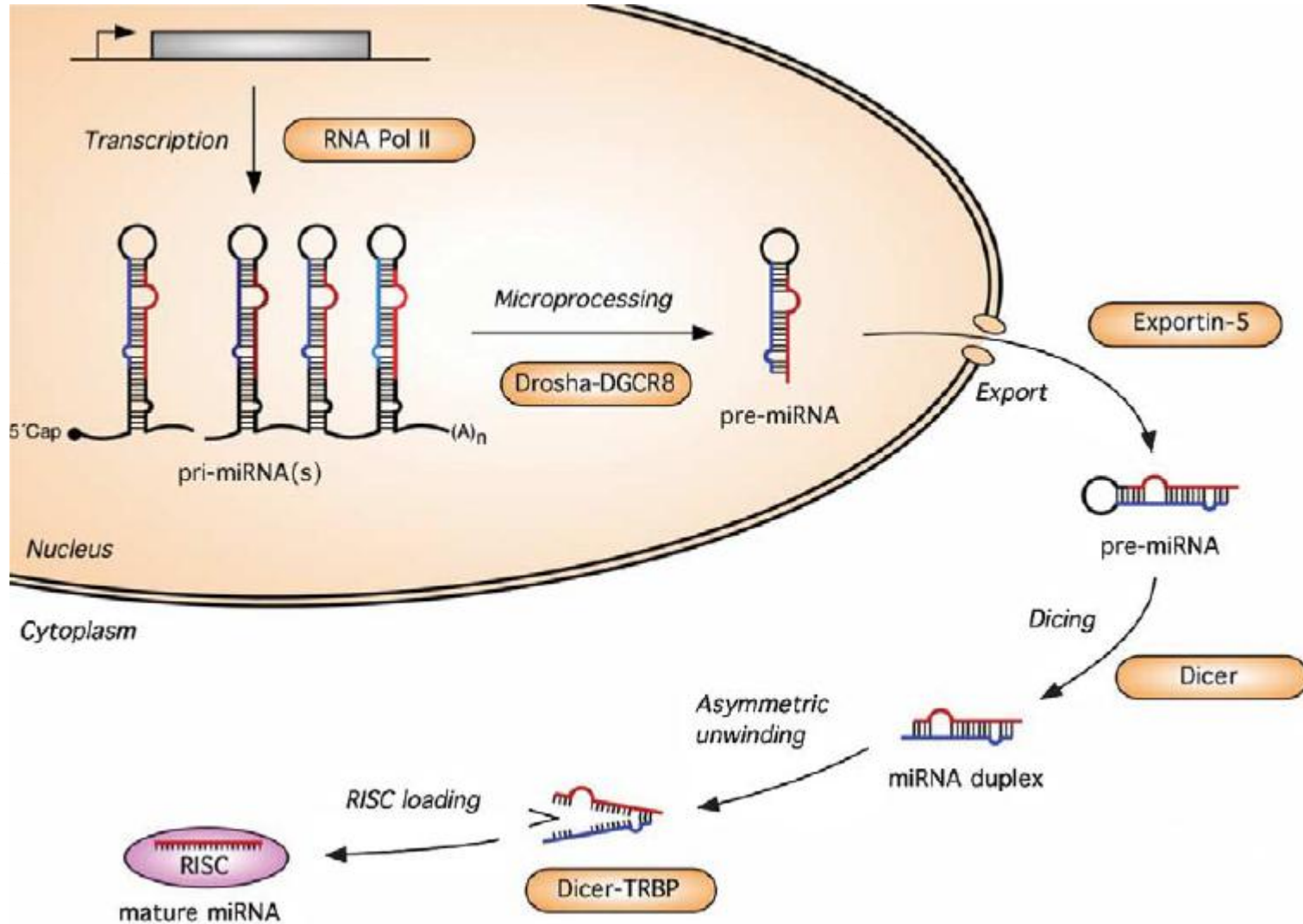
What do microRNAs do?

