TimiRGeN: R/Bioconductor package for time series microRNA-mRNA integration and analysis

Patel K\textsuperscript{1}, Chandrasegaran S\textsuperscript{1} Clark IM\textsuperscript{2}, Proctor CJ\textsuperscript{1}, Young DA\textsuperscript{3}, Shanley DP\textsuperscript{*} \textsuperscript{1}

\begin{enumerate}
\item Campus for Ageing and Vitality, Biosciences Institute, Newcastle University, Newcastle upon-Tyne, NE4 5PL, UK.
\item School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK.
\item Life Science Centre, Biosciences Institute, Newcastle University, Newcastle upon-Tyne, NE1 4EP, UK.
\end{enumerate}

*To whom correspondence should be addressed.

Abstract

Motivation: The analysis of longitudinal datasets and construction of gene regulatory networks provide a valuable means to disentangle the complexity of microRNA-mRNA interactions. However, there are no computational tools that can integrate, conduct functional analysis and generate detailed networks from longitudinal microRNA-mRNA datasets.

Results: We present TimiRGeN, an R package that uses time point based differential expression results to identify miRNA-mRNA interactions influencing signalling pathways of interest. miRNA-mRNA interactions can be visualised in R or exported to PathVisio or Cytoscape. The output can be used for hypothesis generation and directing in vitro or further in silico work such as gene regulatory network construction.

Availability and implementation: TimiRGeN is available for download on Bioconductor (https://bioconductor.org/packages/TimiRGeN) and requires R v4.0.2 or newer and BiocManager v3.12 or newer.

Contact: k.patel5@ncl.ac.uk, daryl.shanley@ncl.ac.uk

Supplementary information: Supplementary data is available at Bioinformatics online.

1 Introduction

microRNAs (miRNAs) are single-stranded functional RNAs, around 16-22 nucleotides long which target specific miRNAs for degradation or translational repression; thus affecting protein levels (Selbach et al., 2008). Targeting is achieved by complementary binding between the 3'UTR of the target mRNA and a 7-8 nucleotide sequence found on the 5'UTR of the miRNA, known as the seed sequence (Bartel., 2004). There is increased clinical interest in miRNAs for several reasons: 1) miRNAs can be tested in animal models to understand human diseases and conditions. An example is miR-140-5p which is up-regulated during chondrogenesis and down-regulated during osteoarthritis (Bartet et al., 2015; Miyaki et al., 2010). 2) miRNAs can be secreted via exosomes into surrounding blood, extracellular matrix and urine (Leidinger et al., 2013; Chaturvedi et al., 2015; Chen et al., 2017). Their presence in body fluids provides valuable non-invasive biomarkers to assess the state of difficult to access tissues such as tumours, brain and bone. 3) Lastly, miRNAs have potential as therapeutic agents as they modulate expression of specific mRNAs (Schwarzenbach et al., 2014).

However, in the laboratory, miRNAs are difficult to study, primarily because a single miRNA can regulate many mRNAs and a single mRNA can be regulated by multiple miRNAs. miRNA-mRNA interactome studies report over 18,000 interactions in HEK293 cells and over 34,000 interactions in human hepatoma cells (Helwak et al., 2013; Moore et al., 2015). A complementary strategy is to use a computational approach. The analysis of longitudinal miRNA-mRNA expression data, construction of Gene Regulatory Networks (GRNs) and subsequent dynamic modelling, is a particularly useful means to gain a better understanding of miRNA-mRNA interactions (Qin et al., 2015; Proctor et al., 2017; Oots et al., 2018). GRNs are useful tools for integrating multi-omic data on mechanistic schematics. Yet, currently there is no computational tool that can handle longitudinal miRNA-mRNA datasets and reduce the volume of data to an extent where GRN construction is possible. This is presented in Table 1.
TimiRGeN offers two functional analysis methods: time dependent or fixed. TimiRGeN can be exported to Cytoscape for further analysis (Camps et al., 2020). miARMa-seq package: DAVID was used for pairwise DE and further analysis (Eisen et al., 2003). miRNet package thereby provides a much-needed function from PathVisio., 2019; Diaz et al., 2018). The miARMa-seq package as well as the miRNet package are capable of handling time series datasets. Several can perform functional analysis, usually utilising GO, Kegg, React (Reactive). DAVID or other tools can reduce the volume of data. Also shown is when each tool was last updated.

