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Cross-linked matrix rigidity and soluble retinoids synergize in nuclear lamina regulation of stem cell differentiation

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Abstract

Synergistic cues from extracellular matrix and soluble factors are often obscure in differentiation. Here, the rigidity of crosslinked collagen synergizes with retinoids in the osteogenesis of human marrow mesenchymal stem cells (MSCs). Collagen nano-films serve as a model matrix that MSCs can easily deform unless the film is enzymatically crosslinked, which promotes the spreading of cells and the stiffening of nuclei as both actomyosin assembly and nucleoskeletal lamin-A increase. Expression of lamin-A is known to be controlled by Retinoic Acid Receptor (RAR) transcription factors, but rigid matrix is needed to induce rapid nuclear accumulation of the RARG isoform and subsequent expression of osteogenic markers. Rigid substrates such as culture plastic are also required for RARG-specific antagonist to increase or maintain expression of lamin-A and for agonist to repress expression. The findings extend to a Progerin allele of lamin-A in iPSC-derived MSCs. Consistent with functional synergy, RARG-antagonist treatment drives lamin-A dependent osteogenesis on rigid substrates, with pre-treated xenografts calcifying to a similar extent as native bone. Proteomics-detected targets of mechanosensitive lamin-A and retinoids underscore the convergent synergy of insoluble and soluble cues in differentiation.

(180 words)
Introduction

Stem cells differentiate in response to microenvironmental cues that derive from surrounding matrix, cell contacts, and soluble factors (Fuchs et al., 2004; Nelson and Bissell, 2006; Engler et al., 2006), but synergy and convergence between cues is under-studied (Fig. 1A). Mesenchymal stem cells (MSCs) are classically isolated by adhesion to rigid plastic but are clearly affected in differentiation by the stiffness of a synthetic gel with matrix ligand (Engler et al., 2006) as well as by many soluble factors, such as retinoic acid (RA) (Swift et al., 2013b) which widely regulates RA receptor (RAR) transcription factors in differentiation (Williams et al., 2009). MSCs in bone marrow contribute to osteogenesis (Park et al., 2012), but they also reside in many tissues within perivascular niches where they contribute to fibrosis and some differentiated lineages (Kramann et al., 2015) as well as neoplasms (Medyouf et al., 2014). Many recent studies of differentiation and disease with various stem cells and gels (eg. Musah et al., 2014) have left unaddressed whether one key physiological modification that should stiffen matrix, namely enzymatic crosslinking, can impact the differentiation effects of equally physiological soluble factors such as RA. Stiffening of bulk matrix by enzymatic crosslinking affects cancer cells in vitro and in vivo (Cox et al., 2013), but differentiation studies at the single cell scale with nano-control of crosslinking could provide deeper insight into synergy of matrix elasticity with a potent soluble factor such as RA.

Collagen-I is not only the most abundant protein in animals and a well-known target of enzymatic crosslinking but is also intrinsically pro-osteogenic (Yener et al., 2008). Cells attach to collagenous matrix and use actomyosin forces to pull on it (Discher et al., 2005), with stiff matrices driving cytoskeleton assembly and cell spreading within hours (Engler et al., 2006). Nuclei flatten and also spread with the cell, and the level of the ‘keratin-like’ nuclear protein lamin-A increases, consistent with lamin-A (but not lamin-B) being high in collagen-rich tissues like bone but low in soft tissues lacking collagen such as marrow and brain (Swift et al., 2013b). Lamin-A mutations affect many stiff tissues as illustrated by defects in muscle, bone, and skin in the accelerated aging syndrome Progeria, whereas soft tissues remain unaffected (Bridger et al., 2007). Such observations suggest matrix-linked regulatory roles for lamins, and recent surprising results for lamin levels in MSCs on gels further show that retinoid regulation occurs only with cells on stiff substrates as opposed to soft gels (Swift et al., 2013b). Promoter-reporter approaches have already shown that the promoter region of the LMNA gene binds RAR transcription factors (Okumura et al.,
so that changes in lamin-A message can be caused by at least some retinoids in culture. However, it remains unclear which of the three known RAR isoforms (RARA, RARB, &/or RARG) and which (if any) isoform-specific soluble factors are relevant to regulation in MSCs – especially when cultured on physiological matrices with enzymatic crosslinking.

Stiffness measurements of the tibia’s osteogenic niche here are followed by meta-analyses of transcriptomes from tissues that span a wide range of stiffness and that suggest general associations of collagen-I with matrix crosslinking, myosin contractile forces, lamin-A, RARG, and osteogenic induction. These tissue-level associations are all observed in culture with naïve MSCs on collagen-I nano-films that are crosslinked rather than pristine, and the results suggest a mapping into gels of suitable effective stiffness. RARG’s nuclear localization in cells on stiff substrates helps to explain the rigidity-dependent regulation of lamin-A expression by soluble RARG agonists/antagonists, and the findings are extended to lamin-A’s ‘progerin’ splice-form in iPSC-derived MSCs from a Progeria patient (Olive et al., 2010). Matrix rigidity synergizes with RARG agonist suppression of normal and progerin lamin-A, and rigidity also synergizes with RARG antagonist to increase lamin-A and to enhance osteogenesis in vitro and in vivo. The approach is likely relevant to fibrosis of the perivascular niche (Kramann et al., 2015), in which a thin layer of matrix is enriched in crosslinked collagen-1 to drive – in the absence of osteogenic soluble factors – a fibrogenic phenotype with high actomyosin contractility, lamin-A, and RARG (Dingal et al., 2015).

Results

Cell-scale stiffness of the osteogenic Niche. In bone formation, MSCs egress from a niche that is likely perivascular and migrate to a pre-calcified surface in differentiation to osteoblasts (Park et al., 2012). In vitro studies show that matrix stiffness directs MSC migration (Raab et al., 2012) as well as differentiation (Engler et al., 2006), and yet no measurements exist for the in situ mechanical properties of osteoid matrix at the micro-scale that a cell adheres to and probes. For cartilage, atomic force microscopy (AFM) has been used by Aebi and colleagues (Stolz et al., 2009) to measure an interstitial elastic modulus $E$ at a scale which approximates that of the matrix surrounding chondrocytes (Guilak et al., 2005) and which proves to be 10-fold or more softer than the macroscopic rigidity of cartilage.
To study the osteogenic niche in bone, mouse tibia was sliced along its length (Fig. 1B), and AFM nano-indentation (Fig. 1C) was applied to the exposed marrow and then to the inner surface of bone after washing away marrow. Samples were fixed and stained for alkaline phosphatase (ALP) activity, which confirmed the presence of osteoblastic cells (Fig. 1D), while high resolution AFM imaging revealed fibrillar matrix (Fig. 1E). Force-versus-indentation data (Fig. 1F) revealed $E$ for marrow to be $\sim 0.1$ kPa (kilo-Pascal), versus a much stiffer bone surface with peaks at 2, 30 and 100 kPa (Fig. 1G). The softest peak is close to $E$ for isolated cells of mesenchymal origin (Titushkin and Cho, 2007; Yourek et al., 2007; Darling et al., 2008; Yim et al., 2010). The 30 kPa and 100 kPa peak could reflect the elastic response and the spatial heterogeneity of the mineralized matrix due to the presence of interfibrillar and extrafibrillar bioapatite (Alexander et al., 2012). Stiffer substances were also detected and likely correspond to calcified bone (~GPa) but cannot be resolved with soft cantilevers. The 30 kPa peak is consistent with $E$ of the osteoid matrix secreted by cultured osteoblasts (Engler et al., 2006). Osteogenesis is thus associated with stiffer tissue than the marrow space that is filled with hematopoietic cells. To address whether such stiff matrix has osteogenic specificity or not, a compositional comparison was made to other collagen-rich tissues.