miRNAs within RISC inhibit translation and cause mRNA degradation

miRNA-mediated translational repression
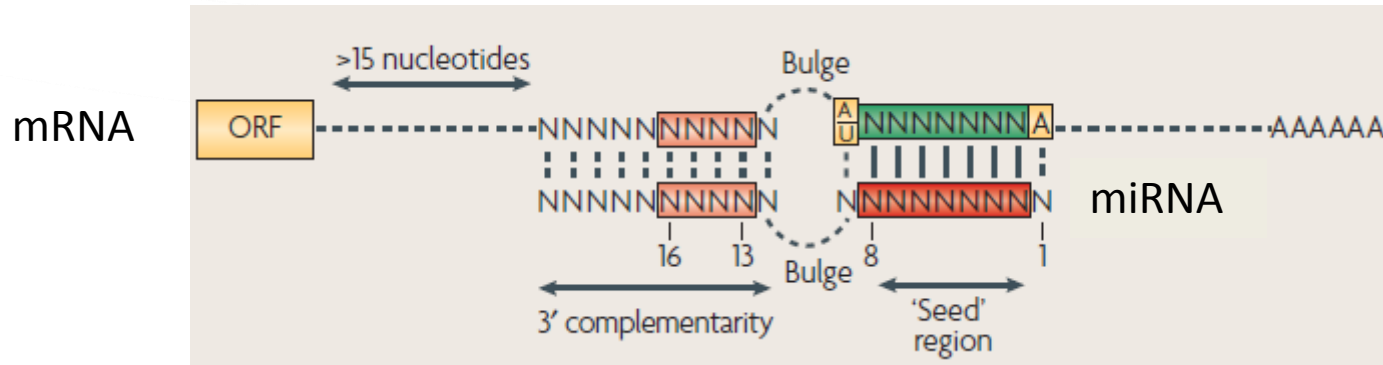
miRNA-mediated mRNA decay

