Bioen 326 2014 FINAL EXAM

Rules: **Closed Book Exam: Please put away all notes and electronic devices**

Reminders: We give partial credit, so include equations and steps.

Equations Provided on the Exam:

- $F_R = \int F_d(x)dx$ or $F_R = \int F_d(\vec{r})d\vec{r}$
- $x_R = \frac{\int xF_d(x)dx}{F_R}$, or $x_R = \frac{\int xF_d(\vec{r})d\vec{r}}{F_R}$
- $\sigma_{av} = \frac{\sigma_x+\sigma_y}{2}$
- $R = \sqrt{\left(\frac{\sigma_x-\sigma_y}{2}\right)^2 + \tau^2}$
- $\sigma_{x\theta} = \sigma_{av} + \frac{\sigma_x-\sigma_y}{2}\cos(2\theta) + \tau\sin(2\theta)$
- $\tau_\theta = -\frac{\sigma_x-\sigma_y}{2}\sin(2\theta) + \tau\cos(2\theta)$
- $\sigma_{1,2} = \sigma_{av} \pm R$
- $\tau_{MAX} = R$
- $\epsilon_x = \frac{1}{E}\sigma_x - \frac{\nu}{E}\sigma_y - \frac{\nu}{E}\sigma_z$
- $\gamma_{xy} = \frac{1}{G}\tau_{xy} = \frac{2(1+\nu)}{E}\tau_{xy}$
- $\sigma_x = \frac{E}{(1+\nu)(1-2\nu)}((1-\nu)\epsilon_x + \nu\epsilon_y + \nu\epsilon_z)$
- $\tau_{xy} = G\gamma_{xy} = \frac{E}{2(1+\nu)}\gamma_{xy}$
- $e = \frac{\Delta V}{V_0} = \epsilon_x + \epsilon_y + \epsilon_z = \frac{1-2\nu}{E}(\sigma_x + \sigma_y + \sigma_z)$
- $\delta = \int_0^L \frac{N(x)}{E(x)A(x)}dx$
- $k_a = \frac{EA}{L}$; $F = k_a\delta$
- $k_t = \frac{GI_p}{L}$; $T = k_t\phi$
- $\int_A y^2 dA = I$
- $I = \frac{\pi}{4}r^4$
- $I = \frac{H^3W}{12}$
- $I = \frac{H^3W}{36}$, (neutral plane is at H/3 from flat edge).
- $\int_A r^2 dA = I_p$
- $I_p = \frac{\pi}{2}r^4$
- $\kappa = M/EI$
- $\sigma_x = N/A$
- $\tau(r) = \frac{T}{I_p}(r)$
- $\sigma_x(x,y) = -\frac{M(x)}{I}y$
- $\tau_{xy}(x,y) = \frac{V(x)}{2I}\left(\frac{H^2}{4} - y^2\right)$, $\max\left(\tau_{xy}(x)\right) = \frac{3V(x)}{2A}$
- $\max\left(\tau_{xy}(x)\right) = \frac{4V(x)}{3A}$
- $\max\left(\tau_{xy}(x)\right) = \frac{4V(x)}{3A}\frac{(r_2^2+r_2r_1+r_1^2)}{r_2^2+r_1^2}$
- $\frac{d^2v}{dx^2} = \frac{M(x)}{EI}$
- $\sigma = \sigma_L = \frac{pr}{2t}$
- $\sigma_C = \frac{pr}{t}$
- $F_c = \frac{EI\pi^2}{L^2}$ (2 pins); $F_c = \frac{EI\pi^2}{4L^2}$ (solid support); $F_c \cong \frac{2EI\pi^2}{L^2}$ (pin and solid support); $F_c = \frac{4EI\pi^2}{L^2}$ (2 solid supports);
- $r = \sqrt{I/A}$; L/r is slenderness ratio
- Voigt $E\epsilon + \eta\frac{d\epsilon}{dt} = \sigma$
- Maxwell $\sigma + \frac{\eta}{E}\frac{d\sigma}{dt} = \eta\frac{d\epsilon}{dt}$
- Kelvin $\sigma + \frac{\eta}{E_S}\frac{d\sigma}{dt} = E_P\epsilon + \eta\left(\frac{E_P+E_S}{E_S}\right)\frac{d\epsilon}{dt}$
- Transforms:
  - $L[\delta(t)\,] = 1$
  - $L[\phi(t)\,] = 1/s$
  - $L[t\cdot\phi(t)] = 1/s^2$
  - $L[e^{-at}\,\phi(t)] = \frac{1}{s+a}$
  - $L[(1-e^{-at})\emptyset(t)] = \frac{a}{s(s+a)}$
  - $L[\sin(\omega t)\,\phi(t)] = \frac{\omega}{s^2+\omega^2}$
  - $L[\cos(\omega t)\,\phi(t)] = \frac{s}{s^2+\omega^2}$
- $k_BT = 4.1e-21\,J$ at room temp.
- $f(r) = \frac{k_BT}{L_p}\left(\frac{1}{4\left(1-\frac{r}{L_0}\right)^2} - \frac{1}{4} + \frac{r}{L_0}\right)$
- $f(r) \approx \frac{3k_BT}{2L_p}\frac{r}{L_0}$
- $r_{rms} = \sqrt{2L_0L_p}$
- $E = \frac{\frac{d^2U}{dr^2}(r_0)}{r_0}$
- $U_0[(r_0/r)^{12} - 2(r_0/r)^6]$
- $K_{eq}^0 = \frac{P_2}{P_1} = \exp\left(\frac{-\Delta G^0}{k_BT}\right)$
- $P_2 = \frac{K_{eq}}{1+K_{eq}}$
- $\Delta G(f) = \Delta G^0 - f\cdot\Delta x(f)$
- $\Delta G(f_{eq}) = 0$
- $\Delta x(f) = x_2(f) - x_1(f)$
- $\Delta x_{1t}(f) = x_t(f) - x_1(f)$
- $K_{eq}(f) = K_{eq}^0\exp\left(\frac{f\Delta x(f)}{k_BT}\right)$
- $k_{12}(f) = k_{12}^0\exp\left(\frac{f\Delta x_{1t}^0(f)}{k_BT}\right)$

