MiR-10b as a marker for disseminated disease in RDEB-linked SCCs

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Introduction. Squamous cell carcinoma (SCC) is the second most common form of skin cancer, and the most common form of nonmelanoma skin cancer (NMSC). When caught early, most SCCs are curable. However, certain sub-types, such as those derived from patients with recessive dystrophic epidermolysis (RDEB), are more difficult to treat [1]. In addition to being difficult to diagnose, SCCs from RDEB patients tend to be significantly more aggressive than SCCs identified in an otherwise healthy population. Because most cancers are treatable, if detected early, there is a great need to shorten the time between diagnosis and treatment. However, current methods, which involve skin biopsies, do not encourage regular screening. In EB patients such screening is especially problematic given that this cohort already has significant problems with wound healing. Consequently, there is a great need to develop less traumatic methods for diagnosing malignant RDEB SCCs. Recent work (currently in review), conducted as part of an international collaboration between the Mellick Laboratory (Ingham Institute for Applied Medical Research), and the Wally Laboratory (EB House) describes the identification of a small RNA (miR-10b) that may drive malignancy in both SCCs and RDEB SCCs (Wimmer *et al*. In review). As part of this larger study, we have continued to investigate the role of miR-10b as a maker of disseminated disease. By adapting methods the 'liquid' (or blood) biopsy we have established the feasibility of identifying miR-10b^{+} SCCs and RDEB-SCCs from a blood biopsy using filtration based circulating tumour cell (CTC) isolation. In future we hope to adapt this methods for the regularly and effective screening of disseminated disease in RDEB.

Approach & Results. Several methods have been developed to analyse the presence of CTCs in the blood of cancer patients [2]. The most notable of these protocols include the CellSearch® methodology, which utilises epithelial cell adhesion molecule (EpCam) and Cytokeratin-with CD45 as an exclusion marker-to extract and identify CTCs from a blood biopsy (**Fig. 1**) [3]. However, in recent years, marker based CTC analysis has been questioned, as the veracity of common marker combinations to label disseminated cancer cells has come into question. This is of particular importance to RDEB patients, because of the differential expression of cytokeratin in SCCs. MiR-10b expression was previously determined to be a hallmark of aggressive SCCs and RDEB-SCCs (**Fig. 2**). Consequently, given that the Mellick laboratory had already established expertise in CTC analysis we determined to begin optimisation of a filtration-based (size-exclusion) protocol (ScreenCell®) [4] to isolate and detect miR-10b^{+} RDEB-SCCs. Importantly, filtration based protocols provide an objective means of isolating CTCs that can be adapted for use of novel disease specific markers.

To first determine if we could distinguish miR-10b^{+} RDEB-SCCs from other blood cells we examined primary cancer cells mixed with blood cells and 'cytospun' onto slides. We then applied the previously developed combined *in situ hybridisation* (*ISH)* and immunohistochemical approach (IHC), previously developed in the Mellick laboratory (**Fig. 3**) [3]. Given that we could distinguish both keratinocytes and SCCs from blood cells, we then proceeded to optimise the protocol for use in analysis of CTCs isolated from the blood using filtration (**Fig. 4**). Not time only did we find that we could use the *ISH-IHC* protocol effectively, but for the first time we have been able to show that RDEB-SCCs could be isolated by size exclusion from blood.