**Proteomics and transcriptomic meta-analysis identify possible factors in MSC osteogenesis.**

Mass spectrometry (MS) proteomics of bone compared to two other stiff tissues, muscle and skin, (Fig. 2A i-iii) showed that all had an abundance of collagen-I (Col1a1 and Col1a2). Collagen-1 is not only the most abundant protein in our bodies (Neuman and Logan, 1950) and a key determinant of tissue mechanics (Swift et al., 2013b), but is also the main scaffold protein of osteoid that acts together with soluble factors in a niche for MSC osteogenesis and bone mineralization (Katz and Li, 1973; Stein et al., 1990). To provide evidence for such a process, human-MSCs were cultured with standard osteogenic soluble factors, engrafted into mouse flanks, and excised after four weeks for profiling by MS. Human-specific peptides were $\sim 10\%$ of all peptides (Fig. S1A) and overall profiles (Swift et al., 2013a; Swift et al., 2013b) compare well to MSC cultures (Fig. 2A iv, v) with a matrix complexity similar to mouse bone rather than mouse skin at the graft site. Interestingly, periostin (Postn) is known to activate the cross-linking enzyme lysyl oxidase (Lox) which stiffens collagen (Maruhashi et al., 2010).
MS profiling of tissues shows stiffer tissues have more fibrillar collagen (with bone > muscle > fat > brain), and so for a diverse set of tissues we conducted a meta-analysis of transcriptomes to ask what transcripts generically associate with collagen-I (COL1A1) in man and mouse (Fig. 2B). A wide range of soft thru stiff tissues was analyzed because MSCs are found in most tissues beyond marrow (Kramann et al., 2015), and MSCs can be induced toward many tissue lineages. Bone transcriptomes were excluded so that the generic nature of any correlations might be assessed in an unbiased way with MSC cultures and osteo-induction. COL1A1 mRNA scaled with protein across many tissues (Fig. S1B), and the top few % of COL1A1-correlated transcripts suggests generic relationships with: (i) collagen crosslinking, (ii) a tensed actomyosin cytoskeleton, (iii) a stiff nucleus with high lamin-A, and (iv) osteogenesis (Fig. 2C). Fibrillar collagens generally correlate with COL1A1 as do enzymes that crosslink collagen, particularly LOX but also tissue transglutaminase (TGM2) to a more limited extent. Smooth-muscle actin (ACTA2) is a well-known marker of cytoskeleton tension (Wipff et al., 2007) as is non-muscle myosin-IIA (MYH9), and both correlate with COL1A1, as does nucleoskeletal lamin-A (LMNA) which we had shown confers nuclear stiffness (Swift et al., 2013b). No correlations are seen with ubiquitous β-actin (ACTB) nor B-type lamins (LMNB1, LMNB2), with the latter consistent with near-constant protein expression across tissues (Swift et al., 2013b). Lamin-A transcription is regulated by RA receptor (RAR) transcription factors (Swift et al., 2013b), but COL1A1 correlates only with RARG, which is the one isoform known to contribute to osteogenesis (Williams et al., 2009). Surprisingly, even though bone is not included in this meta-analysis, COL1A1 shows moderate correlations with the early osteogenic transcription factor RUNX2 and with the late osteogenic marker of bone matrix SPARC (p ~ 0.5).

Skin transcriptomes from mice were analyzed in order to challenge the molecular associations above and to also assess their possible relevance to subcutaneous xenografts (Fig. 2A). RNA-seq data recently produced from both healthy tissue and chemically-induced squamous cell carcinoma (Nassar et al., 2015) shows a near linear relation versus Col1a1 for Col3a1, Lox and Acta2 whereas Actb is constant across both healthy and cancerous skin (Fig. 2D). Myh9 also increases with Col1a1 in healthy tissue but remains constant in cancer. For normal tissue but not cancer, Lmna increases with Myh9, and Rarg (but not Rara) increases with Lmna. Therefore, together with our recent findings that stiff matrix promotes actomyosin contractile stress which stabilizes lamin-A expression in MSCs, the meta-analyses above suggest
a hypothesis to test \textit{in vitro}: if stiff, crosslinked collagen ultimately couples to RARG levels and nuclear localization, then both rigid matrix and RARG-specific soluble factors will jointly modulate lamin-A expression and osteogenesis (Fig. 2E).

**Enzymatic crosslinking stiffens collagen-I nanofilms to drive symmetric spreading of MSCs.** As a minimal culture substrate to use for testing the above working hypothesis, nanofilms of collagen-I were assembled on untreated mica substrates (Cisneros \textit{et al.}, 2007) and the films were either left ‘pristine’ or else enzymatically crosslinked (Fig. 3A). TGM2 was used for crosslinking because it is broadly expressed (Fig. 2C) and required for fibronectin-collagen networks in early matrix genesis (Al-Jallad \textit{et al.}, 2006). TGM2 is also found in bone and in osteoblastic cells associated with matrix mineralization (Heath \textit{et al.}, 2001; Kaartinen \textit{et al.}, 2002). Collagen-I fibrils are only 300 nm long, but self-assembly of these semi-flexible rods produces highly-ordered fibrillar films that – regardless of crosslinking – exhibit in physiological buffers the expected D-spacing of 67 nm (Meek \textit{et al.}, 1979) as well as fibril heights of either 2.2 nm or 1.7 nm (Fig. 3B i, iii; Fig. S2A, B) consistent with staggered tropocollagen.

Nano-film mechanics were altered by collagen crosslinking. Pristine films are anisotropic with higher tensile strength in the long axes than in the perpendicular direction (Friedrichs \textit{et al.}, 2007), and fibrils are attached to the atomically flat and rigid mica substrate only by weak electrostatic interactions so that fibrils are readily displaced and deformed. Lateral forces on fibrils can be exerted by an AFM tip moved across the surface while applying a normal force of ~nano-Newton which cells can easily exert via their adhesions (Discher \textit{et al.}, 2005), (Geiger \textit{et al.}, 2009); subsequent imaging by AFM in tapping mode reveals unraveling of fibrils in pristine films whereas cross-linked fibrils remain intact though slightly bent (Fig. 3B ii, iv). Higher normal forces of 20 nN (Fig. S2A, B) produced local deformations of nanofilms that suggested crosslinking increased film stiffness by at least ~2-fold, consistent with earlier measurements of dialdehyde-crosslinked films (Friedrichs \textit{et al.}, 2007). Slopes of lateral force signal versus distance were likewise ~3-fold higher for crosslinked films compared to pristine films (Fig. S2C, D). As a further check on the effects of TGM2 addition, some MSC xenografts (Fig.2A) that were removed from mice for profiling were treated or not with TGM2 and deformed by micropipette aspiration (Majkut \textit{et al.}, 2013). Crosslinked grafts under
such stress relaxed at a slower rate than untreated grafts (Fig.S1C), which is consistent with a more solid-like response due to crosslinking.