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Cell

Matrix Elasticity Directs Stem Cell Lineage Specification

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SUMMARY

Microenvironments appear important in stem cell lineage specification but can be difficult to adequately characterize or control with soft tissues. Naive mesenchymal stem cells (MSCs) are shown here to specify lineage and commit to phenotypes with extreme sensitivity to tissue-level elasticity. Soft matrices that mimic brain are neurogenic, stiffer matrices that mimic muscle are myogenic, and comparatively rigid matrices that mimic collagenous bone prove osteogenic. During the initial week in culture, reprogramming of these lineages is possible with addition of soluble induction factors, but after several weeks in culture, the cells commit to the lineage specified by matrix elasticity, consistent with the elasticity-insensitive commitment of differentiated cell types. Inhibition of nonmuscle myosin II blocks all elasticity-directed lineage specification–without strongly perturbing many other aspects of cell function and shape. The results have significant implications for understanding physical effects of the in vivo microenvironment and also for therapeutic uses of stem cells.

INTRODUCTION

Adult stem cells, as part of normal regenerative processes, are believed to egress and circulate away from their niche (Katayama et al., 2006), and then engraft and differentiate within a range of tissue microenvironments. The tissue or matrix microenvironments can be as physically diverse as those of brain, muscle, and bone precursor osteoid (respectively, Flanagan et al. 2002; Georges et al., 2006; Kondo et al., 2005, Engler et al., 2004a; Ferrari et al., 1998; Andrades et al., 2001; Holmbeck et al., 1999; Morinobu et al., 2003). Mesenchymal stem cells (MSCs) are marrow-derived and have indeed been reported to differentiate into various anchorage-dependent cell types, including neurons, myoblasts, and osteoblasts (respectively, [Deng et al., 2005; Hofstetter et al., 2002; Kondo et al., 2005], [Pittenger et al., 1999], and [McBeath et al., 2004; Pittenger et al., 1999]). For differentiated cells such as fibroblasts, it is well known that responses to the typical soluble inducers such as growth factors couple to matrix anchorage (Nakagawa et al., 1989). However, with naive stem cells, direct effects of matrix physical attributes such as matrix stiffness have yet to be examined.