How microRNAs target

Bind to complementary sequences on target mRNA transcripts

MicroRNA seed region
- Bases 2-7

- Imperfect base heteroduplexes

• Predicted to target several hundred genes each

• ~60% mRNAs have at least 1 miRNA binding site in UTR
- many have far more

5'UTR and coding region targetting miRNAs

Balance between miRNAs and targets

A miRNA will cause little more than a 2-fold repression in mRNA targets

Many binding sites for multiple miRNAs

- redundancy and cooperation between miRNAs

Conventional RNA logic

5´ UTR   CDS   3´ UTR

AAA

microRNA

ceRNA logic

AAA
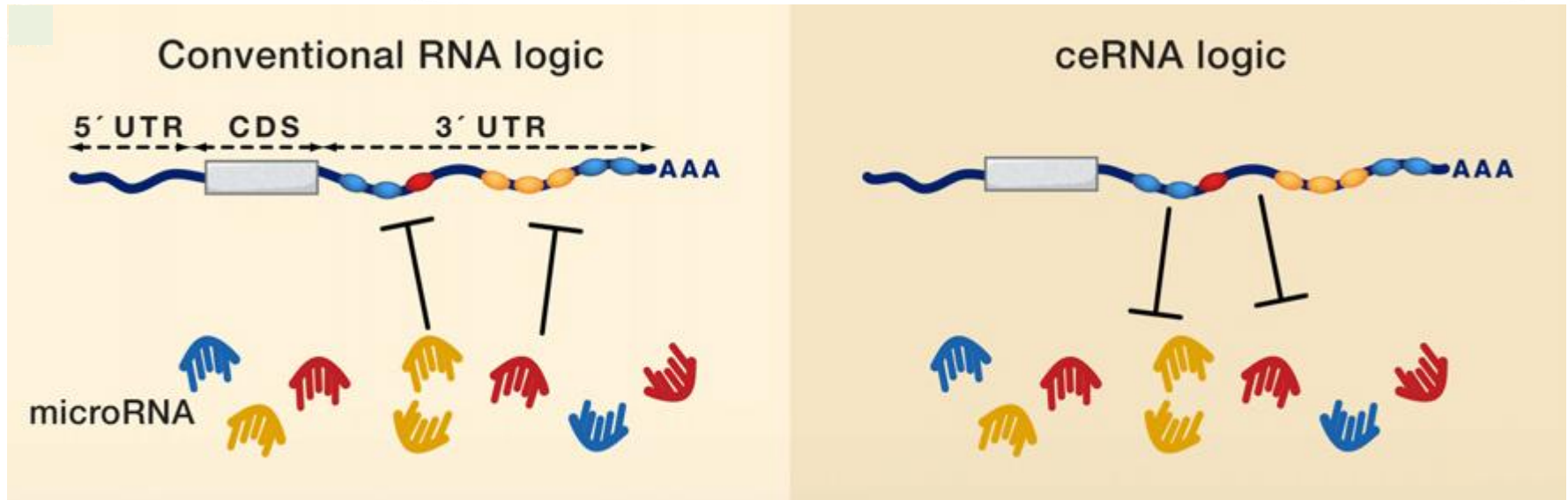
Adapted from Salmena 2011 Cell

Different sets of expressed miRNAs in different cell types and tissues

Competing endogenous RNA logic

- abundance of mRNA targets will influence miRNA regulation
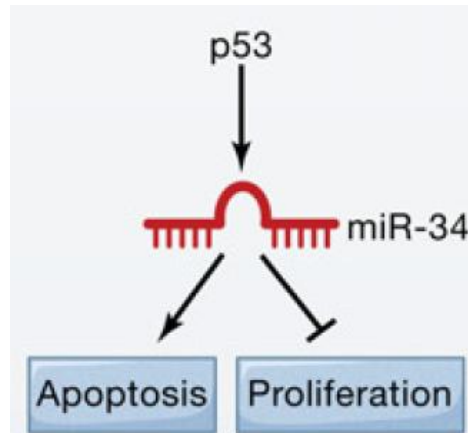concept of sponges, pseudogenes etc.
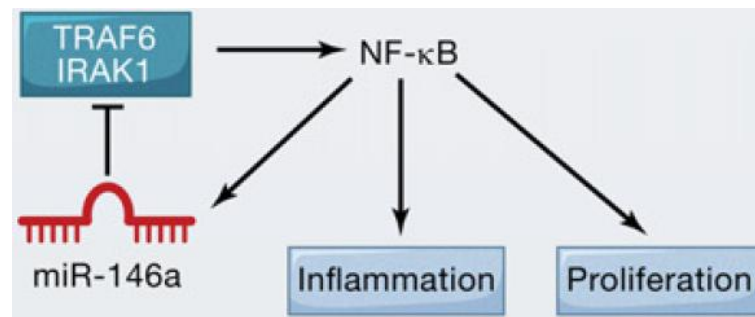
Baseline – fine tune gene expression

Many miRNA knockout mice do not exhibit overt developmental phenotype

- Exhibit abnormal responses to various stress conditions
  - enhance/diminish organisms response to stress
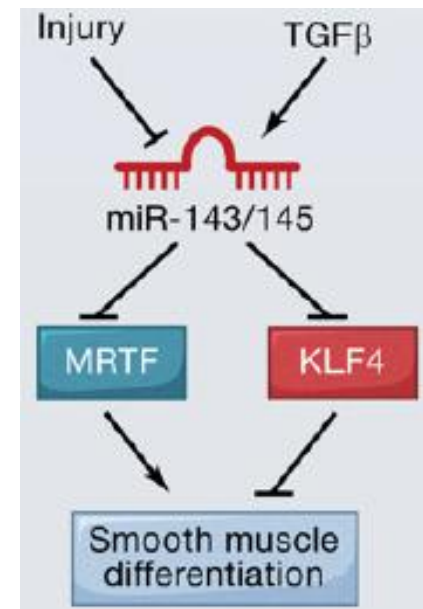 - impact on pathological process

Stress signal mediation/modulation

Negative/positive feedback: signal resolution/phentoype switching

Buffering: signal stability

E.g. microRNAs and disease

In human cancer, miRNA expression profiles differ between normal tissues and the tumours that are derived from them, and differ between tumour types

- Deletion or downregulation oncogene-targetting miRNAs in cancer

- Upregulation of 'oncomiRs' – miR-17-92 polycistron derived miRNAs increased in B cell lymphomas (He Nature 2005)

Polymorphisms in mRNA 3'UTR target sites

- miR-24 site SNP in dihydrofolate reductase gene leads to methotrexate resistance (Mishra PNAS 2007)

- miR-189 site SNP in SLITRK1 associates with Tourette's syndrome (Abelson Science 2005)

 - alternatively polymorphisms may also impact on the biogenesis and miRNA machinery

E.g. miRNA and inherited disease

miR-17-92 polycistron

Example of miRNA mutation responsible for a developmental defect in humans
(de Pontual Nat Genet. 2011)

Hemizygous deletion –
Short stature and digital abnormalities

Serum biomarkers
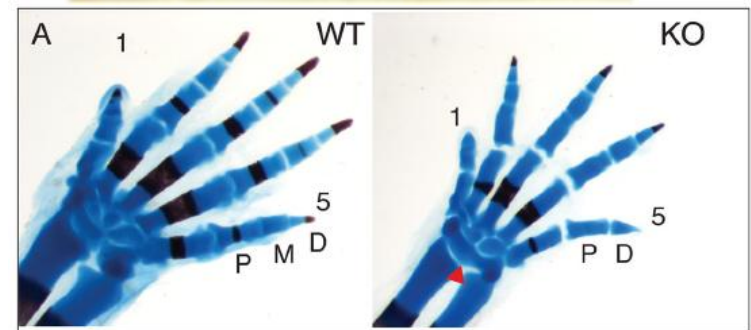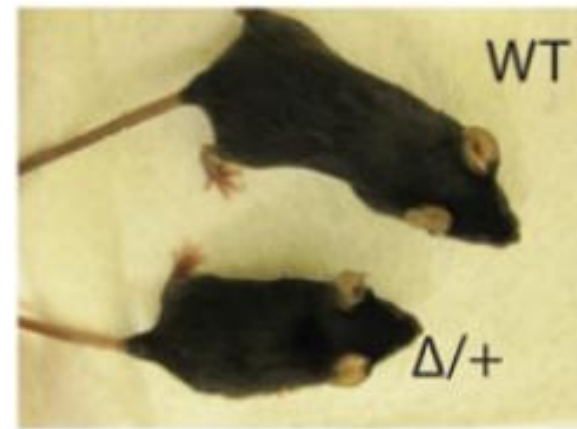
miRNAs present in clinical samples of plasma and serum in a remarkably stable form