Human bone marrow MSCs that were seeded on the nanofilms showed morphology differences within hours. On pristine nanofilms, immunofluorescence imaging showed predominantly spindle-shaped MSCs (Fig. 3C I; Fig. S2C, D), and AFM imaging at sub-micron scales showed cell protrusions and stress fibers aligned with collagen (Fig. S2E). Immunofluorescent collagen showed cells displace the flexible network and bundle fibrils into thick fiber bundles (Fig. 3C ii) that are expected to withstand large tensions as cells adhere and pull (Friedrichs et al., 2007). On crosslinked nanofilms in contrast, MSCs exhibited a distinctly well spread and rounded osteo-like morphology (Fig. 3C iii), and collagen was not detectably displaced by cells (Fig. 3C iv). Pulling forces exerted by a cell on its matrix have an (‘equal and’) opposite action within the cell, in promoting acto-myosin network formation and orientation of stress fibers (Zemel et al., 2010). Indeed, cells grown on pristine collagen films show tight alignment of stress fibers that is absent from cells on crosslinked nano-films (Fig. S2C, D).

**Cell morphologies map responses on nanofilms into equivalent soft or stiff hydrogels.** Synthetic polymer systems of controlled stiffness $E$ are widely reported to drive spreading of diverse cell types (Pelham and Wang, 1997; Engler et al., 2006; Geiger et al., 2009), and such gels provide standards to compare to almost any other new substrate, including a nanofilm. MSCs not only spread more on stiff gels than on soft gels but they also elongate on an intermediate $E_{\text{gel}}$ even though gels are isotropic (Engler et al., 2006; Rehfeldt et al., 2012). The increased cell spread area versus $E_{\text{gel}}$ fits a hyperbolic function independent of substrate material (Rehfeldt et al., 2012), and so spread areas of the same MSCs on nanofilms here can be used to infer an equivalent $E_{\text{gel}}$ felt by cells (Fig. 3D i). MSCs on pristine films thus spread on 10 kPa gels to the same extent, and MSCs on crosslinked films spread the same as on 50 kPa.

With fixed cells on stiff substrates, a large value for nuclear spread area (as a projected area) reflects nuclear flattening (Lovett et al., 2013), and for live cells on the nanofilms our AFM measurements of nuclear height confirm such flattening (Fig.S2F). Consistent with a simple flattening of the nuclear lamina in spreading on stiff substrates, total lamin-B is independent of matrix stiffness (Fig.S2G-i). In contrast, lamin-A increases in the same dual-stained MSCs (Fig.S2G-ii). The increased stoichiometry for lamin-A:lamin-
B versus microenvironment stiffness is consistent with in vitro and in vivo results for cancer cell lines (Swift et al., 2013b) and with mRNA trends for normal tissues (Fig. 2C). Nuclear projected areas are also consistent with the effective elasticities (Fig. 3D ii) as are cell and nuclear aspect ratios and stress fiber alignment (Fig. 3E i,ii). Thus, while AFM nano-scale measurements of the films suggest a >2-fold increase in stiffness after crosslinking, cell and nuclear morphology analyses suggest a ~5-fold increase in the stiffness that cells sense. Importantly, since osteogenesis of MSCs on gels with $E_{gel} > 20-40$ kPa greatly exceeds that of MSCs on 10 kPa gels (Engler et al., 2006; Rehfeldt et al., 2012), osteo-induction was next hypothesized to be highest for the crosslinked nanofilms.

**Crosslinked Nanofilms promote Myosin-II, Lamin-A, RARG, and osteogenesis.**

Cells on soft gels are reportedly softer than cells on stiff gels (Solon et al., 2007; Liu et al., 2014; Pagliara et al., 2014; Staunton et al., 2016), but any cell indentation device such as an AFM in reality measures the effective elasticity of the cell and gel below it, analogous to two springs in series (Staunton et al., 2016). The ~2 nm films here are ~1000-fold thinner than cells and are on rigid substrates, and so moderate indentations reveal a cell’s elasticity without need for complex decoupling of the mechanical contribution of the substrate (Fig. 4A). Force-volume maps of MSCs adhering to either pristine or cross-linked nanofilms thus reveal apparent cell elasticities in the ~kPa range with the nucleus seeming to be stiffer than peri-nuclear regions (Fig. 4B). By probing many MSCs in the nuclear region (~1 μm deep), this apparent nuclear stiffness of cells on cross-linked nanofilms proves ~2-fold higher than for cells on pristine collagen films (Fig. 4C).

To assess the contribution of lamin-A to nuclear stiffness – which could be direct or indirect through other partners and pathways – we knocked lamin-A down by ~50% and found a proportional decrease in apparent nuclear stiffness (Fig. 4D). This is consistent with direct contributions to stiffness of lamin-A in MSCs and other cell types as studied by micropipette aspiration in which measurements are made after cell detachment and actin depolymerization within 1-2 hrs (Harada et al., 2014). However, because it is also known that inhibiting the contractile actomyosin network with blebbistatin decreases ‘Nuclear Tension’ through a decrease in both nuclear area and lamin-A levels in MSCs within hours, inhibition of nuclear tension should also make the nucleus measurably softer (analogous to a tensed rope being laterally stiffer.
until the rope is cut). A ~4-fold reduction in the apparent nuclear stiffness with blebbistatin is indeed measured and is consistent with recent AFM studies comparing isolated nuclei to nuclei in intact cells (Liu et al., 2014). Blebbistatin plus lamin-A knockdown combined did not further decrease the apparent nuclear stiffness in the MSCs, probably because (i) blebbistatin alone tends to quickly decrease lamin-A levels (Buxboim et al., 2014), and (ii) lamin-A knockdown alone reduces the levels of myosin-II in MSCs over days through the SRF pathway (Ho et al., 2013; Swift et al., 2013b; Buxboim et al., 2014; Talwar et al., 2014). In other words, endogenous lamin-A, contractility, and nuclear stiffness are all mechanistically linked – at least in these cells.

MSCs expressing ectopic GFP-lamin-A (Fig. S3A) had an apparent nuclear stiffness (on cross-linked nanofilms) that increased with GFP-lamin-A level. To eliminate contributions of the cytoskeleton, MSCs were detached, the actomyosin cytoskeleton was depolymerized with latrunculin, and micropipette aspiration was used to measure nuclear stiffness in suspended cells. The results demonstrate increased nuclear stiffness with GFP-lamin-A independent of cytoskeleton (Fig. S3B). Nonetheless, time-dependent changes in apparent nuclear stiffness over 2 weeks in culture on cross-linked nanofilms (Fig. S3C) are likely a result of changes in both lamin-A levels and actomyosin tension.

