Current Biology 19, 1511–1518, September 29, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.07.069

Article

Cell-Cycle Control by Physiological Matrix Elasticity and In Vivo Tissue Stiffening

Eric A. Klein,[1] Liqun Yin,[1,3] Devashish Kothapalli,[1,3] Paola Castagnino,[1,3] Fitzroy J. Byfield,[2] Tina Xu,[1] Ilya Levental,[2] Elizabeth Hawthorne,[1] Paul A. Janmey,[2] and Richard K. Assoian[1,*]
[1]Department of Pharmacology, School of Medicine
[2]Institute for Medicine and Engineering
University of Pennsylvania, Philadelphia, PA 19104, USA

Summary

Background: A number of adhesion-mediated signaling pathways and cell-cycle events have been identified that regulate cell proliferation, yet studies to date have been unable to determine which of these pathways control mitogenesis in response to physiologically relevant changes in tissue elasticity. In this report, we use hydrogel-based substrata matched to biological tissue stiffness to investigate the effects of matrix elasticity on the cell cycle.
Results: We find that physiological tissue stiffness acts as a cell-cycle inhibitor in mammary epithelial cells and vascular smooth muscle cells; subcellular analysis in these cells, mouse embryonic fibroblasts, and osteoblasts shows that cell-cycle control by matrix stiffness is widely conserved. Remarkably, most mitogenic events previously documented as extracellular matrix (ECM)/integrin-dependent proceed normally when matrix stiffness is altered in the range that controls mitogenesis. These include ERK activity, immediate-early gene expression, and cdk inhibitor expression. In contrast, FAK-dependent Rac activation, Rac-dependent cyclin D1 gene induction, and cyclin D1-dependent Rb phosphorylation are strongly inhibited at physiological tissue stiffness and rescued when the matrix is stiffened in vitro. Importantly, the combined use of atomic force microscopy and fluorescence imaging in mice shows that comparable increases in tissue stiffness occur at sites of cell proliferation in vivo.
Conclusions: Matrix remodeling associated with pathogenesis is in itself a positive regulator of the cell cycle through a highly selective effect on integrin-dependent signaling to FAK, Rac, and cyclin D1.

Introduction

Soluble mitogens and antimitogens have long been viewed as important regulators of cell proliferation, but it is now accepted that insoluble factors, especially components of the extracellular matrix (ECM), have equally essential roles in the proliferation of most nontransformed cell types. Soluble mitogens and ECM proteins jointly regulate activation of the G1 phase cyclin-dependent kinases (cdks) cdk4/6 and cdk2, which are required for S phase entry [1]. ECM proteins signal by binding and activating the integrin family of cell surface receptors, and many studies have identified integrin-dependent signaling events that support G1 phase progression, especially the induction of cyclin D1 mRNA and protein and the downregulation of the cip/kip family of cdk inhibitors [1]. These effects control activation of cdk4/6 and cdk2, respectively, which in turn catalyze the inactivating phosphorylations of the retinoblastoma protein (Rb) and the related proteins p107 and p130. Rb phosphorylation results in the release of sequestered E2Fs and the induction of E2F-dependent genes required for S phase.

Integrin-dependent signaling events implicated upstream of cyclin D1 and the cip/kips include the activation of ERK MAP kinases, Rho family GTPases, and FAK [1]. However, little is known about whether and how these pathways are activated in physiologically relevant mechanical microenvironments. This gap in knowledge arises at least in part because integrin-regulated events have typically been identified in cultured cells after complete blockade of ECM-integrin binding (e.g., by incubating cells in suspension or on polylysine-coated dishes) or by preventing integrin clustering (e.g., by disrupting the actin cytoskeleton with depolymerizing drugs or inhibitors of Rho GTPase activation or signaling). These approaches result in much more severe changes in integrin occupancy, F-actin polymerization, and Rho GTPase activity than are likely to occur physiologically. Additionally, a pervasive shortcoming of traditional cell biological approaches to the study of ECM function is that cells are typically cultured on nondeformable substrata (culture dishes or glass coverslips), which have little relationship to the elastic (also called "compliant") ECM that cells encounter in vivo. Because a hallmark of ECM-cell interactions is the ability to sense extracellular stiffness, tissue compliance may be an important determinant of downstream adhesion-dependent signaling events.

