Real-time Study of E-Cadherin and Membrane Dynamics in Living Animals: Implications for Disease Modeling and Drug Development

Alan Serrels,[1] Paul Timpson,[2] Marta Canel,[1] Juliane P. Schwarz,[2] Neil O. Carragher,[3] Margaret C. Frame,[1] Valerie G. Brunton,[1] and Kurt I. Anderson[2]

[1]Edinburgh Cancer Research Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom; [2]Beatson Institute for Cancer Research, Glasgow, United Kingdom; and [3]Advanced Science and Technology Lab, AstraZeneca, Charnwood, United Kingdom

Abstract

The ability of tumor cells to invade and metastasize requires deregulation of interactions with adjacent cells and the extracellular matrix. A major challenge of cancer biology is to observe the dynamics of the proteins involved in this process in their functional and physiologic context. Here, for the first time, we have used photobleaching and photoactivation to compare the mobility of cell adhesion and plasma membrane probes *in vitro* and in tumors grown in mice (*in vivo*). We find differences between *in vitro* and *in vivo* recovery dynamics of two key molecules, the tumor suppressor E-cadherin and the membrane-targeting sequence of H-Ras. Our data show that E-cadherin dynamics are significantly faster *in vivo* compared with cultured cells, that the ratio of E-cadherin stabilized in cell-cell junctions is significantly higher *in vivo*, and that E-cadherin mobility correlates with cell migration. Moreover, quantitative imaging has allowed us to assess the effects of therapeutic intervention on E-cadherin dynamics using dasatinib, a clinically approved Src inhibitor, and show clear differences in the efficacy of drug treatment *in vivo*. Our results show for the first time the utility of photobleaching and photoactivation in the analysis of dynamic biomarkers in living animals. Furthermore, this work highlights critical differences in molecular dynamics *in vitro* and *in vivo*, which have important implications for the use of cultured disease models as surrogates for living tissue. [Cancer Res 2009;69(7):2714–9]

Introduction

Animal models have rapidly become essential tools in cancer research; from the determination of basic biological mechanisms to the study of complex human diseases. The analysis of molecular dynamics in an intact host environment, however, remains a major challenge. Fluorescence microscopy has been used to probe molecular dynamics of key proteins within two-dimensional cell cultures (1, 2), but *in vivo* conditions are more restrictive (3–5), and it is unclear to what extent these *in vitro* techniques can be applied *in vivo*. It is therefore important and necessary to develop techniques for the quantification of molecular dynamics *in vivo*.

Cell-cell interactions mediated by E-cadherin are central to maintaining normal tissue epithelial architecture and have been studied in great detail *in vitro* (6). The disruption and deregulation of E-cadherin–mediated cell-cell adhesions in cancer is a critical initiation step in the epithelial to mesenchymal transition associated with an invasive phenotype (7). Alterations in E-cadherin dynamics could therefore serve as an early molecular biomarker of metastasis. In order to study E-cadherin and membrane dynamics in their physiologic context, we have applied two complimentary techniques, photobleaching and photoactivation of fluorescence, to visualize protein dynamics in cell-cell junctions and the plasma membrane in tumor xenografts (8–10). Photobleaching is frequently used for the characterization of molecular dynamics within cultured cells. In the simplest approach, the fluorescence within a small region of the sample is bleached using a laser and the recovery of fluorescence into the bleached region is measured over time (11). Photoactivation is a related technique, in which a nonfluorescent (caged) precursor becomes fluorescent upon activation (10). In both cases, two basic variables are derived, (*a*) the half-time of recovery, an indication of the rate at which probes move in or out of the analysis region, and (*b*) the immobile fraction, an indication of how much of the probe remains trapped and unable to move out of the analyzed region. Using this approach, we have successfully quantified E-cadherin dynamics in a three-dimensional host setting and provided a molecular readout of the early mobilization events in tumor cells. Our results highlight the necessity and advantages of live animal imaging in the study of tumor development and metastasis, and show that photobleaching and photoactivation can be used *in vivo* for the quantification of drug action in the treatment of cancer.