For MSCs cultured on pristine or cross-linked films, immunofluorescence analyses of lamin-A nuclear area, and myosin-IIA in single cells at 2 weeks (Fig. 4E i,ii) generally revealed that LMNA correlated with increased nuclear spreading and with levels of MYH9 (Fig. 4E iii,iv). Importantly, collagen cross-linking caused a 1.5-fold average increase in LMNA, which is consistent with observations of MSCs on soft versus stiff gels (Fig. 4E v,vi) (Swift et al., 2013b; Buxboim et al., 2014). Scatterplots for all of these single-cell analyses always show that some cells on the cross-linked films fall within the response envelopes of cells on pristine films, so that this shared sub-population of non-responding cells at least provides a common basis for extrapolation of the trend. A heatmap of quantified morphologies and protein levels in MSCs on the nano-films and gels of varied stiffness (Fig. 4F) clusters together the cell phenotype on pristine films with that on 10 kPa gels and separately clusters together the cell phenotype on crosslinked films with that on 40 kPa gels, whereas cell phenotypes on soft gels are most distinct. Compared to similar analyses of transcriptomes, this direct alignment of cell phenotypes is more likely to reflect mechanosensitive processes such as protein degradation that sometimes decouple from transcript levels (Dingal et al., 2015).
Immunostaining for the transcription factor RARG that generally correlates with lamin-A in tissues (Fig. 2C) and that can in principle regulate LMNA transcription (Swift et al., 2013b) showed that the total amount of RARG increased on cross-linked films after just 2 days in culture (Fig. 4G i-iii). RARG’s nuclear-to-cytoplasmic ratio in cells on nanofilms also correlated with lamin-A intensity in a cell-by-cell analysis, which is in good agreement with similar trends for cells on gels (Fig. 4G iv-v). Mechanistic links are supported below by short time kinetic studies that use soluble RA ligand.

Based on the surprisingly general association between collagen crosslinking and levels of key osteogenic factors (Fig. 2C), MSCs on the two types of nanofilms were assessed for osteo-induction without addition of any osteogenic soluble factors. RUNX2 is an early osteogenic transcription factor required for osteogenesis (Choi et al., 2001); RUNX2 was mostly nuclear after 1 wk in culture and >2-fold more so on cross-linked nanofilms (Fig. 4H). The results are consistent with matrix elasticity directed lineage specification on stiff gels even in the absence of ‘osseo induction media’ (Engler et al., 2006; Benoit et al., 2008; Chen et al., 2010). SPARC (or osteonectin) is a late marker of osteogenesis and is a bone glycoprotein secreted by osteoblasts to initiate mineralization (Termine et al., 1981); SPARC was also >2-fold higher after 2 wk on cross-linked films. Nuclei in the more osteogenic state were more spread ($p < 0.01$), which once again suggests higher cytoskeletal tension on the stiffer matrix. The findings thus suggest (i) the stiffness of crosslinked nanofilms is osteo-inductive, and (ii) stiffness sensing might be amplified by coupling to soluble factor regulation of the increased nuclear RARG.

**RARG antagonist/agonist respectively increase/maintain or decrease LMNA and Progerin only on stiff substrates.** RA signaling pathways affect many developmental and homeostatic processes. The pan-RAR antagonist “AGN” (AGN193109, see Fig. S4A) was found in our recent study to enhance osteogenesis of MSCs cultured in osteo-induction media (OIM) (Swift et al., 2013b). RARG-specific agonists slow ectopic bone growth in mouse models (Shimono et al., 2012), but antagonists have been unexplored. Here we tested antagonists or agonists that are more specific to RARG (Fig. S4A), and for a wider range of MSCs we first measured the effects on LMNA levels. Whereas agonists tended to decrease LMNA, antagonists tended to increase LMNA on standard rigid culture plastic (Fig. 5A). To clarify the trend on various stiff or rigid substrates, the (antagonist / agonist) effect on LMNA was tabulated across primary MSCs for various
drug pairs and gave 1.8 ± 0.2 for the mean ratio (± SEM) (Fig. 5B, S4B). Donor variability of MSCs is at least one source of variation.

Induced pluripotent stem cells (iPSC) can be a more uniform source of MSCs that turn on expression of lamin-A with loss of pluripotency (Zhang et al., 2011; Talwar et al., 2013). The iPSC-MSCs also have no memory of the many factors in a bone marrow niche. Application of retinoids to iPSC-MSCs gave (antagonist / agonist) effects on LMNA levels that were statistically the same as primary bone marrow MSCs (Fig. 5B). Importantly, Progeria patient derived iPSC-MSCs showed similar effects on lamin-A levels with both RARG-specific and non-specific drugs (Fig. 5C, S5F). Although Progerin is a mutated form of lamin-A protein that causes accelerated aging, expression of the Progerin allele should be unaffected. Immunoblots showed the drugs affect Progerin from the disease-causing allele as much as the normal lamin-A splice-forms (Fig. 5C – lower bargraph, S5E). Retinoid effects are strong in early passages when cultured on rigid substrates where the nucleus is well spread (Fig.S5C). Agonist treatment also resulted in a ~40% increase of nuclei with irregular shapes and showed LMNA enrichment in high curvature regions (tips) (Fig.S5D). Past studies with other cell types showed increased progerin results in misshapen nuclei (Goldman et al., 2004), but cytoskeletal forces in the MSCs studied here could have different effects on nuclei. For all of the different MSCs tested, the findings are nonetheless consistent with retinoids contributing to transcriptional control of LMNA.

To test the functional consequences of low nuclear RARG that was seen in MSCs on pristine collagen nanofilms (Fig. 4F), the RARG-specific antagonist CD2665 was compared to the RARG-specific agonist CD1530 in terms of effects on LMNA in MSCs on the two types of nanofilms. The crosslinked nanofilm produced the expected LMNA ratio for (antagonist / agonist), but the pristine nanofilm – which we had shown was soft (Fig. 3D,E) – gave no significant retinoid effects (Fig. 5D). Lamin-A levels were nonetheless highest in MSCs on cross-linked nanofilms, consistent with (Fig. 4E). For insight into the kinetics of the effect of the RARG-specific antagonist CD2665, MSCs on a rigid substrate were treated with drug and imaged. Within 40 min, nuclear localization of RARG increased (by ~15%; p=0.05) and remained high, while LMNA required 4 hrs to be significantly higher (Fig. S4C). Altogether, these findings suggest a fast-acting cooperation between soluble factor and mechano-sensitive pathways.
To assess the retinoid responsiveness in cooperation with matrix stiffness we examined how Lamin-A level depends on nuclear area when treated with RA agonist/antagonist. First we established the reversibility of nuclear spreading: iPS cells were induced to differentiate into MSCs with well-spread cells and nuclei on gelatin-coated plastic (which is rigid with $E > 100$ kPa based on AFM measurements), but replating onto soft gels (2 kPa) caused a decrease in both nuclear area and its variation (Fig.5E). Parallel analyses of distributions for Lamin-A as well as nuclear area revealed a ‘weak responder’ sub-population of cells on stiff gels that respond like 95% of cells on soft gels. About one-third of cells sense the stiffness and spread with high Lamin-A, and retinoids affect an equal fraction (32% to 36%) in regulating Lamin-A levels (Fig.5F i). The same proved true for bone marrow derived MSCs on pristine and cross-linked films. Importantly, retinoids have no effect on nuclear translocation of RARG in cells on either film, even though the cross-linked matrix drives more RARG into the nucleus and Lamin-A decreases with agonist while increasing slightly with antagonist (Fig.5 F ii).