Strong correlation between miRNA levels in plasma and serum

Correlation between miRNAs in plasma with disease
E.g. cancer serum profiles

Therapeutic microRNA inhibition

antimiRs

Reduce endogenous level of miRNA

- cell permeable, not rapidly excreted, stable in vivo, bind miRNA with high specificity and affinity

    - chemical modifications of oligonucleotides - 2'-O-methyl and LNA

- systemic delivery (dissolved in saline) (IV or SC), reduced level in multiple tissues, extended period of inhibition (weeks)

E.g. dose-dependent lowering of plasma cholesterol in African green monkeys by depletion of mature miR-122 with LNA-antimiR (Elmen Nature 2008)

3. Measuring miRNA expression

Measuring microRNA expression

Applications

Mechanisms of gene regulation

- Identify miRNAs regulating range of cell processes
- Study gene regulation in combination with transcriptome data
- miRNA-mRNA/protein interactions
- Novel miRNAs

Disease mechanisms and biomarkers

miRNAs well preserved in range of specimen types

- Tissue – miRNA profiles of normal and affected tissue – mechanism/biomarkers
- Diagnostic identification of cancers of unknown origin
- Circulating biomarkers – non-invasive biomarkers of disease
- Forensic analysis to distinguish body fluids

Extraction of microRNAs from cells and tissues

miRNA isolation initially same as mRNA isolation

- Chemical extraction - guanidium thiocyanate

Later modification to retain/enrich small RNA fraction

- Solid-phase extraction on silica columns
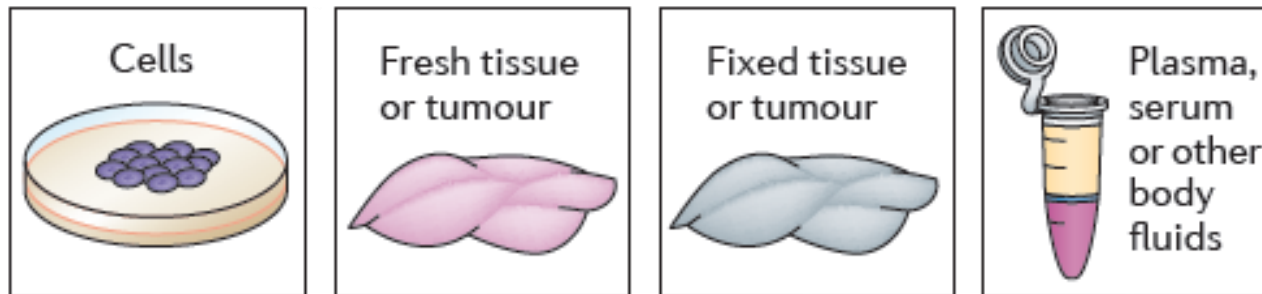
| Yields: | >1ug | >1ug | >1-100ng | <1-10ng |

Most assay platforms distinguish miRNAs from more abundant mRNAs

- enrichment for small RNAs possible

Plasma
- high RNase content
- variables such as centrifugation, white blood cells and red blood cell haemolysis
- miRNAs in vesicles (exosomes/microvesicles) or argonaute-protein complexes

Quality control

Important for reproducibility and accuracy

Routinely assess quality of total RNA with Bioanalyzer (Agilent) or Experion (Bio-Rad) – RIN >7 (RNA integrity score)

miRNA specific challenges
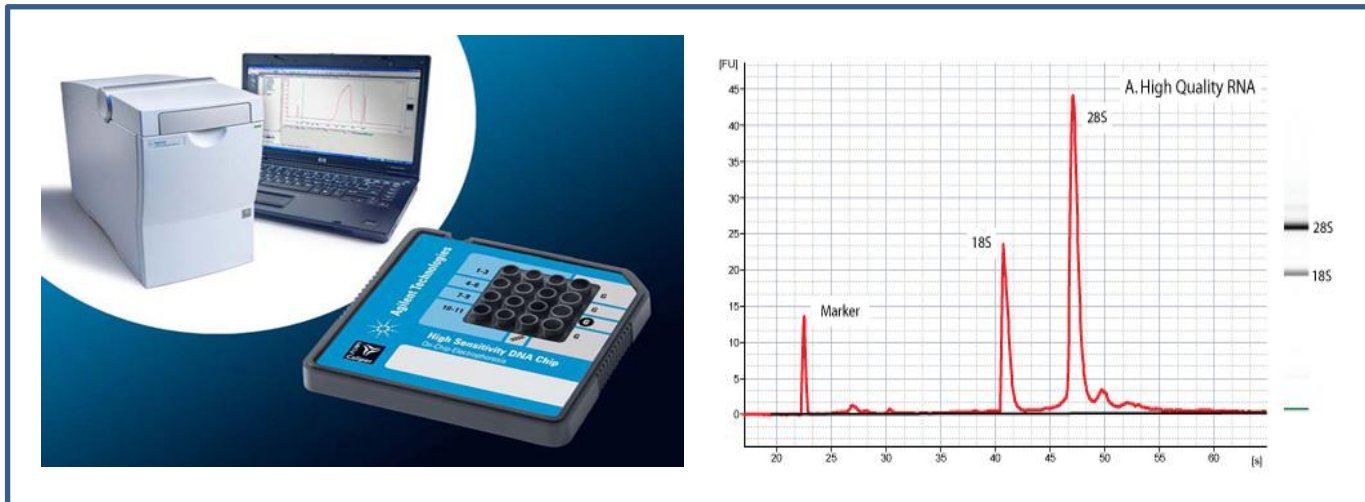
- Short length

