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Construction of therapeutically relevant human prostate epithelial fate map by utilising miRNA and mRNA microarray expression data

Jayant K Rane^{1,7}, Antti Ylipää^{2,3}, Rachel Adamson¹, Vincent M Mann^{4,5}, Matthew S Simms^{4,5}, Anne T Collins¹, Tapio Visakorpi⁶, Matti Nykter^{2,3} and Norman J Maitland^{*,1,5}

¹Department of Biology, YCR Cancer Research Unit, University of York, York YO10 5DD, North Yorkshire, UK; ²Prostate Cancer Research Center, Institute of Biosciences and Medical Technology (BioMediTech), University of Tampere and Tampere University Hospital, Biokatu 8, Tampere 33520, Finland; ³Department of Signal Processing, Tampere University of Technology, Korkeakoulunkatu 10, Tampere 33720, Finland; ⁴Hull York Medical School, University of Hull, Hull HU6 7RX, East Yorkshire, UK; ⁵Department of Urology, Castle Hill Hospital, Cottingham HU16 5JQ, East Yorkshire, UK and ⁶Molecular Biology of Prostate Cancer Group, Institute of Biosciences and Medical Technology (BioMediTech), University of Tampere and Tampere University Hospital, BioMediTech, Biokatu 8, Tampere 33520, Finland

Background: Objective identification of key miRNAs from transcriptomic data is difficult owing to the inherent inconsistencies within miRNA target-prediction algorithms and the promiscuous nature of miRNA-mRNA target relationship.

Methods: An integrated database of miRNAs and their 'relevant' mRNA targets was generated from validated miRNA and mRNA microarray data sets generated from patient-derived prostate epithelial normal and cancer stem-like cells (SCs) and committed basal (CB) cells. The effect of miR-542-5p inhibition was studied to provide proof-of-principle for database utility.

Results: Integration of miRNA-mRNA databases showed that signalling pathways and processes can be regulated by a single or relatively few miRNAs, for example, DNA repair/Notch pathway by miR-542-5p, $P=0.008$. Inhibition of miR-542-5p in CB cells (thereby achieving miR-542-5p expression levels similar to SCs) promoted efficient DNA repair and activated expression of Notch reporters, HES1 and Survivin, without inducing dedifferentiation into SCs.

Conclusions: Our novel framework impartially identifies therapeutically relevant miRNA candidates from transcriptomic data sets.

There are <2000 known human miRNAs, which influence the expression of at least 60% of cell proteins in a tissue-specific manner (Friedman *et al*, 2009), achieving precise orchestration of cell fate decisions. Sequencing of whole genomes, fractionated hierarchical sub-populations, and utilisation of sophisticated computational algorithms have revealed that miRNAs could be

exploitable as both biomarkers and therapeutic targets for a number of diseases, particularly cancers (Catto *et al*, 2011; Iorio and Croce, 2012). However, objective identification and functional validation of candidate miRNAs remain difficult, owing to inherent inconsistencies with miRNA target-prediction algorithms and the promiscuous nature of miRNA-mRNA target relationship.

*Correspondence: Professor NJ Maitland; E-mail: n.j.maitland@york.ac.uk

⁷Current address: Leukaemia and Stem Cell Biology Group, Department of Haematological Medicine, King's College London, Rayne Institute, 123 Coldharbour Lane, Denmark Hill, London SE5 9NU, UK

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To identify cell type-specific biomarker/therapeutically relevant miRNAs in prostate cancer, we have integrated miRNA (Rane *et al*, 2015) and mRNA (Birnie *et al*, 2008) microarray data sets. These data sets profiled miRNA and mRNA expression from patient-derived, human prostate stem-like cells (SC-CD133⁺/α₂β₁^{hi}) and their differentiated progeny: committed basal (CB-CD133⁻/α₂β₁^{lo}) cells (Figure 1A).

MATERIALS AND METHODS

Sample procurement. Patient prostate tissue samples were obtained after written consent and full ethical approval (LREC 07/H1304/121). Tissue collection and epithelial cell culture were performed as described before (Collins *et al*, 2005; Rane *et al*, 2014).

miRNA-mRNA microarray data integration. The details of samples, methods and the platform used for miRNA (accession code: GSE59156) and mRNA (accession code: E-MEXP-993) microarray expression database generation were described before (Birnie *et al*, 2008; Rane *et al*, 2015). The co-expression analysis was performed using Pearson correlation as the distance metric and Ward's linkage method. miRNA target prediction was performed using eight different prediction algorithms on the miRWalk website (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>; Lewis *et al*, 2005; Dweep *et al*, 2011). Target mRNAs not inversely expressed in the appropriate homologous cell type were removed. The pathway enrichment scores were computed on the inversely expressed mRNAs using standard hypergeometric tests. For more details see Supplementary File S1.