| Tool name | Availability | Time | Func analysis | Reduction | Updated |
|-----------|--------------|------|---------------|-----------|---------|
| miARMa-seq | Online | × | × | GO,Kegg | × | 2012 |
| miARMa-seq | Online | × | × | DAVID | × | 2012 |
| miARMa-seq | Online | × | × | GO,Kegg | × | 2019 |
| m RNAseq | SF | × | × | GO,Kegg | × | 2020 |
| m RNAseq | SF | × | × | GO | × | 2009 |
| SpidermR | SF | × | × | GO | × | 2021 |
| ToppMIIR | Online | × | × | GO | × | 2021 |

Many existing tools (Table 1) have particular strengths, but none satisfy the criteria necessary to bridge longitudinal multi-omic data and GRN creation. mi ARMa-seq, miRTarVis+, MAGIA2, Sigermis and SpidermR have substantial miRNA-mRNA integration capabilities but cannot handle longitudinal datasets (Wang et al., 2019; Diaz et al., 2017; Bisognin et al., 2012; Creighton et al., 2008; Carav et al., 2017). Web-based tools such as miNet, miTarVes+ and ToppMIIR have excellent visualisation capabilities but also cannot analyse longitudinal datasets (Fan and Xia, 2014; Li et al., 2017; Wu et al., 2014). DREM2 and miARMa-seq handle longitudinal datasets, but do not reduce the volume of data enough for GRN generation (Schulze et al., 2012; Andres et al., 2016). miRNAHC can use longitudinal data to generate miRNA-mRNA interactions networks, but the networks lack detail on upstream or downstream information, making the output insufficient for GRN generation (Vila-Casadesus et al., 2016). Furthermore, several tools have not been actively maintained so their usability may be diminished.

There is clearly a need for a tool that can integrate, functionally analyse and generate detailed networks from longitudinal miRNA-mRNA datasets, which can then be used to identify GRNs. Here, we present the R Biocgenerate package TimiRGeN, which uses differential expression (DE) data as input to generate small miRNA-mRNA interaction networks. Results from TimiRGeN can be exported to Cytoscape or PathVisio for further bioinformatic analysis (Smoot et al., 2011; Kouton et al., 2015). The TimiRGeN package thereby provides a much-needed means to generate hypotheses from longitudinal multi-omic datasets. To demonstrate the capabilities of the package several datasets were analysed (see methods), including a comprehensive RNAseq time series miRNA-mRNA folic acid (FA) induced mouse kidney injury dataset (Fig. 1) (Crausn et al., 2016; Pellegrini et al., 2016).

| Tool name | Availability | Time | Func analysis | Reduction | Updated |
|-----------|--------------|------|---------------|-----------|---------|
| miARMa-seq | SF | × | × | GO,Kegg | × | 2020 |
| miARMa-seq | SF | × | × | GO | × | 2009 |
| SpidermR | SF | × | × | GO | × | 2021 |
| ToppMIIR | Online | × | × | GO | × | 2021 |

3 Results

3.1 Time point and microRNA specific analysis

Pairwise mRNA and microRNA DE data (Log2FC and adjusted P values) from each time point can be used as input for TimiRGeN. The tool works on RNAseq and microray data, and it has two modes of analysis. The combined mode analyses mRNA and microRNA data from the same time point together, and here each gene from a time point can be filtered for significance independent of all other time points. The separate mode analyses mRNA and microRNA data independent of each other. Separate mode analysis allows for a microRNA or mRNA from a time point to be filtered for significance independent of all other time points and gene types (miRNA or mRNA). TimiRGeN uses WikiPathways for functional analysis, and most are curated by either entrez gene IDs or ensemble gene IDs so TimiRGeN provides both for the user. Neither of these annotation types can distinguish between -3p or -5p miRNAs, thus TimiRGeN also provides adjusted IDs, in case a miRNA-mRNA interaction network is created with both the -3p and -5p versions of a miRNA.