A parsimonious dynamical model of the mechano-chemical effects on the $LMNA$ gene circuit was developed to fit the data (Fig. 6A). We incorporated the recent finding that lamin-A protein is stabilized by cytoskeletal stresses on stiff matrices (Jain et al., 2013). Similar models have been developed for cytoskeletal regulation of Serum Response Factor genes (Jain et al., 2013). Soluble factor effects on RARG translocation for $LMNA$ transcription were incorporated (Fig. 6A), and variation of (agonist / antagonist) effects on lamin-A levels (eg. cell-to-cell variation) were emulated by varying the relative strength of RARG-antagonist / -agonist binding to the $LMNA$ promoter (Fig. 5B, 6B). In particular, increased binding sensitivities (lower $k$'s) increased the modulation by soluble factors of $LMNA$. This matrix-modulated noise analysis (appears novel and) might imply that for individual cells which start more stem-like and less osteogenic, soluble-factor induction of $LMNA$ via RARG is most effective. A matrix elasticity-dependent nuclear localization of RARG ($r_{nuc}$) was also necessary to capture soluble-factor effects on lamin-A levels (Fig. 5C, 6C). The model not only predicted the orthogonality of purely mechanical and purely chemical pathways but also that the combination synergizes to maximally regulate levels of lamin-A protein (Fig. 6D).
**RARG antagonist/agonist respectively increase/decrease osteogenesis in vitro and in vivo.** To assess functional effects of Retinoids on osteogenesis, Alkaline Phosphatase (ALP) activity was assayed as a first readout for osteo-induced MSCs cultured with OIM on rigid substrates. The RARG-specific antagonist CD2665 showed the greatest effect in enhancing ALP staining of cultures by ~12-fold relative to osteo-induced MSC controls not treated with retinoids. CD2665’s effect was also 50% greater than that of the pan-RAR antagonist AGN. Importantly, LMNA knockdown decreased ALP nearly to control levels for the antagonists (Fig. 7A,B). Agonists that were either RARG-specific (CD1530) or pan-RAR (RA) tended to decrease ALP but with little effect of LMNA knockdown. With the RARG-specific antagonist CD2665, single cell analyses show LMNA\(^{\text{high}}\) cells are 6-fold more likely to also be ALP\(^{\text{high}}\) than LMNA\(^{\text{low}}\) cells, and the super-majority of cells (62%) are both ALP\(^{\text{high}}\) and LMNA\(^{\text{high}}\) (Fig. 7C). Knockdown of LMNA increases the ALP\(^{\text{low}}\) subpopulations by >5-fold despite the presence of antagonist. Although retinoids can affect many pathways, given their demonstrated influence on lamin-A levels (eg. Fig. 5) (via promoter-based regulation (Okumura et al., 2004)) and given the evidence that nuclear accumulation of RARG is favored by LMNA\(^{\text{high}}\) (eg. Fig. 4G), it is fully consistent to find differentiation also exhibits synergy between RARG-specific soluble factors and matrix rigidity.

To provide further evidence that the RARG-specific antagonist CD2665 promotes osteogenesis, MSCs were pre-treated with drug (or not) for 3 d, implanted into mouse flank for 2-3 mo per Fig. 2A, and then analyzed for calcification. With live mice, fluorescent-bisphosphonate was injected and imaged, revealing a bone-like intensity for CD2665-treated xenografts that was significantly greater than controls (Figs. S6B, C). Xenograft tissue was removed and stained with alizarin red for calcification. Antagonist gave a calcification signal similar to bone, which is 3-4-fold greater than xenografts from either untreated control or agonist pre-treated MSCs (Fig. 7D). ALP staining showed similar trends for the xenografts (Fig. S6E). The RARG-specific antagonist thus promotes osteogenesis in vitro and in vivo.

**Convergent overlap in synergistic differentiation.** Although cell-to-cell variation can be large (Fig. 7C), robustness of differentiation in noisy systems can be achieved through parallel systems of feedback (Ahrends et al., 2014). Proteomic changes were therefore compared between MSCs treated with AGN versus RA, as characteristic of the soluble factor input, and control versus LMNA knockdown. Based on
detection of three-or-more tryptic peptides-per-protein, 21 proteins (of 121 quantified) varied by more than 1.33-fold in both experiments (Fig. S7A), and most exhibited correlated responses to both perturbations (Fig. 7E). Given the wide-ranging studies of retinoids that rarely if ever mention lamin-A, it seems unsurprising that most pathways regulated by retinoids differ from those affected by lamin-A; this makes their interplay in differentiation that much more biologically significant. Gene ontology and literature analyses of the 21 overlapping proteins nonetheless identified a number of molecules relevant to synergy in osteogenic processes (Fig. 7F), particularly STAT1, MYO1B and PDIA3 (Miyaishi et al., 1998; Balmer and Blomhoff, 2002; Xiao et al., 2004; Tajima et al., 2010; Wang et al., 2010; Roosa et al., 2011; Gao et al., 2012). Further comparison to transcriptional changes after LMNA knockdown suggested additional overlap candidates while adding confidence to common factors STAT1, ALCAM, EHD2, MVP and P4HA2 (Fig. 7G, S7B).

Discussion

Collagen crosslinking, stiffer tissues, and stiffer nucleus

A cell might respond to the rigidity of a single matrix fibril that integrins bind (Watt and Huck, 2013) or a cell might respond to collective matrix properties at a larger scale. Stress-bearing bone, cartilage, and muscle must all have sufficient strength and stiffness at a ‘macroscopic’ scale to sustain the large forces of tissue function (eg. walking). However, the microenvironment that bone-making osteoblasts interact with is collagen-rich and not calcified (Sodek and McKee, 2000), which should make this osteoid much softer than the large-scale properties of rigid bone. MSCs that enter such an osteogenic niche from marrow (such as perivascular niches) (Park et al., 2012) transit from a very soft marrow niche to a stiff but thin collagenous niche – for which the novel, nano-scale measurements here indicate is softer than bone (Fig.1). Osteoid is reasonably approximated in terms of cell-perceived stiffness (Fig. 3) by a crosslinked collagen-I nanofilm, which is more osteogenic for MSCs than a pristine collagen-I nanofilm (Fig.4). Broader significance is suggested by meta-analysis of transcriptomes across many tissues (excluding bone) (Fig.2), with potential relationships emerging between crosslinked collagen, lamin-A which confers nuclear stiffness, RARG which can in principle regulate lamin-A transcription (Swift et al., 2013b), and even osteoinduction. The latter might lend insight into ectopic calcification that is sometimes seen after injury coincident with scars having
an abundance of collagen that is highly crosslinked (Shimono et al., 2012). However, the response of lamin-A to matrix rigidity appears more general and can occur even within hours (Fig.S4) (Buxboim et al., 2014), but whether RARG and downstream pathways are independent or synergize for differentiation is key to understanding signals in a niche.