Expression analysis. miRVana kit (Life Technology, Paisley, UK) was used to extract RNA. miRNA expression was assessed with miScript primer assays (Qiagen GmbH, Hilden, Germany). mRNA expression was assessed by qRT-PCR and normalised first to RPLP0 and then with control expression using respective TaqMan probes (Life Technology). Immunofluorescence studies were performed as described before (Frame *et al*, 2013). For details of antibodies used, see Supplementary Table S1. Nuclear γ-H2AX foci and BRCA1/RAD51 positive nuclei were counted manually.

Transfection of miRNA inhibitor. CB cells were transfected with 100 nM miScript inhibitor for miR-542-5p (miR-542-5p-i) or miScript inhibitor negative control (Qiagen GmbH) using viromer BLUE (Lipocalyx GmbH, Halle (Saale), Germany) for 72 h according to the manufacturer's protocol.

Irradiation of cells and colony-forming efficiency. Irradiation of cells, and colony-forming efficiency were described previously (Rane *et al*, 2015).

Statistical analysis. All experiments were performed on 3 BPH and 3 Pca samples from individual patients. Errors are the standard deviation of mean. The significance was determined using Student's two-tailed *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

RESULTS

miRNA-mRNA microarray database integration. To integrate the miRNA and mRNA microarray data sets, we firstly leveraged the observation that expressed levels of functionally relevant miRNAs and their target mRNAs should be inversely correlated, as mammalian miRNAs are known to suppress not only protein expression, but also the transcript levels of their targets (Guo *et al*, 2010). Having identified differentially expressed miRNAs in SC vs CB cells (*P*<0.05), the targets for each miRNA were selected based on positive target-prediction call by at least three prediction