Materials and Methods

Plasmids. GFP-E-cadherin and PAGFP were kind gifts from Jennifer Stow (Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, Australia) and Jennifer Lippincott-Schwartz (Cell Biology and Metabolism Branch, NIH, Bethesda, MD), respectively. PAGFPFarn2Palm was made by substituting GFP in the pEGFP-N1 vector with PAGFP-Farn2Palm (for cloning, see Supplementary Materials).

Cell lines. A431 cells were obtained from (American Type Culture Collection), and maintained as described. The cell-derived matrix was generated as described previously (12). Cells (2×10^5) were plated onto the cell-derived matrix and cells within colonies or migrating cells were targeted for photobleaching and analyzed as described in the Supplementary Materials.

Photoactivation, photobleaching, and analysis *in vitro* and *in vivo*. A detailed description of these techniques and analysis, as well as drug treatment *in vitro* and *in vivo*, can also be found online in the Supplementary Materials.

Results and Discussion

Photobleaching of GFP-E-cadherin dynamics *in vitro* correlates with cell migration. Cell-cell adhesion turnover is thought

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

A. Serrels and P. Timpson contributed equally to this work.

Requests for reprints: Kurt I. Anderson, The Beatson Institute for Cancer Research, Garscube Estate, Glasgow G61 1BD, United Kingdom. Phone: 44-141-330-2864; Fax: 44-141-942-6521; E-mail: k.anderson@beatson.gla.ac.uk.

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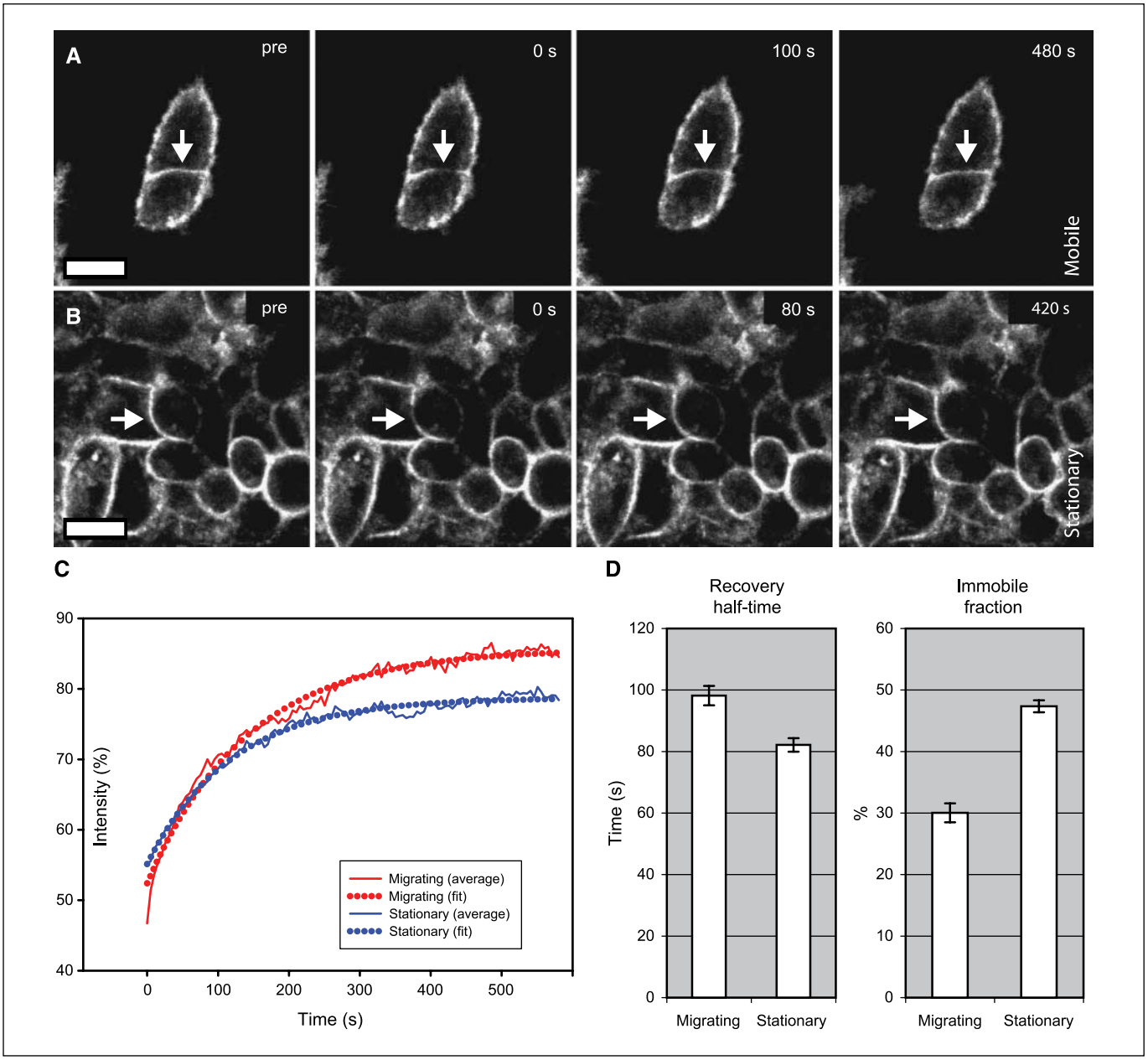

