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Metastasis is linked to mortality in most types of cancer and is therefore a matter of intense investigation around the world. Yet the process by which cells migrate out of tumours is particularly difficult to study because it occurs randomly over large scales of time and space. We have set out to develop fluorescence microscopy approaches to characterise the cellular and molecular dynamics of metastasis in mouse models of cancer.

Our goal is to develop instantaneous read-outs of metastatic potential based on mechanistic understanding of migration. Cell migration comprises steps of protrusion, adhesion and contraction, which are mediated by the actin cytoskeleton. Recently, we have studied the dynamics of E-cadherin in tumour cell migration. Now, for the first time, we have used fluorescence lifetime imaging to study the activation of the small GTPase Rho in mice during mutant p53-driven invasion of pancreatic cancer cells.

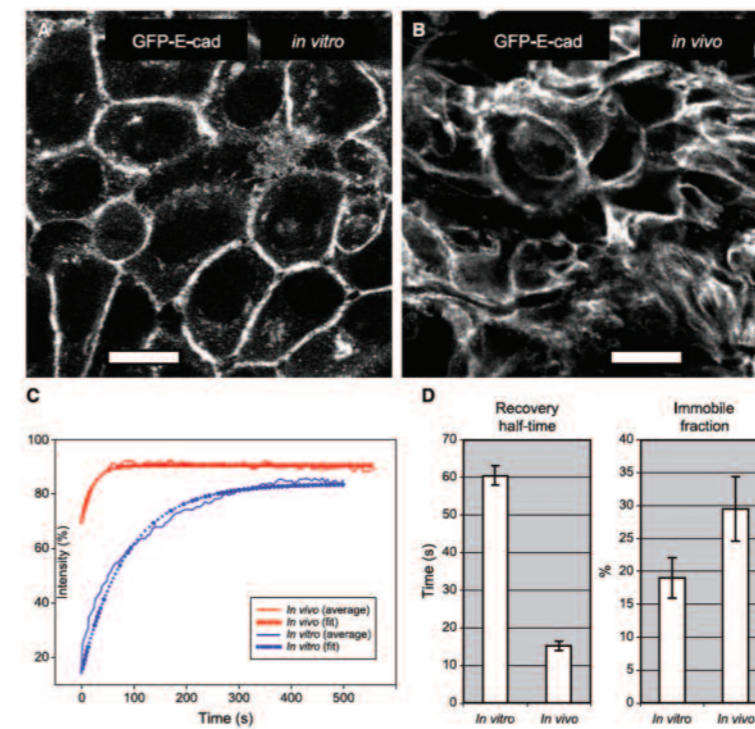
### E-cadherin dynamics in mouse tumours

Photo-bleaching is a technique that can be used to determine the relative fraction of fluorescently labelled molecules that are either stably or transiently incorporated into structures such as cell adhesions. Using photo-bleaching, we first demonstrated that the amount of E-cadherin stably incorporated into cell-cell adhesions is higher in stationary cells and lower in migrating cells. In other words, the immobile fraction of E-cadherin correlates with the level of cell migration. We went on to use photo-bleaching to assay E-cadherin dynamics in living subcutaneous mouse tumours, where it turns out the amount of E-cadherin stabilised in cell-cell junctions is significantly higher than in cell culture. In a parallel approach, we labelled the plasma membrane using a farnesylated, photo-activatable form of GFP (PAGFP-F) that is non-fluorescent until activated with intense irradiation at 405 nm. Using this probe we could show that approximately five times more PAGFP-F is immobilised in the plasma membrane of cells in subcutaneous tumours than in the same cells grown on coverslips.

Finally, quantitative imaging allowed us to assess the effects of therapeutic intervention using dasatinib, a clinically approved Src inhibitor under consideration as an anti-metastatic treatment. We found that dasatinib doubled the immobile fraction of E-cadherin in the junctions of cells in tumours but had no effect on the immobile fraction of cells in culture (Fig. 1). This demonstration of a profound drug effect *in vivo* combined with no effect *in vitro* is particularly relevant to the drug discovery process and highlights the need for early *in vivo* evaluation of drug effects. The specificity of the dasatinib effect on E-cadherin was confirmed using the membrane probe PAGFP-F, which behaved differently than E-cadherin under dasatinib treatment. These results demonstrate the utility of photo-bleaching and photo-activation in the analysis of dynamic biomarkers in living animals. On the basis of these results, we have begun work on a transgenic mouse capable of tissue specific expression of GFP-E-cadherin that will allow us to assess E-cadherin dynamics in a variety of transgenic mouse models of cancer currently in use at the Beatson Institute.