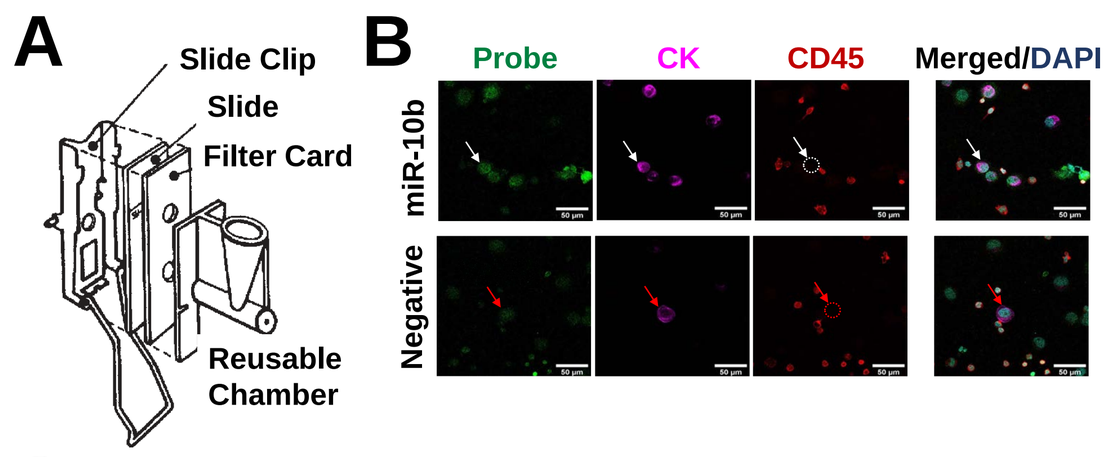

Figure 3 ***A,*** Schematic, filter/funnel for cyto-spinning cells onto slides. ***B*** (20 x resolution) & ***C*** (60 x resolution). Immunofluorescent microscopy of cytospun RDEB-SCCs, stained with **miR-10b** and neg. (scrambled) control. Shown, **miR-10b^{+} cytokeratin (CK)$^{+}$ CD45^{-}** RDEB-SCCs as well as hematopoietic **miR-10b^{+}, CD45^{+}** hematopoietic cells.

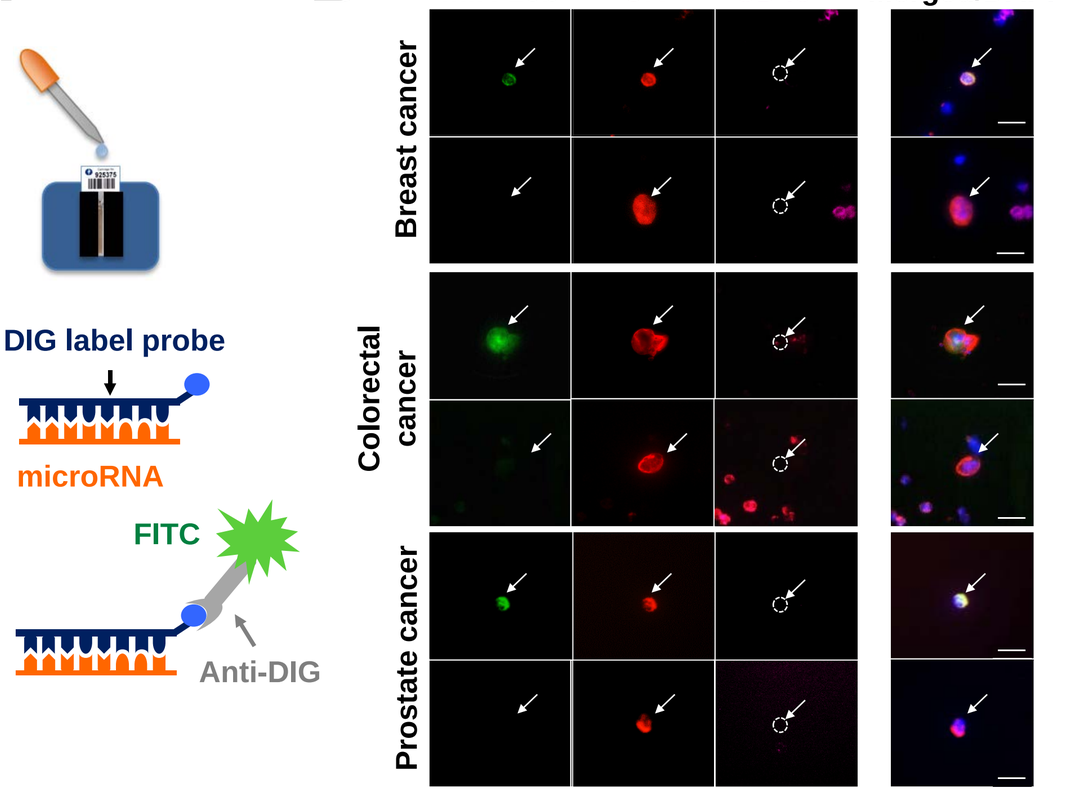

Figure 1 ***A***, Digoxigenin (DIG)-labelled anti-miR-10b locked nucleic acid (LNA) probes detected with **Fluorescein isothiocyanate (FITC)-labelled** anti-DIG antibody, which allows for visualisation of miR expression following CTC isolation using the CellSearch/CellTracks Analyzer®. CTCs can then be identified as either **miR-10b^{+}**, **Cytokeratin (CK)$^{+}$**, **CD45^{-}**; and/or **miR-10b^{-}**, **CK^{+}**, **CD45^{-}** **(*B*)**. Shown results of analysis of blood from prostate, breast cancer colon cancer patients. For a detailed method see Gasch *et al*. *Sci Rep*. 2015 [3].