Differentiated cells ranging from neurons to osteoblasts adhere, contract, and crawl not only within soft tissues such as that of the brain or on top of crosslinked collagen ''osteoids'' in remodeling bone but also in vitro on collagen-coated acrylamide gels and glass (Figure 1A). Such a wide variation in matrix stiffness for differentiated cells is known to influence focal-adhesion structure and the cytoskeleton (Bershadsky et al., 2003; Cukierman et al., 2001; Discher et al., 2005; Engler et al., 2004a; Lo et al., 2000; Pelham and Wang, 1997). Past results with cells committed to a particular lineage, especially fibroblasts, on floating collagen gels and wrinkling-silicone sheets also suggest some responsiveness to the physical state of the matrix (Hinz et al., 2001; Nakagawa et al., 1989; Tomasek et al., 2002; Wozniak et al., 2003), but gel porosity and film topography complicate identification of possible contributions of substrate stiffness. In contrast, tissue-level matrix stiffness is distinct and shown here in sparse cultures to exert very strong effects on the lineage specification and commitment of naive MSCs, as evident in cell morphology, transcript profiles, marker proteins, and the stability of responses.

How might MSCs ''feel'' or sense matrix elasticity and transduce that information into morphological changes and lineage specification? At the molecular scale, matrix sensing first requires the ability to pull against the matrix and, secondly, requires a cellular mechano-transducer(s) to generate signals based on the force that the cell must generate to deform the matrix. Of the cell's cytoskeletal motors, one or all of the nonmuscle myosin II isoforms (NMM IIA, B, and C [Kim et al., 2005]) are candidates, as they are implicated in tensioning cortical actin structures