Some studies have used collagen gels to study the effect of a more compliant matrix on integrin signaling and the cell cycle. These studies showed that human foreskin fibroblasts in free-floating collagen gels have high levels of p27, do not phosphorylate ERK, and do not express cyclin D1 [2, 3]. Others have reported that increased $p21^{cip1}$ or $p15^{INK4B}$ is responsible for G1 phase arrest that occurs when cells are plated on soft collagen gels [4, 5]. FAK autophosphorylation, ERK activity, and Rho activation are also impaired when cells are cultured on or in soft collagen matrices or Matrigels [2, 6]. Although these results suggest that changes in matrix stiffness recapitulate the effects seen upon complete adhesion blockade, collagen gels are much softer than many physiological tissues (elastic moduli of 10–50 pascals [Pa] versus 100–100,000 Pa) [7, 8]. Moreover, changing the stiffness of collagen gels by altering collagen concentration inherently affects the integrin ligand concentration, so observed effects cannot be strictly attributed to changes in matrix elasticity. The same complication exists when cells are cultured in Matrigel, which is also less well defined chemically.

Careful control of substratum elasticity is perhaps best achieved by seeding cells on ECM-coated biocompatible hydrogels because elasticity can be varied independently of matrix concentration [9, 10]. Hydrogels based on polyacrylamide have elastic moduli of ~1,500–150,000 Pa, a range which encompasses the stiffness of most physiological

*Correspondence: assoian@mail.med.upenn.edu
[3]These authors contributed equally to this work