  - primer annealing in reverse transcription and PCR

  - variable GC content impacts annealing reactions in arrays

- No poly(A) tail

  - for enrichment

  - for universal primer binding site for reverse transcription

    - important as only 0.01%
    - pre- and pri- which contain the same sequence

- miRNAs within a family can differ by a single nucleotide
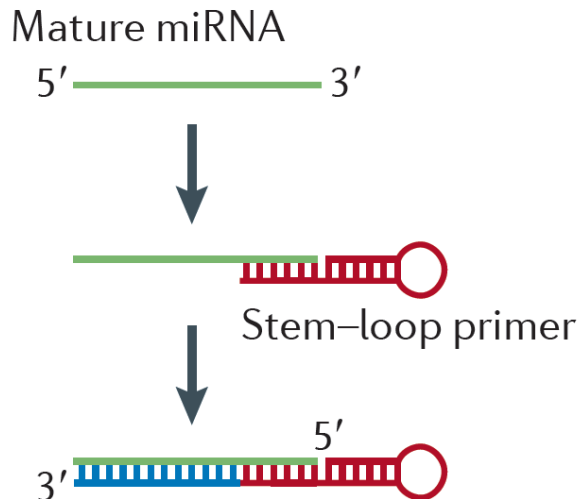
- Sequence length variability – isomiRs

Platforms

1. Real-time qPCR
2. Microarray
3. RNA sequencing

4. Northern blot
5. In situ hybridization

Real-time qPCR

Real-time monitoring of reaction product accumulation by quantitative PCR

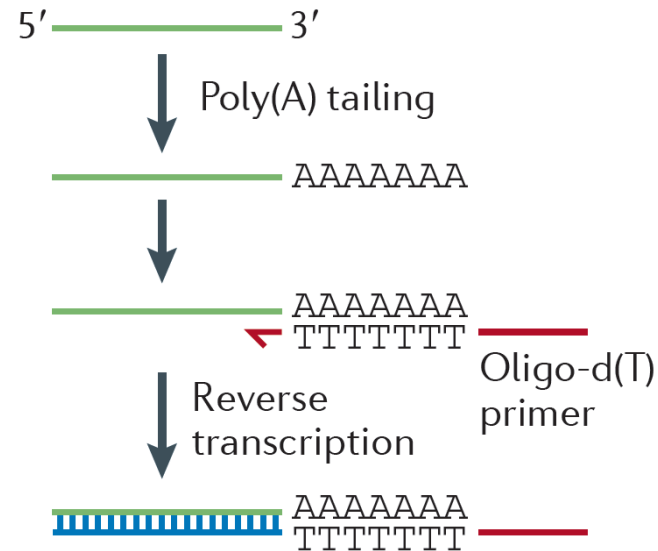- Sensitive and specific
- Absolute quantification possible

Reverse transcription of miRNA to cDNA - 2 approaches:

1. miRNA-specific – stem-loop primer

Mature miRNA

5′ —————— 3′

↓

————— Stem–loop primer

↓

3′ —————— 5′

2. Universal – poly(A) tailing

5′ —————— 3′

↓ Poly(A) tailing

————— AAAAAAA

↓

————— AAAAAAA
TTTTTTT Oligo-d(T) primer

↓ Reverse transcription

————— AAAAAAA
TTTTTTT

Real-time qPCR

Candidate miRNA approach
- pre-designed assays or
- universal RT with specific primer sets

miRNA profiling approach
Parallel high-throughput measurement 100s miRNAs
- pre-plated PCR primers - multi-well plates or microfluidic cards
- customisable content - specific miRNA-set analysis
- comprehensive coverage