Figure 1. Photobleaching of GFP-E-cadherin in migrating or stationary cells. Representative images of migrating (*A*) or stationary cells within a colony (*B*) used to generate fluorescence recovery curves (*C*). Curves represent pooled data (*solid lines*) and the results of exponential curve-fitting (*dotted lines*). *D,* graphs comparing recovery rates and the amount of GFP-E-cadherin trapped in cell-cell junctions between freely moving ($n = 10$) and stationary cells ($n = 4$). *Columns,* mean; *bars,* SE (*bar,* 40 μm).

to play a key role in cell migration and invasion (6, 7). We therefore used photobleaching to assess whether we could quantify differences in E-cadherin dynamics between stationary and migrating cells. A431 carcinoma cells were plated on cell-derived matrix, and cell junctions between collectively migrating or confluent nonmotile cells were targeted for bleaching (refs. 13, 14; Fig. 1*A* and *B*; Supplementary Movies 1 and 2, respectively). Photobleaching recovery curves from migrating or stationary cells were pooled and analyzed (Fig. 1*C*). The half-time of GFP-E-cadherin recovery was reduced by 16 seconds in the junctions of cells restrained in colonies (Fig. 1*D*), and the fraction of GFP-E-cadherin stabilized in cell-cell junctions was 58% higher in confluent cells compared with migrating cells (47.4 ± 1.4% for confluent compared with 30.0 ± 1.5% for migrating cells; Fig. 1*D*). Similar results were obtained comparing mobile cells at the leading edge of a monolayer wound with confluent nonmigrating cells at the rear of the wound (data not shown). The higher level of GFP-E-cadherin trapped at the cell-cell junctions of stationary cells shows a correlation between cell migration and the level of GFP-E-cadherin trapped within cell-cell junctions. We therefore wanted to assess whether the immobile fraction of GFP-E-cadherin could be measured *in vivo* and serve as a biomarker for migration potential.