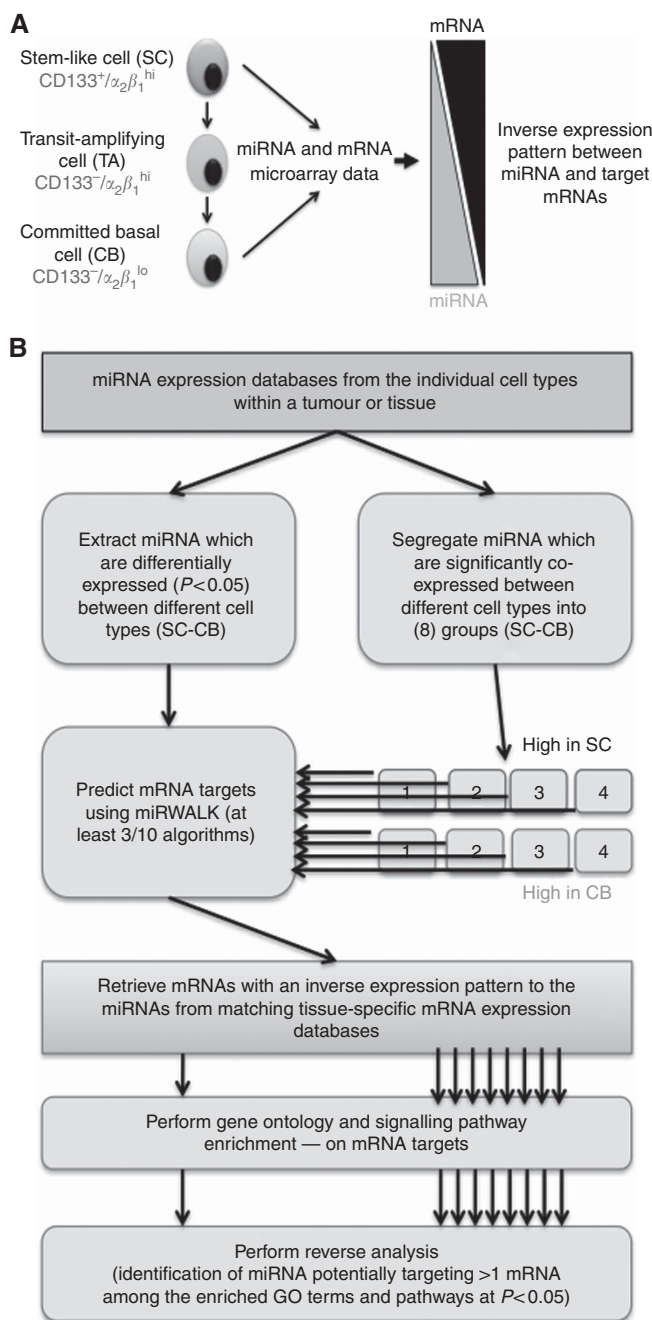


Figure 1. Generation of integrated miRNA-mRNA data sets using expression data from prostate SC and CB cells. **(A)** A schematic of human prostate epithelial hierarchy (left) showing a stem-like cell (SC) with a basal phenotype differentiating into committed basal (CB) cell via transit-amplifying (TA) cell. The nature of inverse expression relationship between miRNA and its target mRNAs is depicted on the right side. For the purpose of data integration, only the expression profiles from SC and CB cells were used. **(B)** Algorithm used to generate integrated miRNA-mRNA data set. Two approaches were taken: one without co-expression analysis on miRNA data and second with co-expression analysis. The eight sub-groups in co-expression analysis were kept separate at each subsequent step in the analysis.

algorithms and on an inverse expression pattern (Figure 1A and B). The analysis implied that signalling pathways/processes (e.g. DNA repair associated with miR-542-5p/99a/100, *P*<0.01) were regulated by a single or relatively few miRNAs, each of which affected multiple genes associated with the same pathway

(Supplementary File S2). To investigate whether diverse miRNAs can have similar pathway-regulating functions, we employed a second approach, where differentially expressed miRNAs (SC vs CB) were K-means clustered into eight distinct sets of co-expressed miRNAs prior to re-running the previous analysis for each cluster separately (Figure 1B and Supplementary File S3). Such co-expressed clusters were expected to have coordinated functions, which should be evident through their targets and their seed-sequence similarity (Lee *et al*, 2004; Supplementary File S4). The first evidence in support of this hypothesis was the observation that miRNAs with known functional associations (e.g., let-7 miRNAs and miRNAs of miR-17-92 family clustered within the same sub-group; Supplementary File S3). Notch signalling has been shown to have a significant role in prostate cancer cell fate (Carvalho *et al*, 2014; Supplementary Figure S1) Therefore it was of interest to further investigate miR-542-5p (identified by both *in silico* analyses), which can regulate Notch pathway-associated protein MAML1 (Supplementary File S4).

Proof-of-principle experiments to validate miRNA-mRNA database predictions. To test the validity of *in silico* predictions from the above two approaches, we measured the functional impact of miR-542-5p inhibition on prostate epithelial fate through

FANCL, POLE1 and MAML1. Little is known about the role of miR-542-5p in prostate cancers, but its predicted targets, FANCL (Rajendra *et al*, 2014) and POLE1 (Palles *et al*, 2013), are vital mediators of DNA repair, whereas MAML1 is a critical transcriptional co-activator of Notch receptors that stimulates transcription of Hes1 (Wu *et al*, 2000). We have previously shown that the SCs from normal prostate and malignant tissues are relatively radio-resistant (Frame *et al*, 2013); however, they can be sensitised to radiation by concurrent inhibition of Notch signalling by the gamma-secretase inhibitor RO4929097 (Tolcher *et al*, 2012; Supplementary Figure S1). As miR-542-5p is suppressed in SC (compared with CB; Figure 2A and B), we hypothesised that this lower expression of miR-542-5p in SCs contributes to radio-resistance. Inhibition of miR-542-5p expression in CB cells (Supplementary Figure S2) resulted in elevated expression of POLE1 and FANCL (Figure 2C). We also observed better cellular recovery (Figure 2D), quicker γ -H2A.X recovery (Figure 2E) and more efficient BRCA1 and RAD51 foci formation (Figure 2F and G) after exposure of the CB cells to 5 Gy radiation. The efficient DNA repair was also accompanied by activation of Notch signalling as demonstrated by upregulation of MAML1 protein (1.8-fold) and Notch targets HES1 (2.1-fold) and Survivin (4.7-fold; Figure 2H). A similar trend was also observed at the