Can be used to measure pri- and pre-miRNAs

Annealing temperature variation overcome by commercial vendors
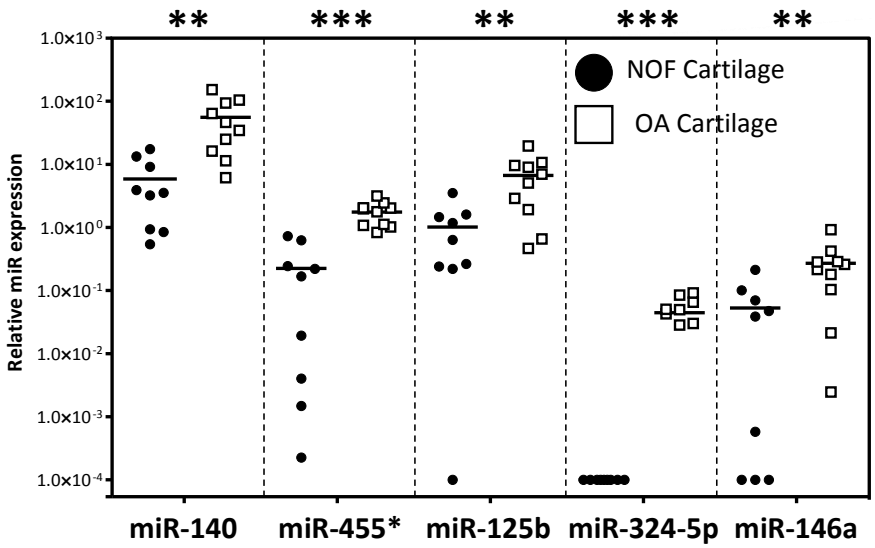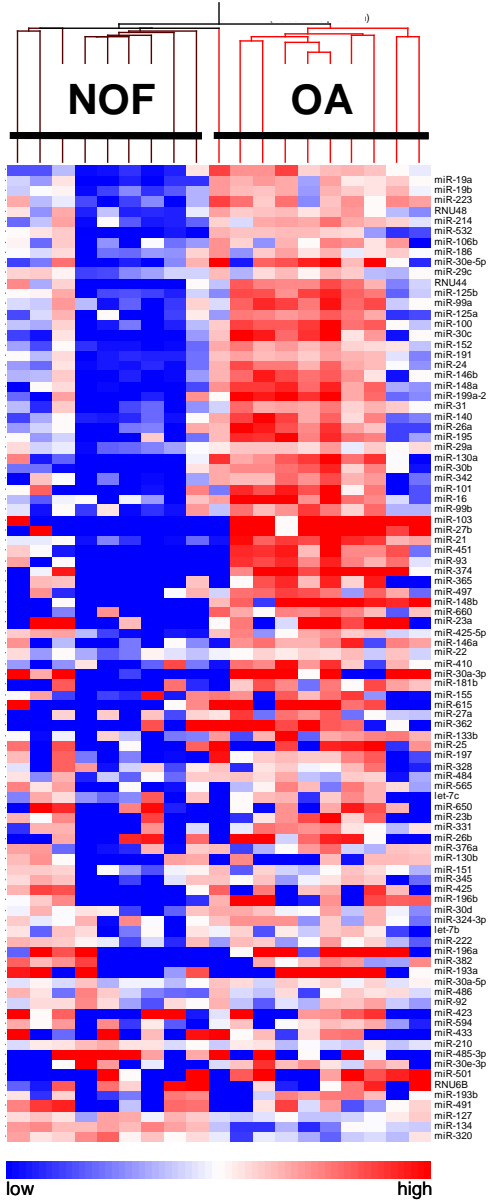- LNA incorporation to standardise annealing temps

Low input RNA quantity (1ng-1ug)

Cost $$

Limitations: specificity, contamination

E.g.

Work performed by Hannah Elliott

Microarray

Parallel analysis large number defined miRNAs - ~1000 human miRNAs

- Tool to survey expression and dysregulation in tissue

1. Fluorescent labelling of miRNA
2. Hybridization to DNA probes on arrays

3. Washing and scanning of array
4. Data extraction and processing

miRNA enrichment may increase sensitivity – small RNAs =0.01% all RNAs
Probe design impt as miRNAs short therefore hybridization temps vary – modifications e.g. LNA incorporation to standardise

E.g. Screen miRNA levels in plasma from patients vs. healthy controls

Microarray

Medium input quantity (200ng – 1ug)

Cost – $

Limitations

- Restricted linear range of quantification and no absolute quantification

- Also lack of specificity for miRNAs with closely related sequences

    - Nanostring nCounter may overcome this

- Microarrays may differ in miRbase content of miRNAs and probe modifications causing differences in results across array platforms – like mRNA arrays

- Requires confirmation by other detection methods

E.g.
miRNA expression
during MSC
chondrogenesis

RNA sequencing

Find novel miRNAs and expression profiles in samples

1. Enrichment of small RNAs
2. miRNA conversion to cDNA library
3. Followed by 'massively parallel' sequencing

e.g. Illumina

e.g. Roche, ABI

Measures relative quantification

Precise identification of miRNA sequences

Unlimited - identify known and novel miRNAs

- criteria: length, genomic precursor origin, hairpin (-3p and -5p), species conservation