Photobleaching of GFP-E-cadherin shows fundamental differences in E-cadherin mobility *in vivo*. To examine

GFP-E-cadherin dynamics *in vivo*, cells were grown as subcutaneous tumors in nude mice and compared with GFP-E-cadherin in the junctions of confluent cells cultured *in vitro*. In both cases, GFP-E-cadherin was similarly localized to the cell periphery (Fig. 2*A* and *B*). Cell-cell junctions could be imaged up to ~70 μm deep in tumor tissue and effectively bleached at ~20 μm, which enabled cells completely surrounded by others to be targeted for bleaching, analogous to the confluent cells targeted *in vitro*. Initial observation of time-lapse movies suggested that the rate of recovery was much faster *in vivo* than *in vitro*. Analysis of pooled recovery curves (Fig. 2*C*) confirmed that the half-time of recovery was four times faster *in vivo* (15.3 ± 1.2 seconds *in vivo* compared with 60.5 ± 2.5 seconds *in vitro*; Fig. 2*D*). Furthermore, the fraction of GFP-E-cadherin stabilized in cell-cell junctions was 1.5 times greater *in vivo* (Fig. 2*D*). These results show that the mobility of the tumor suppressor E-cadherin, a key protein implicated in the early stages of invasion, is substantially different *in vitro* and *in vivo*. Moreover, these differences have profound implications for cancer research, in which the behavior of E-cadherin in cultured disease models is used to elucidate basic biological mechanisms which are extrapolated back into human disease (6, 7).

Photoactivation shows fundamental differences in plasma membrane dynamics *in vivo*. Protein mobility within the plasma membrane also plays a fundamentally important role during cell migration (12, 15). We therefore examined whether plasma membrane dynamics might also differ in the intact host environment compared with cell culture models. To answer this question, we used photoactivatable GFP anchored to the plasma membrane through the farnesylated and doubly palmitoylated membrane targeting sequence of H-Ras (PAGFP-Farn2Palm; ref. 16). Cells were stably transfected with the membrane probe, cultured *in vitro* or *in vivo*, and targeted for photoactivation. *In vitro*, the activated fluorescence was observed to undergo rapid and virtually complete lateral diffusion (Fig. 3*A*; Supplementary Movie 5). However, initial observations *in vivo* suggested