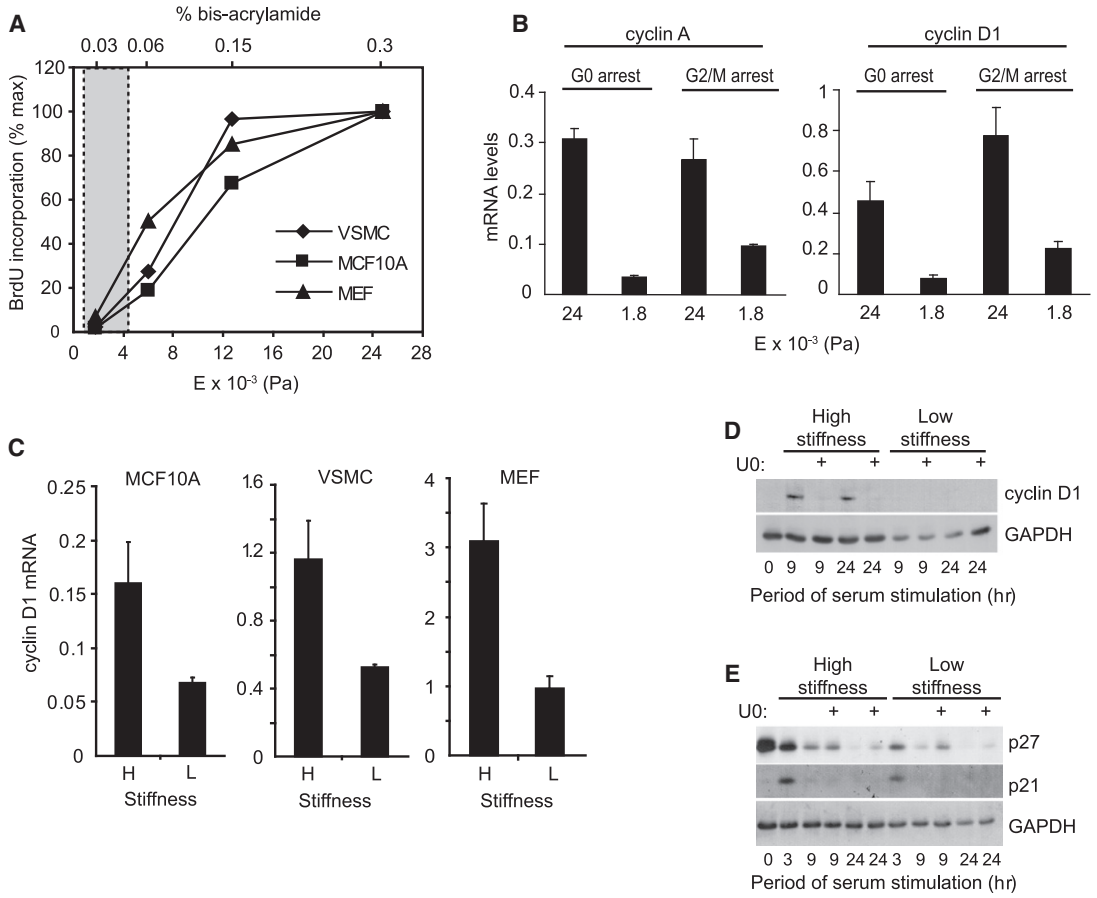

Figure 1. Selective Effect of Extracellular Matrix Stiffness on Cyclin D1 Gene Expression

(A) Serum-starved cells were stimulated with mitogens, incubated with bromodeoxyuridine (BrdU), and reseeded on hydrogels made with a constant 7.5% acrylamide. Bis-acrylamide varied from 0.03% to 0.3%. After 24 hr (MCF10A cells and mouse embryonic fibroblasts [MEFs]) or 48 hr (vascular smooth muscle cells [VSMCs]), cells were fixed and BrdU incorporation was determined. The graph compiles results from an individual experiment for each cell type and shows percent maximal BrdU incorporation compared to the stiffest hydrogel. The shaded area highlights the range of elastic moduli measured in mouse mammary glands and arteries as determined by milliprobe indentation and atomic force microscopy (AFM) (see Table S2).

(B) MEFs were synchronized at G0 (by 48 hr serum starvation) or at G2/M (by treatment with 5 μg/ml nocodazole for 24 hr). The cells were reseeded on hydrogels and stimulated with 10% fetal bovine serum (FBS). RNA was isolated 24 hr after reseeding and analyzed by qPCR for cyclin A or cyclin D1 mRNAs. Data in (B) and (C) are mean ± standard deviation (SD) of duplicate PCR reactions.

(C) Serum-starved cells were reseeded on high-stiffness (H) and low-stiffness (L) hydrogels with mitogens. Cyclin D1 mRNA was measured by qPCR at times corresponding to optimal induction (12 hr for MCF10A cells, 24 hr for VSMCs, and 9 hr for MEFs).

(D and E) Serum-starved MEFs were pretreated with dimethyl sulfoxide (vehicle) or U0126 (U0) prior to reseeding on hydrogels and stimulation with 10% FBS. Reseeded cells were collected at the indicated times and analyzed by western blotting.

tissues [10]. Cells plated on soft hydrogels show a decrease in cell number [9, 11], but the molecular events that underlie cell-cycle control by matrix stiffness remain undefined. In this report, we combine biophysical measurements of tissue elasticity with a molecular analysis of the cell cycle on compliance-appropriate hydrogels to elucidate the subcellular effects of matrix stiffness on cell proliferation.

Results

A Small Subset of Adhesion-Dependent Signaling Events Accounts for Cell-Cycle Control by Matrix Stiffness

We adapted the use of deformable matrix protein-coated acrylamide hydrogels to a molecular analysis of the cell cycle. Quiescent mouse embryonic fibroblasts (MEFs) were plated on fibronectin (FN)-coated hydrogels having elastic moduli within the physiological range [7, 12]. Serum-stimulated cell-cycle entry was barely detected when MEFs were seeded on low-stiffness FN substrata (elastic modulus < 2000 Pa), and the degree of S phase entry increased with matrix stiffness until optimal cycling was obtained at ~24,000 Pa (Figure 1A). Remarkably, the same range of matrix stiffness regulated S phase entry in MCF10A mammary epithelial cells (Figure 1A), vascular smooth muscle cells (VSMCs; Figure 1A), and osteoblastic cells (see Figure S1 available online). Thus, the effect of matrix stiffness on G1 and S phase progression is widely conserved and independent of the rigidity of individual tissue microenvironments. Mitogenesis (assessed as mRNA induction of cyclin A, an E2F target required for S phase entry and progression) was dependent on matrix stiffness regardless of whether cells entered G1 phase from G0 or G2/M (Figure 1B), indicating that both cell-cycle reentry from quiescence and subsequent cycling are controlled by ECM stiffness. Subsequent experiments used hydrogels prepared at