RNA sequencing

Limitations

Higher input quantity (2-10ug)

Expression analysis in infancy therefore significant bioinformatic challenge

Sequence specific bias in cDNA library preparation – overcome recently

Cost $$$
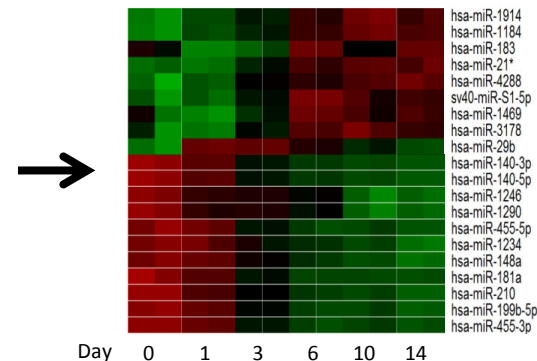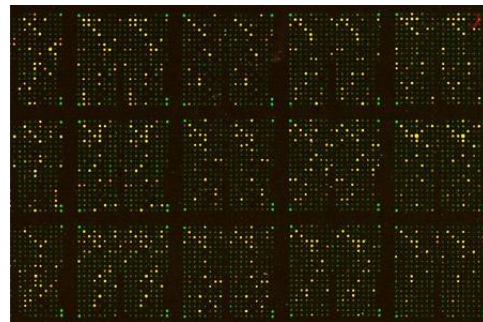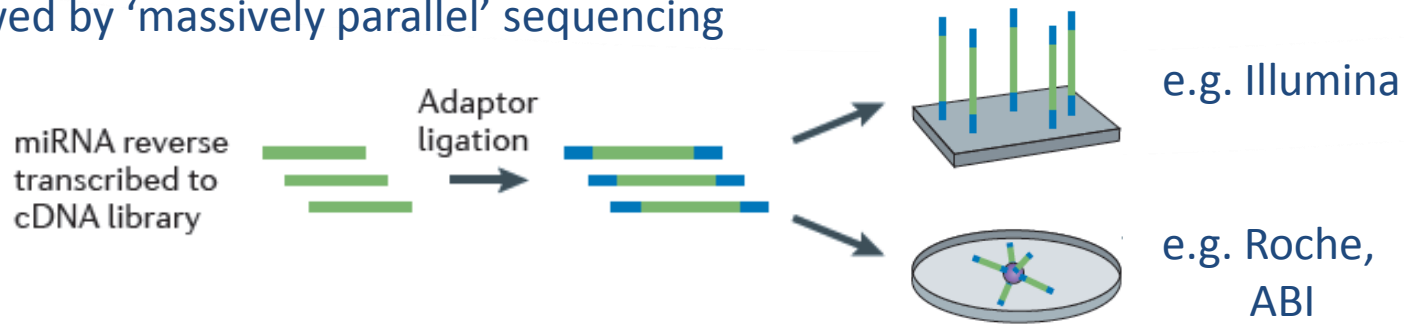
In situ hybridisation

Address 'when' and 'where' a miRNA is expressed

Long probes for pri-miRNAs

Mature miRNAs - small size - technically challenging

LNA-based probes

- high binding affinity for short RNAs
- sensitive and specific detection of miRNAs
- wide range of sample sources

Yamashita JBC 2010

Exiqon

Northern blot
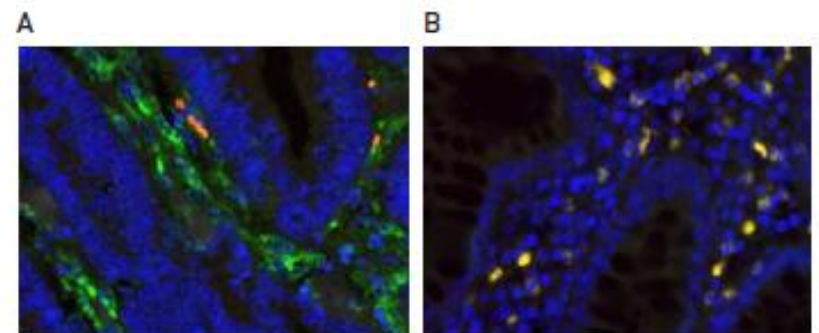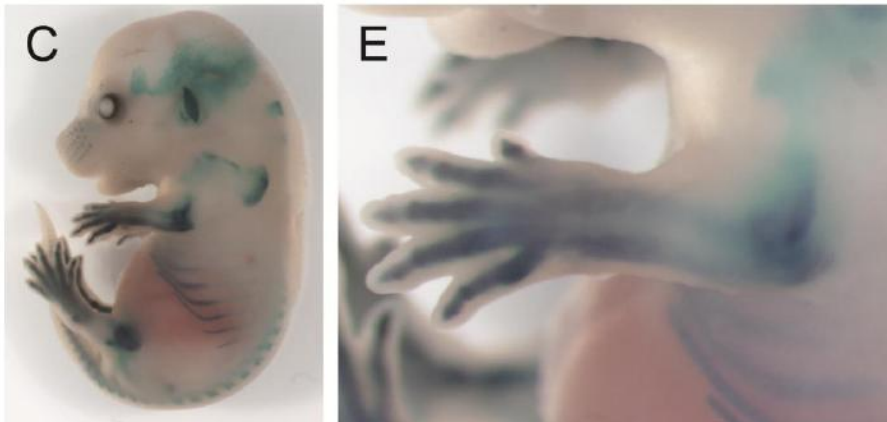
Widely used to visualise specific microRNAs