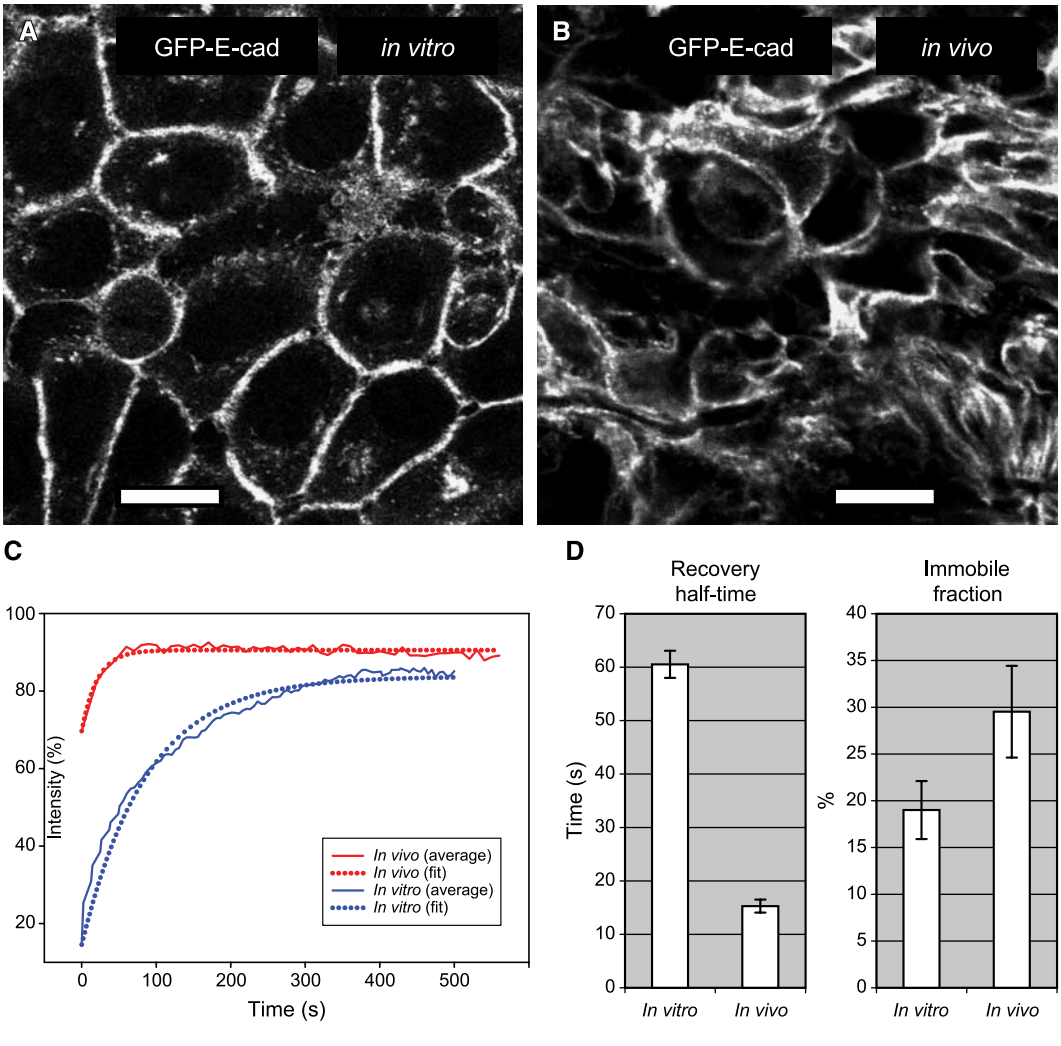

Figure 2. Photobleaching of GFP-E-cadherin *in vitro* vs. *in vivo*. GFP-E-cadherin localization *in vitro* (*A*) and *in vivo* (*B*). *C*, fluorescence recovery curves following photobleaching of GFP-E-cadherin performed *in vitro* (*blue*) and *in vivo* (*red*). *D*, graphs comparing recovery rates and the amount of GFP-E-cadherin trapped in junctions *in vitro* (n = 12) and *in vivo* (n = 15). *Columns*, mean; *bars*, SE (*bar*, 20 μm).