With crosslinked collagen-I, if a cell adheres to and pulls on even one fibril, bending of that fibril will also require bending of adjacent fibers (Fig.3), which means that the cell should feel a stiff film. With synthetic gels that are also modified with adhesive ligands such as collagen, molecular-scale mechanisms of force transmission remain unclear (Watt and Huck, 2013). Nonetheless, gel stiffness generally drives cell spreading that requires actomyosin stress fiber formation (Discher et al., 2005), and the MSC nucleus also becomes more spread and flattened with lamin-A levels increasing while B-type lamins remain unchanged (Fig.S2F,G). Crosslinked collagen-I films and stiff gels cause similar MSC responses (Fig.3), but only the crosslinked nanofilms on rigid mica permit probing of nuclear stiffness (as opposed to indenting nuclei into gels beneath a cell), helping to show nuclear stiffening parallels matrix stiffening (Fig.4). Nuclear stiffening’s dependence on lamin-A and actomyosin is consistent with the pathway for lamin-A accumulation in MSCs on stiff substrates: actomyosin tension opposes lamin-A phosphorylation and degradation that occurs in cells on soft gels (Buxboim et al., 2014). Downstream pathways are reasonably clear, since we had shown (Swift et al., 2013b) that the lamin-A binding protein SUN2 co-immunoprecipitates with RARG and that overexpressed SUN2 decreases both nuclear RARG and lamin-A. We can therefore infer that high lamin-A levels favor nuclear retention of SUN2 and RARG, which primes the latter for transcriptional control of LMNA. Consistent with these aspects of mechanism worked out with cells on gels, crosslinked nanofilms here indeed increase nuclear RARG relative to MSCs on pristine films (Fig.4), and addition of RARG-specific antagonist increases nuclear accumulation of RARG and lamin-A levels within hours (Fig.S4). Whereas pan-RAR agonists and antagonists suggest roles for RAR’s,(Swift et al., 2013b) RARG-specific agonists and antagonists provide functional evidence for this isoform distinct from RARA and RARB (Fig. 5, S4). Our mathematics for the mechanochemical gene circuit thus couples matrix elasticity regulation of lamin-A protein to RARG soluble factor effects on lamin-A expression. Importantly, RARG antagonist/agonists are ineffective on MSCs on soft matrix in both experiment and calculation (Fig. 5).
Implications for Progerin and Aging

Microenvironments trigger and direct stem cell differentiation, but microenvironments can change in aging and so can lamin-A. For example, collagen-I rich skin (Fig. 2A) stiffens 2-3 fold in human aging, (Diridollou, 2001), and Progerin in addition to normal lamin-A has been detected in skin biopsies and fibroblasts of normal donors above the age of 80 (Dahl et al., 2006; McClintock et al., 2007). The potential rejuvenating effects of RA on aging tissues such as skin has many complexities (Mukherjee et al., 2006), and the results here for Progerin as well as normal lamin-A (Fig. 5B, S5) suggest additional complexity because such soluble factors are generally applied to cells adhering to matrix or another cell that can influence cell responses as extreme as differentiation. Furthermore, RAR antagonists might naturally derive from vitamin-A (Eroglu et al., 2012) as is the case for RA, and so the antagonist/agonist balance or imbalance in a given microenvironment or niche could contribute to levels of both normal lamin-A and Progerin. Levels can have dramatic effect on health since homozygous lamin-A knockout mice all die 3 weeks post-partum with major heart and cardiovascular defects (similar to progerin mice that die) whereas heterozygotes maintain normal heart function at least 20-fold longer (Kubben et al., 2011). Beyond retinoids and lamins, responses of cells on rigid coverslip glass or tissue culture plastic to other drugs that converge on mechanosensitive pathways could be different from the same cells on soft, tissue-like matrix.

Translatable potential versus matrix-associated growth factors

Matrix-associated growth factors such as TGFβ superfamily members have potent effects on stem cells, and include BMP2 effects on bone formation (eg. (Hayashi et al., 2009)). However, the release of such matrix-bound factors depends in part on matrix mechanics and cell contractility (Wipff et al., 2007), which of course ties such factors to matrix pathways that are distinct from purely soluble factors such as retinoids (Fig. 1A). Implants that are simply loaded with recombinant human BMP cause ectopic bone (Carragee et al., 2011). RARG antagonist pre-treatment of human bone marrow MSCs leads to human bone formation in vivo even without addition of BMPs (Fig. 7D) and could prove a useful alternative for bone tissue engineering applications. Importantly, whereas matrix inputs might only affect cells while they are in contact with matrix (Schellenberg et al., 2014), short periods of soluble factor pre-treatment of stem cells can have long-term effects such as in vivo osteogenesis over many weeks. Retinoids can certainly drive epigenetic
programs (Gudas, 2013), and matrix mechanics could exert similar effects especially given the mechanosensitivity of lamin-A and regulation of chromatin by lamina associated domains (Dechat et al., 2008).
Materials and methods

AFM on primary tissue and cells on films. Tissue samples or mica discs with cultured cells were attached to a glass dish fully immersed in media at 37 °C and indented with soft cantilevers with nominal spring constants of 0.01 N/m when probing bone marrow or 0.05 N/m for osteoid and cells. Before each experiment, cantilevers were calibrated using a thermal fluctuations method. The distribution of Young’s moduli of bone tissues was evaluated from measurements at multiple locations in tibia samples from 6 different mice. Silverman’s test (B.W., 1981) for multimodality in R package “silvermantest” available at https://www.uni-marburg.de/fb12/kooperationen/stoch/forschung/rpackages was used with a null hypothesis that the logarithm of the data has at most 2 modes. The p-value of the test was < 0.01, which provides strong statistical evidence for at least 3 modes. The Sneddon modification to the Hertz model for conical tip was used to calculate the apparent Young’s modulus, assuming indentations to an isotropic, homogenous material. The depth of indentations was determined using the procedure we described earlier in (Buxboim et al., 2010) and it is between 400 nm to 1 µm, and the fitting range was obtained by minimizing the fitting error assuming a Poisson ratio of 0.5. All measurements were performed within hours of mouse sacrifice in order to preserve the in vivo properties of the tissue. Cells on collagen films were probed in force volume mode with a resolution of 128x128 indentations curves per frame with scanning frequency of 0.2 Hz. Topographical images of fixed cells were taken in contact mode.

Preparation of thin collagen films. Rat tail collagen solution (Corning CB354249, 200 µL at 1 µg/mL in 50 mM glycine buffer, 200 mM KCl, pH 9.2) was deposited on freshly cleaved mica disks at room temperature (RT) for self-assembly. After 30 min. liquid was gently aspirated and disks were washed with DPBS. The collagen films were cross-linked with tissue transglutaminase (Sigma-Aldrich T5398, concentration 30 µg/mL in of 50 mM TRIS buffer, 5 mM CaCl₂, 2 mM dithiothreitol at pH 7.4). The films were immersed in the solution and agitated for 4 hrs. at 37 °C before washing with DPBS.