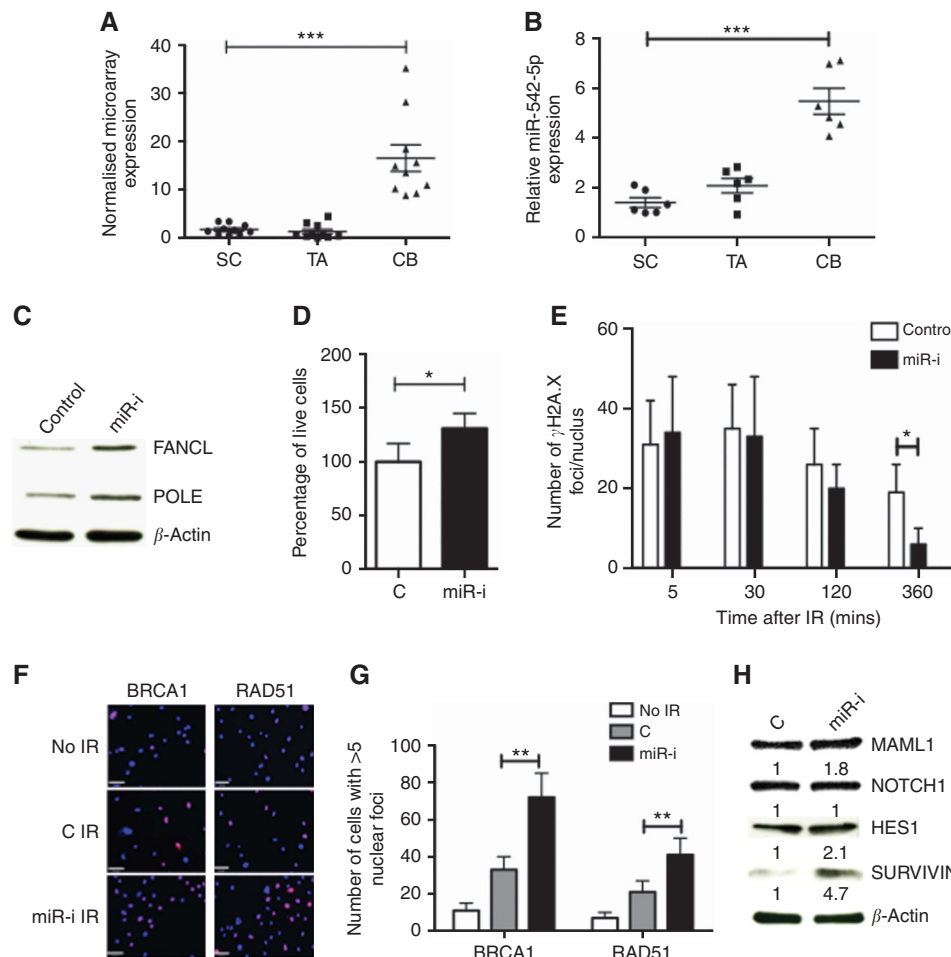


Figure 2. Proof-of-principle experiments to validate miRNA-mRNA database predictions using miR-542-5p. (A) miR-542-5p expression in miRNA microarray data set and (B) qRT-PCR validation on independent samples. CB cells were then transfected with 100 nm miR-542-5p-inhibitor ('miR-I') or negative control ('C') for 3 days. The expression of FANCL and POLE proteins was assessed by western blot (C). These cells were irradiated with 5 Gy IR before counting live cell count after 48 h of IR (D), γ -H2AX immunofluorescence foci/nucleus recovery (E), BRCA1 and RAD51 nuclear positivity 2 h after IR (F) Blue: DAPI and lilac: BRCA1/RAD51, and quantification is shown in G. Protein expression changes in MAML1 and Notch-associated effector proteins HES1 and Survivin (H). Error bars = 60 μ m. Each experiment represents mean of 3 BPH and 3 PCa and plotted as mean \pm s.d. * P <0.05, ** P <0.01, *** P <0.001 (Student's *t*-test). Abbreviations: SC=stem-like cells; TA=transit-amplifying cells; CB=committed basal cells; BPH=benign prostatic hyperplasia; PCa=treatment naive G1-7 prostate cancer; miR-I=miR-542-5p inhibitor; C=inhibitor negative control.