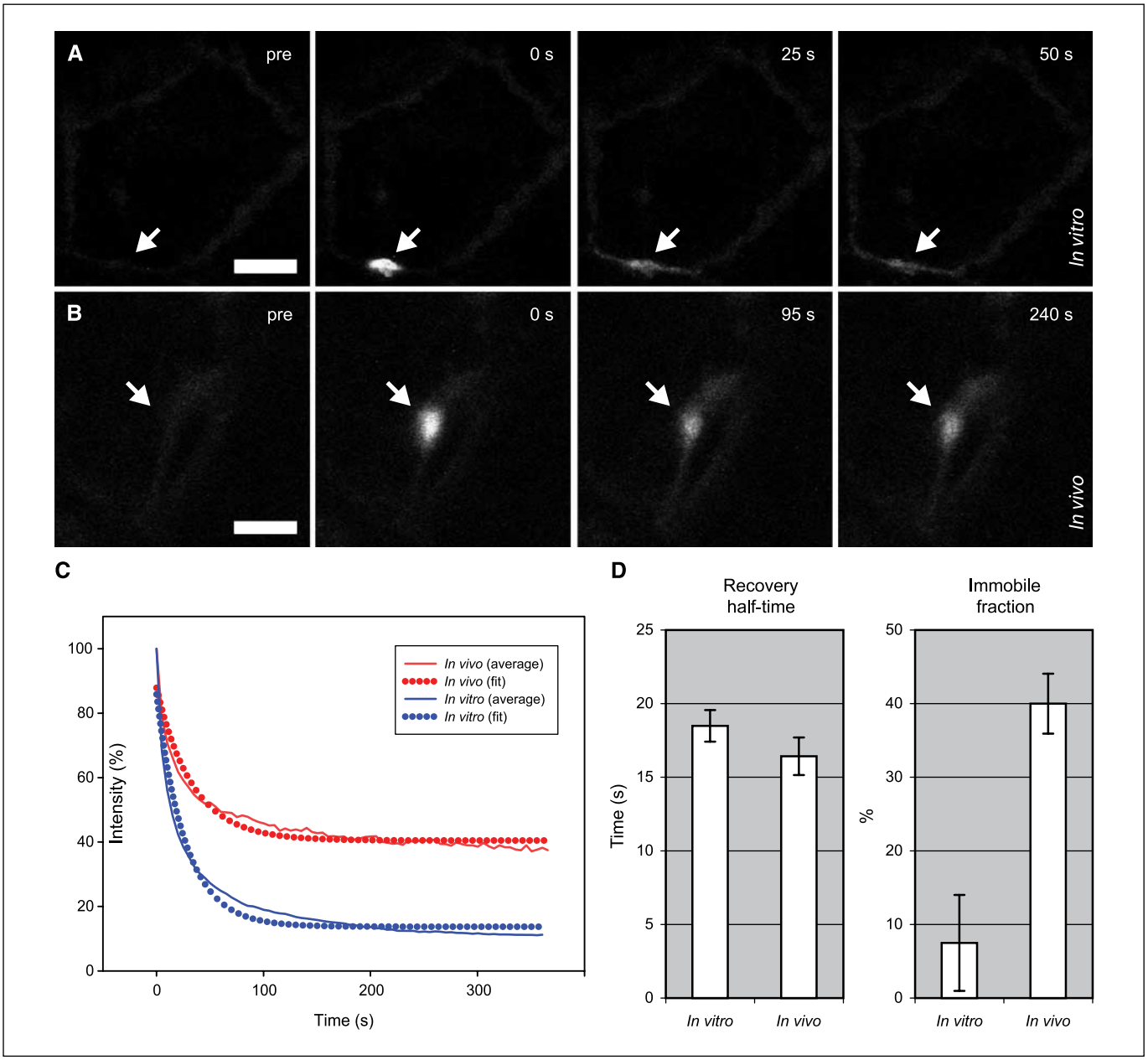

Figure 3. Photoactivation of membrane probe *in vitro* vs. *in vivo*. *A* and *B,* representative time series demonstrating *in vitro* and *in vivo* photoactivation of PAGFP-Farn2Palm. *C,* fluorescence decay curves following photoactivation of membrane probe *in vitro* (*blue*) and *in vivo* (*red*). *D,* graph comparing the rate of fluorescence loss and the amount of membrane probe trapped in the membrane *in vitro* (n = 40) and *in vivo* (n = 11). *Columns,* mean; *bars,* SE (*bar,* 10 μm).

that like E-cadherin, a significant proportion of the membrane probe remained trapped in the site of activation (Fig. 3*B*; Supplementary Movie 6). Analysis of pooled PAGFP-Farn2Palm decay curves (Fig. 3*C*) revealed a similar half-time of recovery *in vitro* and *in vivo* (Fig. 3*D*). However, the immobile fraction of the membrane probe was more than five times greater *in vivo* than *in vitro* (40.00 ± 4.1% *in vivo* compared with 7.5 ± 6.5% *in vitro*; Fig. 3*D*). Thus, in a similar manner to E-cadherin, we found a substantial increase in the fraction of the membrane probe trapped *in vivo* compared with *in vitro* (compare Figs. 2*D* and 3*D*). The altered dynamics of both E-cadherin and the plasma membrane *in vivo* may occur as a result of different environmental cues within the host such as interactions with stromal cells and the extracellular matrix and are currently under investigation.