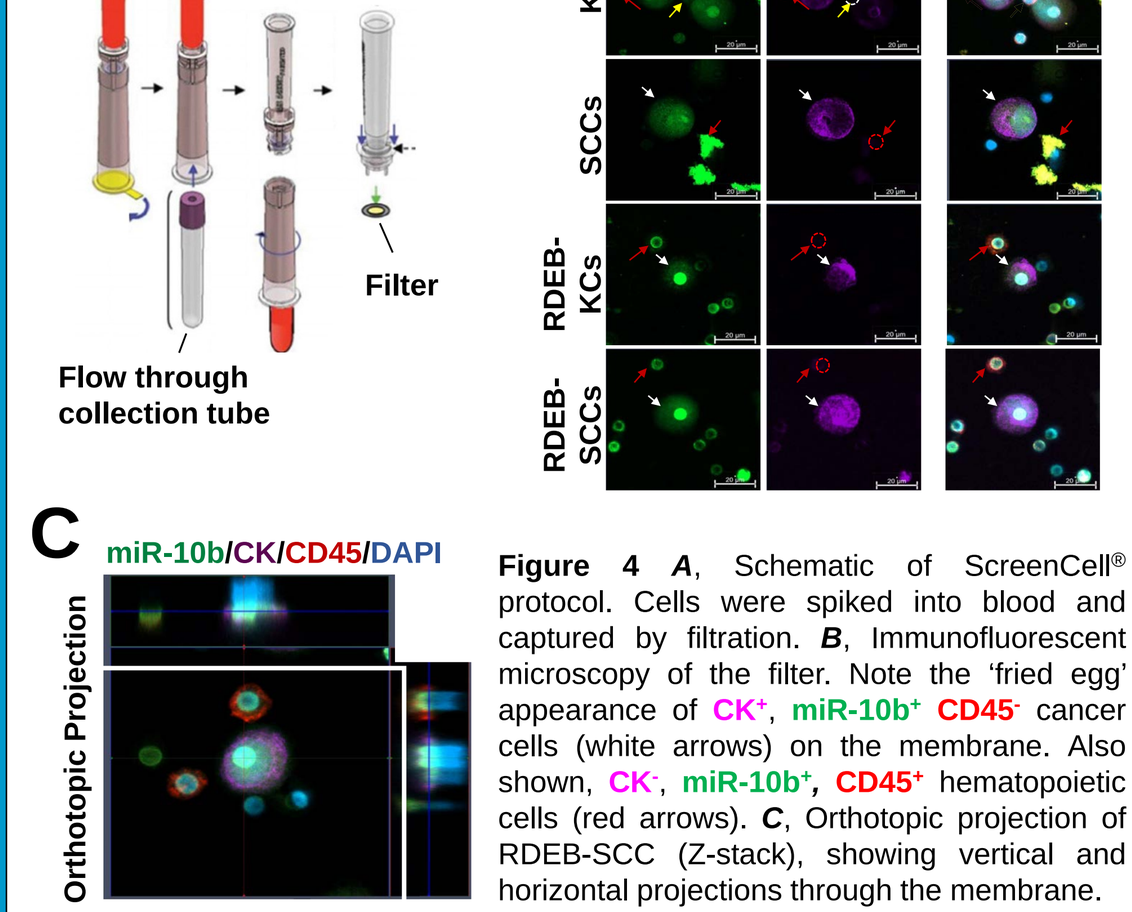

Figure 4 ***A***, Schematic of ScreenCell® protocol. Cells were spiked into blood and captured by filtration. ***B***, Immunofluorescent microscopy of the filter. Note the 'fried egg' appearance of **CK^{+}**, **miR-10b^{+}** **CD45^{-}** cancer cells (white arrows) on the membrane. Also shown, **CK^{-}**, **miR-10b^{+}**, **CD45^{+}** hematopoietic cells (red arrows). ***C***, Orthotopic projection of RDEB-SCC (Z-stack), showing vertical and horizontal projections through the membrane.