AFM of thin films. Collagen films were imaged in DPBS at RT in tapping mode with driving frequency close to the resonance frequency of the cantilever. Fibril deformation was performed in a direction perpendicular to the fibril orientation in contact mode with predefined force and trajectory. After scratching, films were reimaged in tapping mode.
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Fig. 1. The bone microenvironment defines mechanical and molecular properties that influence the trajectory of stem cell differentiation. (A) The fate of a stem cell on a Waddington-like landscape is potentially influenced by matrix properties in synergy with soluble factors. One example is osteogenesis of bone marrow mesenchymal stem cell (MSC). However, differentiation might also include subpopulations that are not responsive to either stimuli or their combination. (B) Mouse trabecular bone was cut and opened
to expose the tissue inside for probing by AFM. (C) Bright field image showing bone marrow probed \textit{in situ} by AFM. The bone marrow was subsequently washed away and the remaining tissue and osteoid re-examined: (D) Bone tissue stained for alkaline phosphatase (ALP) activity; (E) Contact mode AFM image of the exposed bone, showing ECM fibers as part of osteoid. (F) A typical force-indentation curve of bone marrow (pre-wash), fitted with a modified Hertz model for a conical probe (red). (G) A comparison of the distributions of Young’s moduli $E$ measured by AFM at different locations in the pre-washed marrow, which is soft, and exposed osteoid, which is typically stiffer. All experiments $n \geq 3$ (Mean + S.E.M.).
Fig. 2. Proteomic and transcriptomic profiles of mouse tissue and human xenografts suggest the key components in a model of the microenvironment-to-osteogenesis signaling cascade. (A) Whole
mouse tissues (i, bone; ii, muscle; iii, skin) were profiled by mass spectrometry (MS) proteomics and the ratios of mean ion currents used to estimate the fractional compositions of the respective extracellular matrices. In all cases, the matrix was dominated by collagen-I. Human MSCs were also engrafted into mouse flank, excised after four weeks and examined by MS. Subsequent analysis allowed separate identification and quantification of mouse and human peptides (Fig. S1A) (Swift et al., 2013a; Swift et al., 2013b). The human peptides in the xenograft (iv) showed some similarity to the endogenous bone profile, and most of the detectable matrix proteins were also present in pre-engraftment MSCs (indicated by an asterisk). Mouse peptides in the xenograft (v) suggested comparison to skin, perhaps unsurprising given the location of the graft, and also showed likely remnants of the Matrigel component of the initial injection. 

(B) Plot showing the Pearson correlation between primary matrix component $COL1A1$ mRNA and other genes quantified in soft tissues of mouse and man (genes with common annotation, $n \approx 15000$), sorted by the mean Pearson coefficient in mouse and man (red line). (C) Pearson correlation between $COL1A1$ and transcripts for fibrillar collagens, cross-linking enzymes, acto-myosin cytoskeleton proteins, nuclear lamina proteins, retinoic acid receptor (RAR) and osteogenic transcription factors. Many of these key components were in the top few percent of correlations with collagen-I, as seen by comparison to Fig. 2B. (D) RNA-seq data from mouse skin of normal or induced squamous cell carcinomas (SCCs) (Friedrichs et al., 2007) revealing some power law relations between many of the factors analyzed in panel-(C). (E) Gene circuit model of how extracellular factors, mechanical (matrix composition and cross-linking) and molecular (soluble factors such as retinoic acid, RA) can influence MSC osteogenesis. Matrix stiffness induces stress in the acto-myosin network, which feeds into levels of nucleoskeletal protein lamin-A (LMNA) (Buxboim et al., 2014); LMNA regulates its own transcript through retinoic acid binding factor RARG, which in turn can be modulated by soluble agonists and antagonists to the RA pathway (Swift et al., 2013b). Both LMNA, through regulation of the SRF pathway (Ho et al., 2013; Swift et al., 2013b; Buxboim et al., 2014; Talwar et al., 2014), and the RA pathway conceivably contribute towards an osteogenic endpoint, but the extent of overlap between these pathways is not known (Fig. 1A). All experiments $n \geq 3$ (Mean + S.E.M.).
Fig. 3. Analysis of cell morphology and protein organization in MSCs cultured on pristine and cross-linked collagen-I films shows the equivalence of cross-linking to increased matrix stiffness. (A) Thin collagen films allowed an in vitro study of the effects on MSC morphology of culture on (i) pristine and (iii) cross-linked collagen-I films, ii) shows the nuclear height on the two films. (B) AFM amplitude mode topographical images of molecular films of highly ordered collagen-1 fibrils, showing D-periodic structure, self-assembled on hard substrates in the absence (i, ii) and presence (iii, iv) of cross-linking. Collagen fibrils were deformed by using the AFM stylus in lithography mode to apply a low dragging force across the film
(ii, iv), thus showing the flexibility of the fibrils and the deconstruction of the ribbons to monomers in the absence of cross-linking (ii). (C) Immunofluorescence images of MSCs cultured for 24 hrs on (i) pristine and (iii) cross-linked collagen-I films with staining against F-actin (red) and DNA (Hoechst, blue). (ii, iv) Staining against collagen showed that cross-linked films are not as severely deformed as the native ones. The random orientation of the dense regions of collagen protein suggests that the films are mechanically anisotropic, despite the topographically isotropic orientation of the fibrils evident from AFM images. (D) Comparison of matrix $E$ dependent cell morphologies of MSCs cultured on thick, isotropic polyacrylamide (PA) gels or thin collagen-I films. Plots of (i) cellular and (ii) nuclear spread area were fitted to hyperbolic functions with free parameters obtained from cells on gels of controlled stiffness (blue) and cells on thin films (red squares), with the unknown effective stiffness felt by the cells on thin films are superimposed to obtain the effective stiffness (10 kPa for pristine and 50 kPa for cross-linked collagen-I films). (E) Plots of cellular aspect ratio (i, left axis), order parameter (i, right axis), and nuclear aspect ratio (ii) as functions of the gel matrix elasticity also showed consistency between the morphology of pristine and cross-linked films with 10 kPa and 40 kPa PA gels, respectively. All experiments $n \geq 3$ (Mean + S.E.M.).
Fig. 4. Influence of matrix mechanics on osteogenic pathways: effect of collagen cross-linking on nuclear elasticity and protein expression. (A) AFM was used to probe the stiffness profiles of MSCs cultured on a rigid substrate, thus allowing an in situ readout of cellular elasticity without having to deconvolute effects of substrate deformation. (B) Force-volume mode elasticity maps of living cells cultured for 6 days on (i) pristine and (ii) cross-linked collagen-1 films, showed matrix cross-linking caused a 2-fold increase in the Young’s modulus of the nuclear region (highlighted with dotted circles). (C) Plot of Young’s moduli obtained from force-indentation curves at the position of the nucleus, averaged from ~ 60 curves per cell and from 7-13 individual cells per sample, cultured on pristine of cross-linked collagen films. (D)
Relative contributions to the normalized stiffness of the nuclear region from the nuclear lamina and cortical tension in the acto-myosin network can be appreciated by treatments with siLMNA and blebbistatin, respectively (averaged from ~60 force-indentation curves measured at different locations within the nuclear region of 4 - 7 cells, cultured on plastic). (E) MSCs cultured for 2 weeks on cross-linked collagen-1 films have 1.5-fold higher levels of LMNA. Immunofluorescence images of LMNA (green) and myosin-IIA (MYH9, red), on (i) pristine and (ii) cross-linked collagen-I films. Quantitative image analysis showed that (iii) increased levels of MYH9 correlated with greater nuclear spread area and (iv) higher levels of LMNA (n > 115 cells; significantly different in both dimensions, p < 0.01) v-vi) LMNA and MYH9 levels in MSCSs on gels are shown for comparison. (F) heatmap summary and hierarchical clustering of cellular and nuclear parameters as well as protein expression on 0.3 kPa, 10kPa, 40 kPa gels and both pristine and crosslinked collagen films from various experiments. The dendogram indicates cells on 10kPa gels and pristine films cluster together as do cells on 40 kPa gels and crosslinked films whereas cells on soft 0.3 kPa gels are distinct from the others. v) LMNA intensity on gels vi) MYH9 intensity on gels. (G) Comparison of the cellular location of transcription factor RARG in MSCs cultured on (i) pristine and (ii) cross-linked collagen-I films. (iii) A quantification of RARG levels showed a significant increase on cross-linked substrate (n > 50 cells). (iv) A cell-by-cell analysis showed that cross-linked collagen films favor higher LMNA and a correlated increase in the nuclear-to-cytoplasmic ratio of RARG (n > 30 cells; significantly different in both dimensions, p < 0.01). v) Average nuclear-to-cytoplasmic ratio of RARG on gels. (H) RUNX2 and SPARC were also compared in MSCs cultured on (i) pristine and (ii) cross-linked collagen-I films. Quantification of the images (iii, correlating results from two separate experiments) showed that cross-linked substrates favored a greater nuclear localization of transcription factor RUNX2 and a greater expression of osteogenic marker SPARC. Nuclear spread area is also highest on cross-linked films as predicted from the fits of Fig. 3D i. All experiments n≥3 (Mean + S.E.M.).
Fig. 5. Influence of soluble factors on RA pathway implicated in osteogenesis: convergence of mechano-chemical effects. (A) MSCs cultured on plastic with drugs that perturb the RA pathway (control is ethanol/DMSO vehicle only; drug treatments at 1 µM, except adapalene and CD437 at 0.1 µM; see Fig. S4A for summary of drug properties). LMNA was quantified by immunostaining after four days. (B) Although the magnitude of response varied across MSCs from different donors, RA pathway antagonists consistently increased LMNA level with respect to the agonist on a range of stiff substrates (rigid plastic, stiff polyacrylamide gels, collagen thin-films; see Fig. S4B for full table); iPS derived MSCs and progeria patient derived iPS – MSCs gave similar response (bottom of the table). (C) (Antagonist / agonist) effects on LMNA levels from immunofluorescence of iPS-MSCs and Progeria patient derived iPS-MSCs relative to control. The immunoblots (lower bar graphs) showed the Progerin from the disease-causing allele was affected as much as the normal lamin-A spliceforms. (D) LMNA was quantified by immunofluorescence in MSCs treated for 3 days with RARG-specific RA pathway antagonist CD2665 or agonist CD1530 while being cultured on pristine or cross-linked collagen-I films. Consistent with earlier reports (Swift et al., 2013b), a significant differential response between agonist and antagonist is apparent only on a ‘stiff’ (cross-linked) substrate. (E) (i) The spread area of nuclei in iPS-progeria derived MSCs depends on matrix stiffness and is reversible. Early passage, P1, MSCs spread less as P2 on soft gels than on stiff gels or plastic. (F) Nuclear area and
lamin-A in iPS-progeria derived MSCs on soft gels of 2 kPa shows 95% of cells are within ±2 σ of untreated cells even after treatment with RA agonist/antagonist. These cells are ‘weak responders’ to retinoids, and the same range of small nuclear areas identifies weak responders to retinoids on stiff gels of 50 kPa. However, about one-third of cells are mechanosensitive to stiffness, with elevated Lamin-A levels that also change in response to retinoids. (ii) Nuclear RARG in bone marrow derived MSCs on pristine and cross-linked films increases (for responding cells) with matrix stiffness, but retinoids have no effect on RARG even though Lamin-A tends to decrease with agonist and increase with antagonist.