Dasatinib treatment enhances the stability of E-cadherin in cell-cell junctions *in vivo* but not *in vitro*. Drug discovery involves the validation of drugs and targets *in vitro* to be used for therapeutic intervention *in vivo*. The high attrition rates of compounds entering clinical trials (17) suggests that drug discovery may be particularly sensitive to the kinds of differences in molecular dynamics *in vitro* and *in vivo* that we have observed here for E-cadherin and the membrane probe. We therefore addressed whether photobleaching could be used to

compare the effects of pharmacologic intervention *in vitro* with *in vivo*. This approach could provide valuable *in vivo* preclinical data for validating mechanisms of action and guiding dosing regimes. We selected dasatinib, a dual Src/Abl tyrosine kinase inhibitor under consideration for use as an anti-invasive drug in epithelial tumors (18). Although the mechanism of action is not fully understood, it may include effects on E-cadherin dynamics known to be regulated by Src tyrosine kinase activity (19, 20).

Cells were cultured *in vitro* and *in vivo*, and treated with dasatinib (20) prior to photobleaching of GFP-E-cadherin. Dasatinib treatment caused no change in E-cadherin levels (Supplementary Fig. S2); however, photobleaching analysis revealed that dasatinib treatment shortened the rate of GFP-E-cadherin recovery both *in vitro* and *in vivo* (Fig. 4*A*). Remarkably, photobleaching analysis also revealed that drug treatment had no effect on the immobile fraction of E-cadherin *in vitro* but doubled the fraction of E-cadherin trapped in cell-cell junctions *in vivo* (Fig. 4*B*). This striking increase in the immobile fraction of E-cadherin *in vivo* (from 29.5 ± 4.9% to 65.4 ± 2.3%) is significant in relation to our earlier finding that more GFP-E-cadherin is immobilized in junctions between confluent nonmigrating cells within colonies or at the rear of a wound (Fig. 1*D*; results not shown). This result lends support to a possible mechanism of dasatinib as an anti-invasive drug through the stabilization of cell-cell adhesion, and would be missed by cell culture–based screening assays typically used in drug discovery.

To assess the specificity of the E-cadherin response to dasatinib treatment, E-cadherin recovery dynamics were compared with membrane recovery dynamics *in vitro* and *in vivo*. Dasatinib had no effect on either the half-time of recovery or the immobile fraction of the membrane probe *in vitro* (results not shown). *In vivo*, the half-time of recovery was similar for both GFP-E-cadherin and the membrane probe. However, dasatinib treatment had significantly different effects on the immobile fraction of GFP-E-cadherin and the membrane probe *in vivo*: drug treatment *increased* the immobile fraction of GFP-E-cadherin by a factor of 2 but *decreased* the immobile fraction of the membrane probe by a factor of 1.6 (from 40.00 ± 4.1% to 24.7 ± 4.0%; Fig. 4*C*). This highlights the selectivity of dasatinib for E-cadherin *in vivo* independent of membrane dynamics.

In summary, we have shown the first use of photobleaching and photoactivation to monitor molecular dynamics in living tumors. Our results show that fundamental cellular properties such as the mobility of cell-cell adhesion components or plasma membrane proteins are different *in vitro* and *in vivo*. Photobleaching offers the potential to probe the spatial and temporal dynamics of cell-cell junctions in a context-dependent manner; for example, depending on the proximity to migratory stimuli. Such *in vivo* analysis enables the quantification of subtle early changes in protein behavior in response to therapeutic intervention, which might be missed using cell culture models. The altered mobility of the membrane targeting sequence of H-Ras *in vivo* highlights the potential that other membrane-targeted signal transduction molecules may behave differently than expected *in vivo*. Our data critically highlight a shortcoming of conventional drug discovery, the use of cell culture models which fail to recapitulate the behavior of cells in living organisms, and emphasize the importance of early *in vivo*