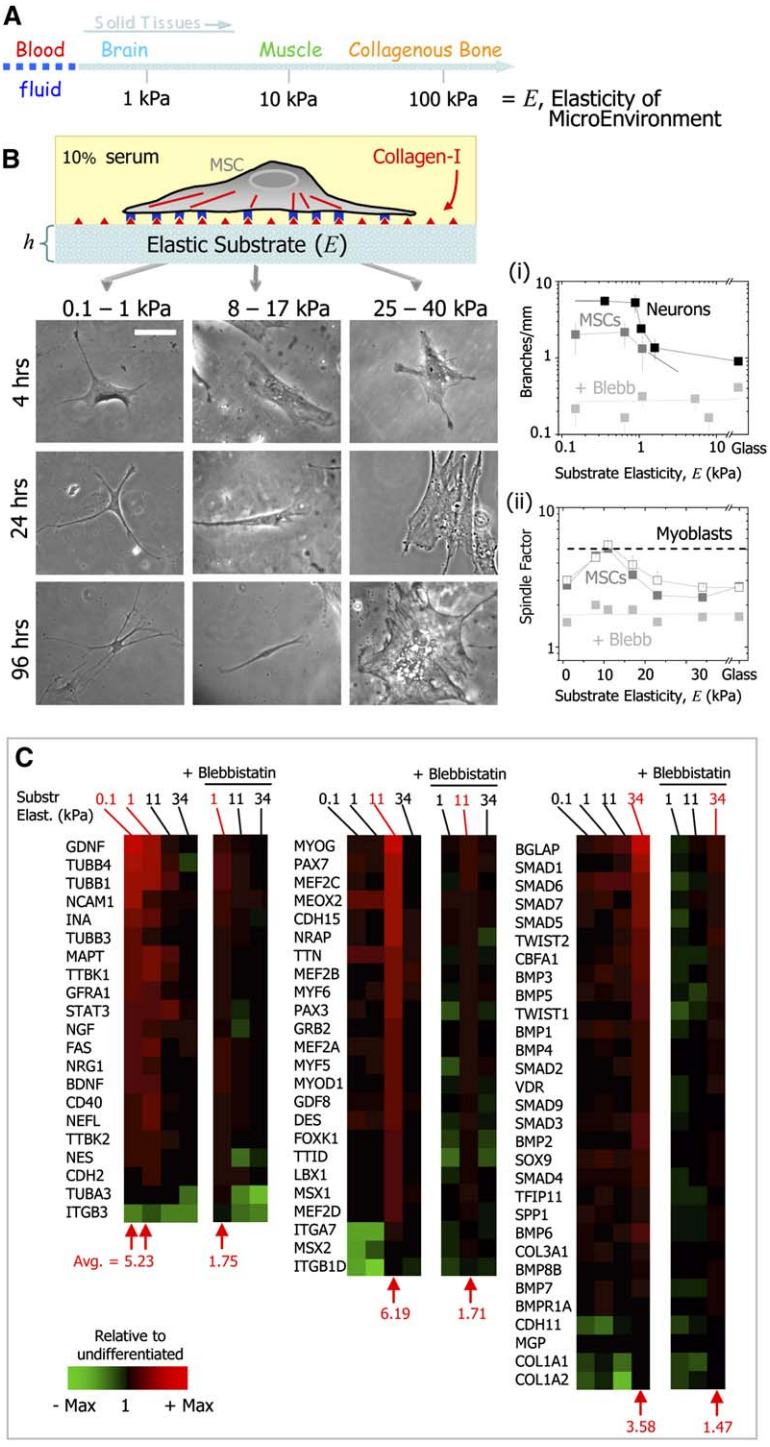

Figure 1. Tissue Elasticity and Differentiation of Naive MSCs

(A) Solid tissues exhibit a range of stiffness, as measured by the elastic modulus, *E*.

(B) The in vitro gel system allows for control of *E* through crosslinking, control of cell adhesion by covalent attachment of collagen-I, and control of thickness, *h*. Naive MSCs of a standard expression phenotype (Table S1) are initially small and round but develop increasingly branched, spindle, or polygonal shapes when grown on matrices respectively in the range typical of $\sim E_{brain}$ (0.1–1 kPa), $\sim E_{muscle}$ (8–17 kPa), or stiff crosslinked-collagen matrices (25–40 kPa). Scale bar is 20 μm. Inset graphs quantify the morphological changes (mean ± SEM) versus stiffness, *E*: shown are (*i*) cell branching per length of primary mouse neurons (Flanagan et al., 2002), MSCs, and blebbistatin-treated MSCs and (*ii*) spindle morphology of MSCs, blebbistatin-treated MSCs, and mitomycin-C treated MSCs (open squares) compared to C2C12 myoblasts (dashed line) (Engler et al., 2004a).

(C) Microarray profiling of MSC transcripts in cells cultured on 0.1, 1, 11, or 34 kPa matrices with or without blebbistatin treatment. Results are normalized to actin levels and then normalized again to expression in naive MSCs, yielding the fold increase at the bottom of each array. Neurogenic markers (left) are clearly highest on 0.1–1 kPa gels, while myogenic markers (center) are highest on 11 kPa gels and osteogenic markers (right) are highest on 34 kPa gels. Blebbistatin blocks such specification (<2-fold different from naive MSCs).

(McBeath et al., 2004; Wang et al., 2002). These actin structures are in turn linked to focal adhesions that provide the pathway of force transmission from inside the cell to the elastic matrix (Beningo et al., 2001; Tamada et al., 2004) and associated with the focal-adhesion complexes are a number of well-known signaling molecules that are well-placed to act as the mechano-transducers (Bershadsky et al., 2003; Alenghat and Ingber, 2002). With MSCs here, we demonstrate that one or all of the NMM IIA–C are likely to be involved in the matrix-elasticity sensing that drives lineage specification.

The resistance that a cell feels when it deforms the ECM is measured by the elastic constant, *E*, of the matrix or microenvironment. For microenvironments of relevance here,

that of the brain (Flanagan et al., 2002) is considerably softer than muscle (Engler et al., 2004a), and muscle is softer than collagenous osteoid precursors of bone (measured here). The wide range of microenvironment elasticity highlighted in Figure 1A is central, we show, to predicting specification of MSCs. Matrix elasticity is mimicked in vitro here with inert polyacrylamide gels in which the concentration of bis-acrylamide crosslinking sets the elasticity (Pelham and Wang, 1997), and adhesion is provided by coating the gels with collagen I, which is known to support myogenic and osteogenic differentiation (Engler et al., 2004a; Garcia and Reyes, 2005). Using this well-defined, elastically tunable gel system (Figure 1B), as opposed to wrinkling films or degrading collagen gels (Hinz et al., 2001; Wozniak et al., 2003), we provide the first evidence with sparse cultures of MSCs that matrix can specify lineage toward neurons, myoblasts, and osteoblasts—all in identical serum conditions. We document the matrix regulation of key lineage markers and myosins, including NMM IIs, which—when inhibited with blebbistatin (Straight et al., 2003)—blocks differentiation. We also show that soluble induction factors tend to be less selective than matrix stiffness in driving specification, and cannot reprogram MSCs that are precommitted for weeks on a given matrix. Finally, by controlling gel thickness, h, we establish how far stem cells can feel and thus physically define their microenvironment.