3.2 Filtering data with time based functional analysis

TimiRGeN offers two functional analysis methods: time dependent pathway enrichment and temporal pathway clustering analysis. Both use the WikiPathways package an API for the WikiPathways database to find signalling pathways of interest (Slenter et al., 2018).
Fig. 1. Pipeline of the TimiRGeN R package: The FA miRNA-mRNA data are input and filtered for significantly expressed genes for each time point. From here, one of two methods can be used to find WikiPathways of interest. A) time dependent pathway enrichment to find enriched pathways at each time point. The enriched pathways are ranked in descending order of adjusted P values on bar plots. Results from day 1 and day 14 are shown. Or B) temporal clustering where global trends of the pathways over time are clustered. Two clusters are shown here. Each line is a pathway and the colour represents how well a pathway fits into a cluster. Ranking from highest to lowest are: red, orange, yellow. miRNA-mRNA interactions within a selected signalling pathway can be predicted by filtration of miRNA-mRNA pairs using databases and correlation. C) Filtered miRNA-mRNA pairs can be viewed in R. Nodes are pink for miRNAs or blue for mRNAs and edges are colour coded by correlation over time. D) Behaviour of genes within the miRNA-mRNA interaction network can be viewed across the time course and genes which pass a threshold (≥ 1.5 in this example) are highlighted. E) The genes can also be hierarchically clustered to identify trends. F) Expression changes within the clusters can be plotted. These line plots include a grey line (data points) and a red line (smooth spline). G) A selected miRNA-mRNA pair (mmu-miR-181c-5p and Plau) can be analysed using cross-correlation analysis. H) The selected mRNA (red) and miRNA (blue) can also be displayed over the time course. The data is scaled and interpolated over a spline and the correlation is displayed. I) Regression analysis can be performed on a selected miRNA or mRNA. Plau was selected, so its expression over time is predicted based on the chosen miRNAs that target it. In this example mmu-miR-181c-5p is selected to predict the behaviour of Plau. Expression values of Plau are displayed as red dots and the predicted expression of Plau is displayed as a dashed blue line. R² and Pvalue are shown. J) Regression analysis can also be performed between a miRNA-mRNA pair. The OR (odds-ratio) between the two time series can be calculated, along with the 95% CI (confidence intervals). Correlation, R², Pvalue, OR and CI are rounded to 2 decimal places. Network data can be exported to PathVisio or Cytoscape.

Fig. 2. miRNAs influencing anti-fibrosis factor Tnfa and pro-fibrosis factor Igf1: This GRN shows how FA may be down-regulating let-7c-5p, let-7e-5p, let-7g-5p, miR-18a-5p, miR-26b-5p, miR-29a-3p, miR-29c-3p, miR-365-3p and miR-98-5p, which are all predicted to target pro-fibrosis factor Tgfb. Reduction of Tnfa will increasing levels of pro-fibrosis factor Tgfb.
3.2.1 Time dependent pathway enrichment method
Overrepresentation analysis from clusterProfiler is applied to time series data (Yu et al., 2012). Hypergeometric tests are performed to contrast the number of genes found in common between each time point (after filtering for significantly differentially expressed genes) and each species specific WikiPathway. This produces a list of enriched pathways for each time point (Fig.1A). Alternatively, if the separate mode of analysis is applied, enrichment analysis is performed for each time point per gene type. The background/universe used to perform overrepresentation analysis can be adjusted by the user e.g. probes in a microarray or all known genes within a cell type.

3.2.2 Temporal pathway clustering method
Temporal pathway clustering (Fig.1H) utilises Mfuzz (Kumar et al., 2007). Supervised soft clusters are created based on temporal patterns which stem from the number of genes found in each time point (after filtering for significance) and each species specific WikiPathway. This will show global trends within the dataset. Pathways are assigned fitness scores for each cluster, from 0-1, and these can be filtered to find highly correlating pathways in clusters of interest. If the separate mode is used, temporal pathway clustering is performed for each gene type individually.