### FLIM-FRET imaging of Rho activation during mutant p53-driven invasion of pancreatic cancer cells

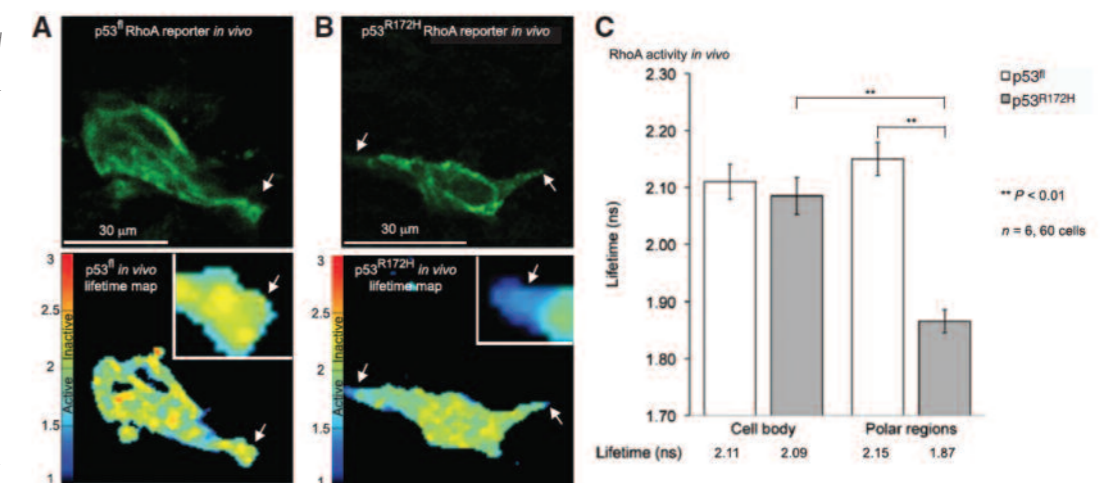
Pancreatic cancer is one of the most lethal forms of human cancer, with an overall five-year survival rate of less than five percent. Initiating KRAS mutations occur in approximately 90 percent of human pancreatic ductal adenocarcinoma (PDAC), while p53 mutations arise in 50 to 75 percent of human pancreatic cancer. We are using a mouse model of pancreatic cancer driven by mutant forms of



**Figure 1**  
E-cadherin dynamics assayed *in vitro* and *in vivo* using photo-bleaching. GFP-E-cadherin incorporates into the junctions of cells grown on glass coverslips (A) or in subcutaneous tumours (B). Quantitative assessment of photo-bleaching recovery curves (C) leads to different values for half-time of recovery and immobile fraction (D) between cells on coverslips and in tumours. Treatment with the Src inhibitor dasatinib had no effect on the *in vitro* immobile fraction of E-cadherin but doubled the immobile fraction *in vivo*. Bar in A and B is 20 microns.

**Figure 2**  
FLIM-FRET imaging of RhoA activity *in vivo*. PDAC cells expressing a GFP-RFP variant of the Rho-Raichu probe were injected subcutaneously and allowed to develop into tumours. PDAC cells either expressed a mutant form of p53 (R172H) or had p53 expression ablated. Fluorescence lifetime imaging was performed using a LaVision TRIM scope with 16-channel TCSPC detector under multi-photon excitation. Intensity images (top panels, A and B) show cell shape and localisation of the probe. Lifetime images (bottom panels, A and B) show that RhoA activity is highest at the tips of cellular processes extending outwards from the cell body (blue corresponds to lower activity, red corresponds to higher activity). Quantification of lifetime images (C) demonstrates that the activity of RhoA is similar for both cell types within the cell body but that RhoA is more active in the poles of cells expressing mutant p53.

KRAS and p53 that develops into invasive PDAC. Previous work has demonstrated that p53 mutation, rather than loss, can drive metastasis of pancreatic cancer and there is evidence to suggest that the small GTPase RhoA, a master regulator of cell migration, may act downstream of p53 to drive invasion. Fluorescence resonance energy transfer (FRET) biosensors based on the Raichu model have been adapted to study the activation of many small GTPases, including RhoA, in cell culture. Raichu biosensors work by changing conformation in response to activation of the GTPase, which changes the proximity between two fluorescent proteins and thereby the FRET signal. The use of FRET-based biosensors is technically challenging and has not previously been applied to the study of protein dynamics in mouse tumours. However, there is much evidence to suggest that the behaviour of cells in tumours is critically different than the behaviour



of cells under artificial culture conditions, especially in response to drug treatment. Therefore, we have endeavoured to study the activation of RhoA and response to drug treatment *in situ* using cells derived from the KRAS/p53 PDAC model.

We first established cell lines expressing GFP-RFP variants of the Rho-Raichu probe in both mutant p53 and p53 deletion PDAC cell lines. Probe response was characterised in cell culture using drug treatments and a variety of dominant negative and constitutively active mutants. We next used organotypic cultures, consisting of a rat tail-collagen gel contracted by primary human epidermal fibroblasts, as an intermediate model system to assess invasion. This form of three-dimensional collagen matrix is more realistic than cell culture but more amenable to experimental manipulation than the tumour microenvironment. Mutant p53 cells invaded rapidly into the collagen matrix, whereas p53 deletion cells remained on the surface of the gel. Fluorescence lifetime imaging of the two cell types indicated that invasion was associated with activation of RhoA, especially in the poles of invading cells, whereas RhoA was not active in non-invasive cells. Finally, the same cells were subcutaneously injected and allowed to form tumours in mice. Again, we found that higher RhoA activity was found in the poles of mutant p53 cells (Fig. 2). Dasatinib has been shown to prevent metastasis of mutant p53-driven cells from the pancreas to the liver, so we examined the effects of dasatinib treatment on RhoA activation. Interestingly, we found that dasatinib treatment selectively inhibited RhoA activation at the cell poles but did not reduce the average activation level within the cell body. This is the first demonstration of drug action within a sub-cellular region *in vivo*.

**Publications listed on page 76**