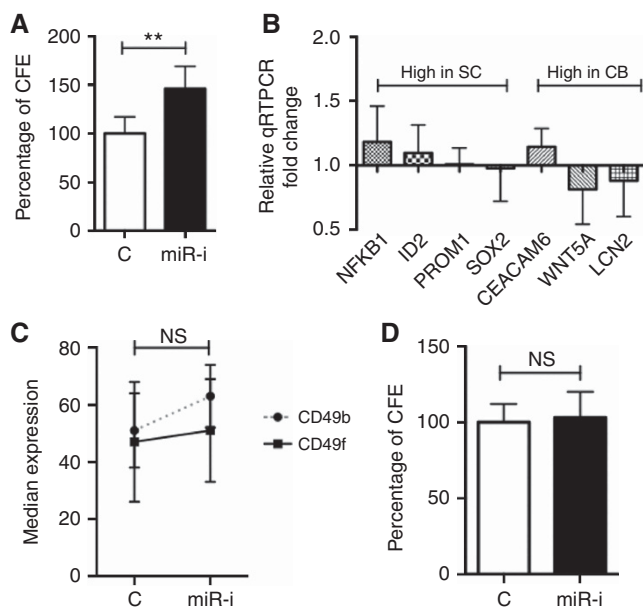


Figure 3. Effect of miR-542-5p inhibition on differentiation state of prostate epithelial cells. **(A)** Colony-forming efficiency (CFE) of miR-542-5p inhibitor (or negative control)-transfected CB cells after exposure to 5 Gy radiation ($n = 3$ BPH and PCa each). Before radiation, CB cells were transfected with miR-542-5p inhibitor for 72 h. CB cells transfected with miR-542-5p inhibitor (or negative control) for 72 h and subjected to qRT-PCR analysis for known SC/CB associated genes **(B)**, FACS analysis for CD49b/f **(C)** and colony-forming efficiency **(D)**. Each experiment represents the mean of 3 BPH and 3 PCa and plotted as mean \pm s.d. (except **C**). $**P < 0.01$ (Student's *t*-test). Abbreviations: SC=stem-like cells; CB=committed basal cells; BPH=benign prostatic hyperplasia; PCa=treatment naive G1-7 prostate cancer; miR-I=miR-542-5p inhibitor; C=inhibitor negative control; NS=not significant.

mRNA level, especially for Notch effector *HES1* and Survivin (Supplementary Figure S3).

Absence of a stem-like phenotype in miR-542-5p inhibited CB cells. A substantial (50%) increase in colony-forming ability (Figure 3A) was also noted in miR-542-5p inhibited CB cells after radiation treatment (5 Gy). As we have shown that SCs are more radio-resistant than CB cells (Frame *et al*, 2013), the observed efficient DNA repair after miR-542-5p inhibition in CB cells could either be due to the direct effects of *POLE1*, *FANCL* and *MAML1* overexpression, or the result of dedifferentiation of CB into SCs (or both). However, inhibition of miR-542-5p did not result in any detectable dedifferentiation of CB cells (Figure 3B–D). Thus, miR-542-5p inhibition in CB cells resulted solely in efficient DNA repair and Notch activation, as predicted by our miRNA-mRNA data integration data sets.

DISCUSSION

The central theme of this work was to devise a means for the identification of functionally relevant miRNAs and their target mRNAs. Our approach selects only tissue-specific mRNA targets in the specific cellular context, such as SC fate determination in present study. As the reduction in target mRNA expression may not always be evident (as we have assumed), our investigation may have underestimated the overall influence of miRNAs in prostate carcinogenesis. However, the causal relationship between some of the identified miRNA and their target pathways/

processes, for example, miR-99a/100 regulation of DNA repair (Mueller *et al*, 2013; Rane *et al*, 2015), clearly demonstrates the validity of our approach. Furthermore, the importance of studying miRNAs in a specific cellular phenotype has recently been underscored by the discovery that the expression changes in miR-143/145 in purified mouse colon stromal cells, rather than epithelial components (as previously assumed from unfractionated tissue/tumour biopsies) has functional relevance in regenerating mouse intestine (Chivukula *et al*, 2014). This pathway/process-based approach also provides an initial framework to address the long-standing issue of co-operative or functionally inter-related nature of miRNA targets in a practical setting. The database integration methods we have described can be readily adapted for any other tissue types to generate functionally relevant and therapeutically viable candidates, as exemplified here by miR-542-5p. In combination with alternative approaches such as large-scale miRNA functional screens, in an appropriate cellular environment (as we demonstrate), our approach should bring miRNA-driven therapeutics closer to direct clinical application.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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