3.3 Filter miRNA-mRNA interactions from a signalling pathway of interest
After a signalling pathway has been selected for further analysis, the TimiRGeN pipeline will extract each mRNA that is found in common between the selected pathway and the input mRNA data. Each of these mRNAs are assumed to be potential targets of every miRNA in the input data. This results in a miRNA-mRNA interaction matrix which can be used to filter out miRNA-miRNA interactions that are not likely to occur by using correlations and miRNA-mRNA interaction databases TargetScan, miRDB and miRtarBase (Agarwal et al., 2015; Chen et al., 2020; Huang et al., 2020). Correlations are calculated for changes over time (Log2FC or average expression) between a given miRNA and a given mRNA. The default method is Pearson, but users can also select between Spearman or Kendall. Since miRNAs negatively regulate mRNAs, highly negative correlation values from miRNA-mRNA pairs could be used to identify miRNA-mRNA interactions that are likely to regulate the selected pathway. Users can define a correlation threshold to filter for miRNA-mRNA interactions. The default setting for maximum correlation is -0.5. Three miRNA target databases are also usable to filter for miRNA-mRNA interactions. This includes two predictive target databases (TargetScan and miRDB) and one functional database (miRtarBase) that has had all functional support labelled as ‘weak’ removed. Predictive databases TargetScan and miRDB were selected because, although they have differences in their prediction methods, they share usage of 3’UTR-seed site complementarity and seed site conservation to predict miRNA-mRNA interactions (Peterson et al., 2014). Comparisons between different miRNA-mRNA prediction methods find that 3’UTR-seed site complementarity identify the most true positive miRNA-mRNA interactions (Mazière et al., 2007; Zhang and Verbeek., 2010). Interactions found or not found in the three databases will be represented as 1 or 0 respectively. Users have the option to choose which combination of databases they wish to mine information from and they can choose the number of databases which an interaction needs to be mined from to be filtered. The default setting for the minimum number of databases needed to filter a miRNA-mRNA interaction is 1. Once correlation and databases have been used to filter for miRNA-mRNA interactions which may be affecting the signalling pathway of interest, they can be displayed in an internal R network (Fig 1C). Resulting genes found in the miRNA-mRNA interaction network can be viewed over the time course. Here genes that pass a user defined threshold can be highlighted (Fig.1D). The genes can also be sorted into hierarchical clusters shown by a dendrogram, from which clusters can be plotted to show the behaviour of the genes (Fig 1E-F). A heatmap which is compatible with the dendrogram can also be generated (S Fig.1).

3.4 Longitudinal miRNA-mRNA pair analysis
The TimiRGeN R package has a suite of longitudinal analysis approaches for analysing predicted miRNA-mRNA interacting pairs. This includes several correlation and regression based methods which are commonly used to analyse longitudinal datasets (Ding and Bar-Joseph., 2007). Cross-correlation analysis is a useful method to determine similarity between two time series (Fig 1G). If the time series is of sufficient length, the metric can be used to identify delays and further filter for miRNA-mRNA interacting pairs with interesting dynamics (Jung et al., 2011; Lakshmipathy et al., 2007). miRNA-mRNA pairs can also be plotted in a time series line plot. This plot can be scaled and interpolated over a spline (Fig 1H). Two types of regression analysis can also be performed. Firstly, a linear model is generated from a selected gene (miRNA or mRNA) and any number of its predicted binding partners. The combination of miRNA-mRNA interactions are left for the user to define. The longitudinal behaviour of the selected gene is predicted based on the binding partners used in the linear model. The predicted simulation and the gene data are plotted along with the R2 value and Pvalue (Fig 1I). This type of regression prediction is useful in cases where a miRNA is targeted by multiple miRNAs or if a miRNA targets multiple miRNAs. Next, a linear model can be created from a single miRNA-mRNA pair. The odds-ratio is calculated from the regression coefficient. This measures the likelihood of one gene influencing the behaviour of another gene and has previously been used as a metric to determine miRNA-mRNA relationships (Jayawal et al., 2009). 95% confidence intervals are calculated which give a range where there is a 95% certainty of the mean of the data being within the range (Fig 1J) (Szramlas., 2010). Selecting a miRNA-mRNA pair to investigate can be made easier by plotting a heatmap which orders the interacting pairs by descending correlation (S Fig 2). Statistics generated from correlation and regression analyses may be overestimations if too few time points are found within the input data. Thus the tool will generate an error if fewer than three time points are detected and warnings if fewer than five time points are detected.

3.5 Output of the TimiRGeN package and exportation of data from R
TimiRGeN is an open-ended tool that exports to networking software PathVisio and Cytoscape for further in silico analysis. The TimiRGeN R package produces two data files for upload onto PathVisio. A file which includes a single result type, e.g. Log2FC, from each time point and gene IDs. This can be uploaded into PathVisio to show how the genes in a signalling pathway of interest change over the time course. Also a file which contains all filtered miRNAs can be uploaded into PathVisio. The second file requires the user to install the MAPPBuilder app in PathVisio (Kamorn et al., 2015). With this, changes over time in a miRNA integrated signalling network of interest can be visualised to show how the miRNAs may be influencing the signalling pathway. This type of display is ideal for bottom-up GRN construction (S Fig 3). Filtered miRNA-mRNA interactions can also be exported to Cytoscape for improved visualisation and alternative analysis via Cytoscape apps (Smoot et al., 2013). The enhanced graphics of Cytoscape are especially useful to visualise large numbers of miRNA-mRNA interactions (S Fig 4).
3.6 Data from non pairwise DE
The FA kidney injury dataset had pairwise DE performed using the zero time point as the denominator. This type of pairwise analysis is recommended for time series datasets with <8 time points, however longer time series datasets may be more suitable for DE without using the pairwise approach e.g. over a cubic spline, muSigPro or the LRT method with DESeq2 (Conesa et al., 2006; Spies et al., 2019). In these cases, users are recommended to filter out significantly differentially expressed genes from averaged count or expression data, and to use this as input for TimiRGeN. Pathway enrichment can be used to identify the most enriched pathways from the whole time course or temporal clustering can first cluster genes based on temporal behaviour. From here, genes can be sorted based on clusters, and then pathway enrichment can be used to identify enriched pathways from each temporal cluster. An alternative pipeline is shown in Fig.5 and this is explained in section 5 of the vignette.