RESULTS

Cell Morphology Suggests Lineage Specification Is Directed by Matrix Stiffness and Dependent on Nonmuscle Myosin II

On soft, collagen-coated gels that mimic brain elasticity ($E_{brain} \sim 0.1$–1 kPa) (Flanagan et al., 2002), the vast majority of MSCs adhere, spread, and exhibit an increasingly branched, filopodia-rich morphology (Figure 1B). Branching densities after 1 week in culture approach those of primary neurons on matrigel-coated gels (Flanagan et al., 2002), and the dynamics of outward extension with branching is clearly opposite to DMSO-induced retraction of the cell body that can leave pseudoextensions behind (Neuhuber et al., 2004). MSCs on 10-fold stiffer matrices that mimic striated muscle elasticity ($E_{muscle} \sim 8$–17 kPa) lead to spindle-shaped cells similar in shape to C2C12 myoblasts (Engler et al., 2004a). Stiffer matrices (25–40 kPa) that we show below mimic the crosslinked collagen of osteoids (Garcia and Reyes, 2005; Kong et al., 2005) yield polygonal MSCs similar in morphology to osteoblasts. Analyses of cell morphologies (Figure 1B; plots i and ii) show that matrix-dependent shape variations of MSCs are similar to differentiated cells. It is important to also note in these plots and elsewhere below that the results with stiff acrylamide gels extrapolate to those with collagen-coated, rigid glass; this is expected if substrate elasticity is a key variable of importance. Furthermore, since the inhibition of proliferation by mitomycin-C (open squares, Figure 1B; plot ii) has little impact on average cell shape, the morphology results are consistent with lineage development being a population-level response to substrate elasticity.

As introduced above, nonmuscle myosin II is likely to be involved in exerting force through focal adhesions in mechanisms of sensing matrix elasticity. All of the NMM II isoforms are inhibited by blebbistatin, which does not inhibit any other myosin found in MSCs (see below), other than myosin VI (Limouze et al., 2004). Addition of blebbistatin during plating blocks branching, elongation, and spreading of MSCs on any substrate (Figure 1B; plots); however, addition of blebbistatin 24 hr postplating does not significantly reverse cell shape or spreading on E_{muscle} gels after the cells have already spread and adopted a spindle morphology (e.g., 24 hr per Figure 1B). Less specific and less potent myosin inhibitors such as BDM (at ~mM concentrations) are already known to block neuronal motility as well as the sensitivity of differentiated cells to substrate elasticity (Pelham and Wang, 1997), but blebbistatin is far more selective and potent (Straight et al., 2003). It inhibits actin activation of NMM II ATPase activity (at ~μM concentrations) and blocks migration and cytokinesis in vertebrate cells without affecting MLCK. Crystal structures show inhibition of actin-activated ATPase activity by blebbistatin (Allingham et al., 2005) requires a specific alanine (or serine) residue that is found only in class II and VI myosins (Limouze et al., 2004; Straight et al., 2003). We confirm below that MSCs express the three NMM IIs and myosin VI, but we implicate NMM IIs and the cytoskeleton as critical to differentiation.

To reinforce this conclusion and to rule out a role for myosin VI in matrix sensing, we repeated the above experiments with the myosin light chain kinase (MLCK) inhibitor, ML7 (Dhawan and Helfman, 2004). Of the myosins found in MSCs thusfar (see below), regulatory light chain phosphorylation via MLCK is only used to activate the NMM IIs. ML7 will block activation of these as well as smooth muscle myosin isoforms but will not affect activation of any other myosins in MSCs. Results with ML7 prove below to be identical to those seen with blebbistatin, and so NMM II activity appears to be necessary for matrix elasticity-driven lineage specification.

RNA Profiles Indicate Lineage Specification on Matrices of Tissue-like Stiffness

Transcriptional profiles of neurogenic, myogenic, and osteogenic markers—from early commitment markers through mid/late development markers—prove consistent with indications from morphology. On the softest gels, MSCs show the greatest expression of neurogenic transcripts (Figure 1C, left column; Table S3). Neuron-specific cytoskeletal markers such as nestin, an early commitment marker, and β3 tubulin, expressed in immature neurons, as well as the mature marker neurofilament light chain (NFL) (Lariviere and Julien, 2004) and the early/midadhesion protein NCAM (Rutishauser, 1984), are all upregulated. In terms of a simple average across various key