Measure pri- and pre-miRNAs
 - variations in processing in disease

1. Fractionation by high % gel electrophoresis
2. Transfer to membrane
3. Fixing and probing with miRNA specific probe
4. Radioactive/non-radioactive visualisation

Time consuming and requires large input RNA

Hwang PNAS 2008

Data analysis
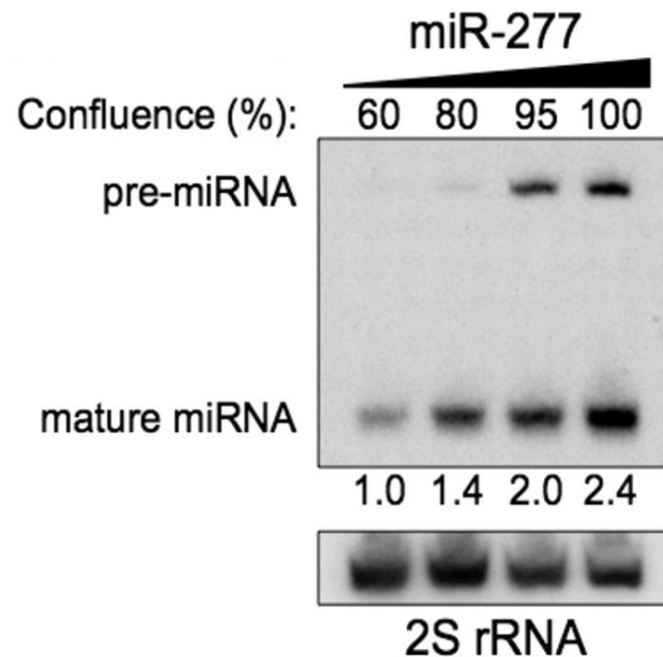
Data processing

- e.g.ROX dye normalisation for qPCR

- e.g. different scanning settings for arrays

- e.g. different software for sequencing alignments

Data quality assessment

- internal controls distributed across plates/arrays for qPCR/microarrays

- reference standards across plates to avoid batch effects

- samples mixed

- data-handling checks and recording for traceability

Data analysis

Data normalisation – to identify relevant biological differences

qPCR data – endogenous control snRNAs or invariant miRNAs - accounts for RNA input and quality variations
- limited by assumption of control non-variation and identification of controls

high-throughput data - normalisation to majority of miRNAs which remain invariable = global mean miRNA expression normaliser
- spiked in synthetic controls

sequencing normalisation – in infancy, problem due to lack of independence
i.e. limited sequence reads, examine overall frequency distribution of reads

Differential expression calculation
- between groups comparison – fold differences and statistical significance
- qPCR best dynamic range and accuracy and absolute quantification
- array best for discovery and cost but needs follow up confirmation by qPCR
- RNA-seq. caveat for highly variable samples

Follow up – target identification

Function defined by gene targets

Bioinformatic prediction

- online programs: e.g. Targetscan, miRanda
- algorithms based on criteria:
    seed region complementarity, conservation, thermal stability, secondary structure, position in 3'UTR, multiple target sites

***In vitro* UTR analysis** – clone 3'UTR of target into reporter

- overexpress/inhibit miRNA and mutate target site
- in cell type of interest to assess whether regulation actually occurs

Transcriptome/proteomic analysis

- majority of protein level changes are due to mRNA level changes
- mRNA/protein analysis following miRNA regulation

Biochemical assays

- argonaute pull down assays to identify miRNA-target mRNAs
- HITS-CLIP (high throughput sequencing to crosslinking immunoprecipitation)

Examine phenotypic changes in culture/organism in response to miRNA regulation

In vitro manipulation

- transfection miRNA mimics and inhibitors (antimiRs)
- 'soaking up' - sponge/decoys vectors containing many target sites

Genetic manipulation

- *in vivo* transgenesis or genetic deletion
- care needed for intronic miRNAs and polycistronic miRNAs

Therapeutic inhibition in vivo

Summary

- **microRNAs critical regulators of disease**

- **A number of platforms with many commercial vendors to profile expression of miRNAs in a variety of specimen types**

- **Well defined experimental procedures to understand microRNA function**

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