3.7 Datasets with multiple interventions
More complex datasets may include interventions other than time. In these cases, TimiRGeN should be used for each individual time series and then the results can be contrasted between different interventions. This requires the same signalling pathway to be explored in each time point. As an example, the "Lung fibrosis" pathway was analysed in the FA and UUO datasets. A pipeline is shown in S Fig.6 and section 6 of the vignette provides detail.

3.8 Hypothesis generation with TimiRGeN
To demonstrate the tools ability to generate biologically relevant hypotheses, the FA mouse kidney injury dataset was analysed with TimiRGeN (Fig.1). Findings from the analysis were used to hypothesise how FA can induce fibrosis. A GRN was constructed to formalise the hypotheses (Fig.2). Investigation of these results can be used to ratify the miRNA-mRNA interactions predicted by TimiRGeN and make a stronger case for experimental validation. FA injection is known to cause acute injury conditions in the kidneys, resulting in a reversible chronic kidney disease (CKD) like condition (Craciu et al., 2016; Pellegrini et al., 2016). During the 14 day time course, a number of different processes occur, such as inflammatory response, scar tissue forming, wound healing, cytokine activity (Leask and Abraham., 2004). TimiRGeN analysis highlights several of these processes and GRNs were generated to represent how miRNAs may be influencing fibrosis factors (Fig.2) and scar tissue forming by collagen synthesis (S Fig.7-S Fig.10). The GRN presented in Fig.2 indicates that IGF1 acts as a miRNA sponge. Many of the presented miRNA-Igf1 interactions have been reported, including miR-18a, miR-98, miR-365 and miR-26b (Liu et al., 2017; Hu et al., 2013; Sun et al., 2019; Liu et al., 2016). let-7e-5p has been reported to target Igf1, and TimiRGeN predicted other let-7 family genes let-7e-5p and let-7g-5p also target IGF1 (Liu et al., 2018). Finally, miR29 family members are predicted to target IGF1, and research indicates that IGF1 is a miR-29 family sponge (Gao et al., 2016). It is unknown why IGF1 may be a miRNA sponge, but IGF1 is known to induce collagen production, which contributes to kidney fibrosis and CKD (Hung et al., 2013). Exploration of IGF1 as a miRNA sponge in kidney injury conditions could be beneficial for therapeutics for CKD. Overall, this case study highlights that the TimiRGeN R package can be used to identify biologically relevant miRNA-mRNA interactions from potentially tens-of-thousands of possible miRNA-mRNA interactions. The ability to reduce the volume of big multi-omic data is an important feature of TimiRGeN and one which could lead to making miRNA research easier and faster for users. Further analysis on a breast cancer dataset is also found in the supplementary data (S Fig.11-S Fig.16).

4 Conclusion
As recognised in Bar-Jones et al. (2012), generation of complex transcriptomic datasets will continue, so computational biologists will need more sophisticated and up-to-date software to analyse these datasets (Bar-Joseph et al., 2012). We have presented a novel R/Bioconductor package which aims to help researchers find direction when working with large longitudinal multi-omic datasets. Overall, TimiRGeN is a useful new tool which could become a part of miRNA-mRNA data analysis pipelines.

Supplementary data
Supplementary data contains additional work. 1) Extra figures not shown in Fig.1. 2) Alternative pipelines for non pairwise DE analysis and multivariate datasets. 3) Alternative analysis of the FA kidney injury dataset. 4) A complete workflow for a breast cancer study. Including identification of a suitable dataset, processing and performing analysis with TimiRGeN to build a GRN which identifies miRNAs that influence TGF-beta driven tumour fibrosis. 5) Links to TimiRGeN R scripts for reproducibility, vignette and a download link are also found in this file.

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Conflicts of interest
None.

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