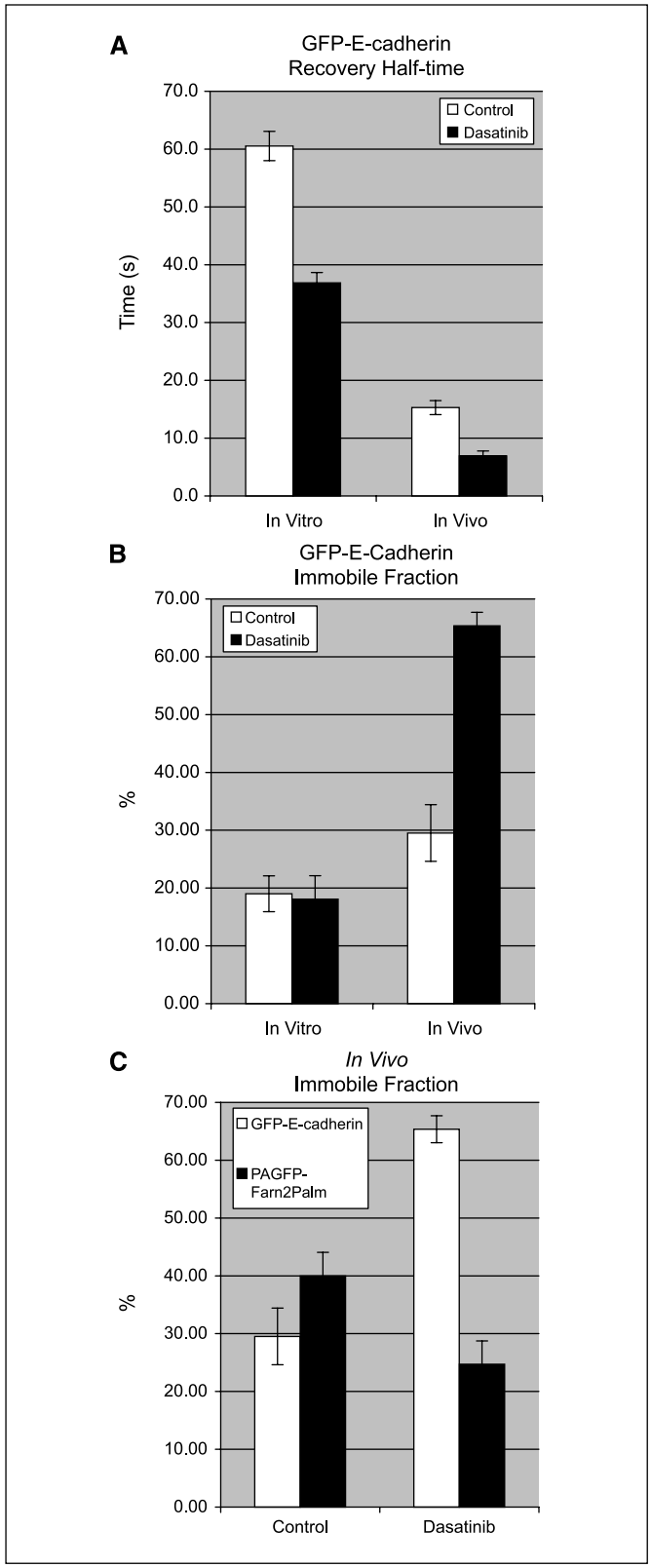

Figure 4. Dasatinib treatment enhances the amount of GFP-E-cadherin trapped in junctions *in vivo* but not *in vitro*. *A*, graph comparing fluorescence recovery rates of GFP-E-cadherin +/− dasatinib *in vitro* ($n = 12$) and *in vivo* ($n = 15$), derived from pooled recovery curves. *B* and *C*, graph comparing the amount of GFP-E-cadherin or membrane probe trapped in cell-cell junctions +/− dasatinib *in vitro* ($n = 20$) and *in vivo* ($n = 171$) derived from pooled recovery curves. *Columns,* mean; *bars,* SE.

preclinical testing in the drug discovery pipeline. Finally, the adaptation of these techniques for real-time live animal imaging can be extended to the analysis of other key dynamic biomarkers or oncogenes during the study of cancer progression and should provide an in-depth understanding of molecular behavior in a spatial, temporal, and now, contextual setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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