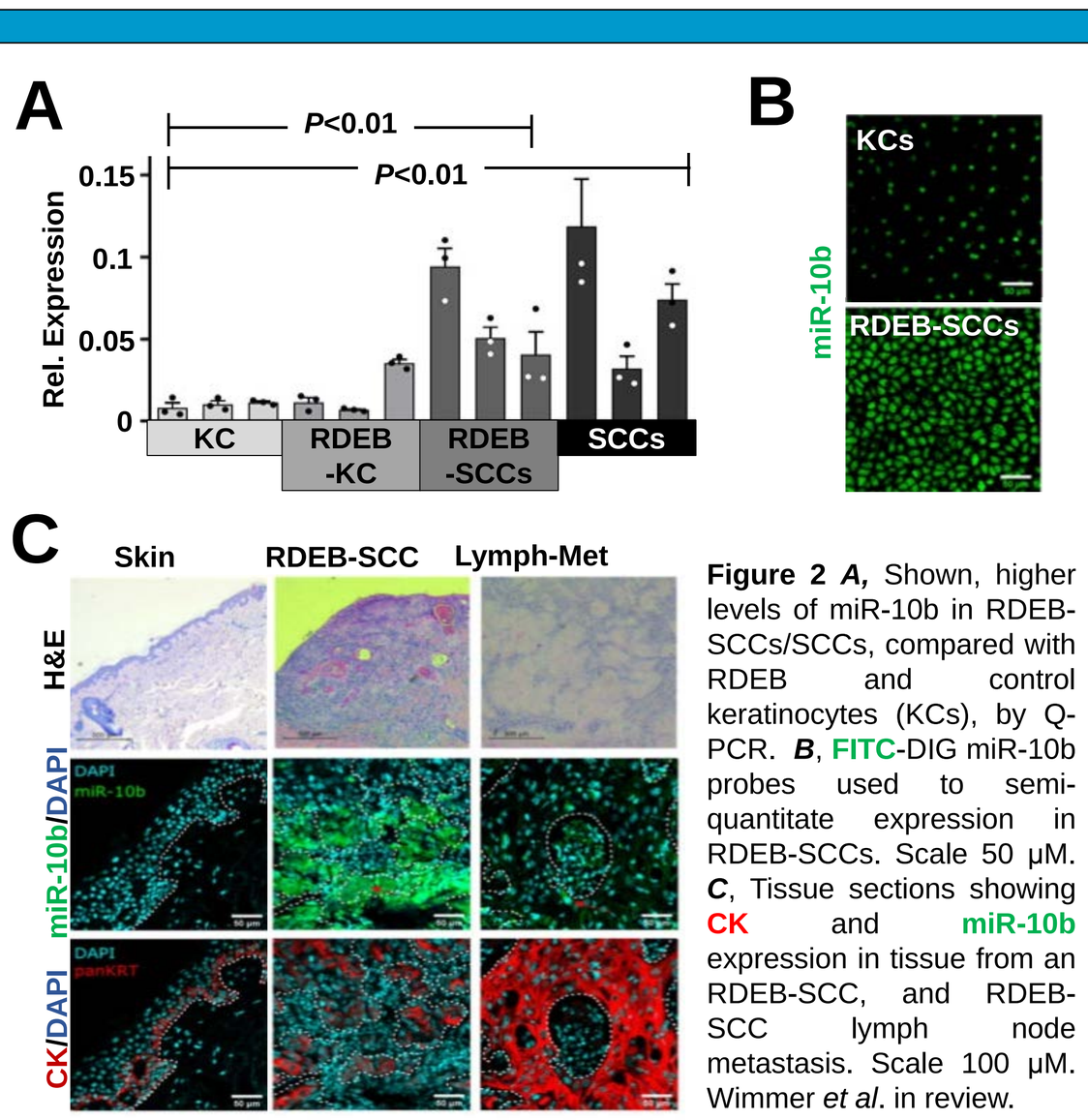

Figure 2 ***A,*** Shown, higher levels of miR-10b in RDEB-SCCs/SCCs, compared with RDEB and control keratinocytes (KCs), by Q-PCR. ***B***, **FITC**-DIG miR-10b probes used to semi-quantitate expression in RDEB-SCCs. Scale 50 μM. ***C***, Tissue sections showing **CK** and **miR-10b** expression in tissue from an RDEB-SCC, and RDEB-SCC lymph node metastasis. Scale 100 μM. Wimmer *et al*. in review.

Conclusions. Recent advances have significantly improved quality of life for patients with RDEB. However, at the same time screening and treatment of RDEB linked SCCs has not significantly improved. For all intent and purpose by the time RDEB SCCs are diagnosed they have often already spread and are difficult to treat. Consequently, there is a desperate need for more efficient diagnostic methods. The liquid biopsy holds significant potential because it allows identification of cancer cells in the blood. Unfortunately, current liquid biopsy based analysis developed for epithelial cancers may not be appropriate for RDEB-SCCs. By adapting filtration based methods and using more effective markers of malignancy, such as miR-10b, we hope to provide a tool that can be used to identify early signs of cancer spread. In addition, the use of these same filters allows, which can be dried, stored and transported prior for analysis, will greatly expand the use of liquid biopsy based diagnostics in EB. More work needs to be done, however, it is hoped that by further refining analysis of the liquid biopsy we can identify malignant cancers earlier, so that appropriate life extending treatments can be employed.

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