Fig. 6. Waddington-inspired model of mechano-chemical effects on lamin-A levels. (A) Lamin-A (l) levels increase with matrix elasticity (E_{matrix}) and influences cytoplasmic RARG (r_{cyto}) shuttling into nucleus (r_{nuc}), where its binding on LMNA (L) promoter as a repressor can be enhanced or abrogated by soluble agonist (ag) or antagonist (ant). Transcription rate of LMNA is a function of both soluble (α_{soluble}) and matrix (α_{matrix}) factors. Soluble effects are, in turn, modulated by the levels of RARG-antagonist (r_{nuc,ant}) and RARG-agonist (r_{nuc,ag}) complexes, whose dissociation constants are represented by k_1 and k_2, respectively. Building from our previous model where lamin-A’s tension-inhibited degradation, K, is key to its scaling with matrix elasticity E, we extended the model to capture the convergence of mechanical and chemical effects on
lamin-A. Model parameters were specified by recapitulating experimental results in Figs. 5B and 5D: $\alpha = 0.35; \beta = 3; \gamma = 1; \delta_0 = 2.5; \eta = 4; k_1 = 0.5; k_2 = 0.3; n = 4; \kappa = 0.6$. (B) Sensitivity analysis of varying binding strengths of RARG-antagonist ($k_1$) or -agonist ($k_2$) complexes to lamin-A expression response ($E = 0.99$, $\{ant, ag\} = \{1,0\}$ or $\{0,1\}$). (C) For pristine (relative $E = 0.22$) and cross-linked (relative $E = 0.99$) films, antagonist (ant) and agonist (ag) values set to 1.0. Error bars are standard deviations derived from simulating 100 cells with $k_1$ and $k_2$ randomly generated as values within the range [0.4, 0.6] and [0.2, 0.4], respectively. (D) The model predicts two paths from a high-lamin state (blue sphere) to low lamin-A states (red spheres) by reducing matrix $E$ (green arrows) or adding soluble agonist (red arrows). All experiments $n \geq 3$ (Mean + S.E.M.).
Fig. 7. A proteomic comparison of the effects of LMNA knockdown and drug perturbations to the RA pathway allows identification of factors common to both pathways. (A) MSCs were cultured on plastic for one week with combinations of osteo-inducing media (OIM), siLMNA and drug perturbations to the RA-pathway: (i) ALP staining of scrambled RNA and OIM; (ii) RARG-specific antagonist CD2665 and OIM; (iii) siLMNA, CD2665 and OIM. (B) ALP activity is enhanced in the presence of RA antagonists, but this effect is abrogated by LMNA knockdown. (C) A cell-by-cell analysis of LMNA level correlated to local ALP staining in MSCs cultured on rigid substrate with OIM and CD2665, with or without siLMNA, showed that higher LMNA levels positively correlate with greater osteogenesis (Fig. S5A). The percentages indicate...
the number of points (blue, without siLMNA; gray, with siLMNA) in quadrants defined by the means of the siLMNA points. (D) Antagonist to RARG also increased osteogenesis in vivo: MSCs were engrafted into mouse flank, excised after four weeks and stained with alizarin red, an indicator of osteogenic calcification: (i) untreated MSC graft; (ii) CD2665 pretreated MSC graft; (iii) mouse femur; (iv) quantification of staining. These experiments suggest that CD2665 pretreatment increased the bone-like character of the grafted MSCs (Figs. S5B-D). (E) MSCs were cultured on plastic for eight days with drug perturbation to the RA-pathway, or subjected to LMNA knockdown. Cells were analyzed by MS proteomics: of 121 proteins quantified (with greater than three peptides-per-protein), 53 varied by more than 4/3-fold in the RA-perturbation experiment and 36 varied by the same degree in the siLMNA experiment (Fig. S6A). 21 proteins varied in both experiments, and of these, 14 were positively correlated. (F) Table of correlated protein changes with a heatmap representation of fold-change in RA-pathway and siLMNA experiments. See Figure S7 for references. (G) Cartoon representation of proteins involved with overlapping pathways towards osteogenesis. All experiments n≥3 (Mean ± S.E.M.).