Tissue matrix arrays for high-throughput screening and systems analysis of cell function

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Cell and protein arrays have demonstrated remarkable utility in the high-throughput evaluation of biological responses; however, they lack the complexity of native tissue and organs. Here we spotted tissue extracellular matrix (ECM) particles as two-dimensional (2D) arrays or incorporated them with cells to generate three-dimensional (3D) cell-matrix microtissue arrays. We then investigated the responses of human stem, cancer and immune cells to tissue ECM arrays originating from 11 different tissues. We validated the 2D and 3D arrays as representative of the in vivo microenvironment by means of quantitative analysis of tissue-specific cellular responses, including matrix production, adhesion and proliferation, and morphological changes after culture. The biological outputs correlated with tissue proteomics, and network analysis identified several proteins linked to cell function. Our methodology enables broad screening of ECMS to connect tissue-specific composition with biological activity, providing a new resource for biomaterials research and further understanding of regeneration and disease mechanisms.

Tissues and organs in the body are composed of cells and their surrounding ECM, generated by self-assembly and cellular processing. Tissue specificity is determined by the unique composition of the tissue—from hundreds of different biomolecules—and supramolecular structures that interact physically, chemically and biologically with cells to regulate cellular-level functions2–5. Ongoing research continues to elucidate how the structural and compositional properties of the ECM influence resident cells6,7. Despite the use of tissue-derived materials in the clinic, detailed mechanistic information on how tissue ECMS directly influence cell behavior or repair processes is largely unavailable.

Strategies based on arraying DNA, RNA, single ECM proteins or synthetic biomaterials allow high-throughput in vitro screening of cellular functions and biological outputs on diverse substrates8–12. For example, 2D microarray libraries of synthetic polymers have been used to delineate the optimal scaffold composition for lineage-specific stem cell differentiation8,9. ECM proteins have been integrated with synthetic hydrogels to identify combinations that stimulate stem cell osteogenesis in three dimensions13–15. Individual and combinatorial screening of purified proteins in microarray formats has suggested mechanisms of cell-protein interactions10 and identified candidate cell-protein interactions that correlate with cancer metastasis11. All of these previous arrays started with simple building blocks, such as polymers or proteins that can be tested in a combinatorial manner. However, cells in the body exist in tissues and organs with a complex ECM that includes hundreds of different molecules ranging from nanometer-sized fibrils to micrometer-sized units that can modulate cell behavior16.

Tissue ECMS have been used for regenerative medicine and wound healing in humans17–19, typically to match ‘like with like’20–23—for instance, stem cells cultured on liver ECM to create new liver tissue21. However, broader screening of tissue ECM properties may elucidate more general biological functions and novel therapeutic entities. In order for the understanding and use of tissue-derived biomaterials to advance, high-throughput screening tools are needed to probe variability in ECM composition and complex cell-matrix interactions in vitro. This would allow the intricate mechanisms of cell-material response and repair processes to be teased apart to explain how these materials can be used to influence cell behavior in vivo.

Here we developed 2D and 3D tissue ECM arrays for screening biological responses to tissue-specific scaffold microenvironments. We processed tissues to remove soluble tissue components and then mechanically fragmented the matrix to create tissue ECM microparticles that retained the proteomic complexity of the natural ECM in a medium compatible with array fabrication (spotting). We characterized a range of cell-matrix interactions at cellular and functional levels, including mineralization, cell adhesion and proliferation, gene expression, and changes in cell morphology, using stem cells, cancer cells and macrophages.
We then correlated tissue ECM array outputs with proteomic composition to build networks that highlighted candidate proteins responsible for tissue-specific differences in cell function.

RESULTS
Fabrication of 2D and 3D tissue ECM arrays
We established a physiologically broad tissue ECM data set by harvesting 11 different porcine tissues and organs (spleen, small intestine, bladder, bone, brain, cartilage, heart, kidney, liver, lung and adipose); chemically treating the tissues using a combination (unless noted otherwise) of acid, detergent and DNase; and then lyophilizing them to mechanically break them down into microparticles using a cryomill. The tissue ECM particles contained numerous intact proteins reminiscent of the original tissue (Supplementary Fig. 1a), a feature that is lost with alternative methods of enzymatic digestion.

Array spots (3-mm diameter) consisted of tissue matrix particles spotted on acrylamide-coated glass (Fig. 1a) with a layer of purified soluble type I collagen to promote homogeneous particle binding, as confirmed by scanning electron microscopy (Supplementary Fig. 1b). Collagen controls included acid-soluble type I collagen protein without the addition of tissue ECM particles and insoluble collagen particles that match the structure of processed tissue ECM particles. We prepared up to 40 spots of tissue ECM per array (Fig. 1b and Supplementary Fig. 2a) and then seeded cells uniformly on the array using a confined flow device (Supplementary Fig. 2b), which limited cell spot-seeding variability to 13% of the mean per row and column (n = 10 rows, n = 4 columns per 40-spot array) (Supplementary Fig. 2c). Spot locations and seeding direction were organized so that replicates of each tissue were distributed evenly throughout the seeding area to limit the effects of flow patterns in the seeding device (Supplementary Fig. 2a).

3D systems provide an additional means to evaluate and predict cell-tissue interactions in an environment and may allow for better prediction of some in vivo behaviors. With this in mind, we also developed hanging droplet arrays of 3D tissue ECM spheroids in which each spheroid contained 10,000–20,000 cells and ECM particles at a concentration of 0–10 ng per cell in 40 µl of culture medium. Spontaneous cell-matrix assembly resulted in the formation of large agglomerations after 24 h in culture, with continued self-assembly over the course of 2–6 d (Fig. 1c). Tissue particle-to-cell ratios were optimized to maximize tissue ECM content without disruption of compact spheroid formation or cell viability (Supplementary Fig. 3a–c). Compact spheroid formation and microtissue size were consistent across all ECM types tested at concentrations of 2 ng per cell or less after 6 d of culture. Cells were viable at ECM concentrations of up to 2 ng per cell but decreased at higher particle concentrations for some tissues.

To form 3D spheroids, we seeded human adipose-derived stem cells (hASCs) with ~17,000 cells and 16 µg of tissue particles to create compact spheroids that had a uniform diameter of ~460 µm (±40 µm; n = 8) despite having different tissue particle compositions (purified type I collagen particles, bone, brain, cartilage, adipose, lung or spleen). To enable high-throughput morphological, histological and immunohistochemical analyses of the 3D microtissue arrays, we developed a method similar to the tissue microarray technology used in tumor pathology. We covered cell-tissue spheroids that were arranged in a microarray mold with agarose gel to fix the location of the microtissues and make possible sectioning of the spheroids. Microtissue cross-sections showed a relatively uniform distribution of cells and tissue particles throughout each spheroid (Fig. 1d).

Characterization of tissue ECM arrays
We then characterized the physical and biochemical properties of the tissue-specific ECMs to understand tissue-specific attributes and to confirm the reproducibility of the array fabrication.

Physical properties. After complete drying, spot surfaces for brain, bladder and small intestine were generally smooth, whereas those for other tissues, such as spleen, bone and liver, showed more variation in roughness and texture (Supplementary Fig. 1b). The elastic moduli of the dry tissue spots were two orders of magnitude higher and demonstrated less variation between tissue types than stiffness values reported for fresh hydrated tissue (Supplementary Fig. 1c). For example, after being processed into particles and spotted in 2D, cardiac tissue spots were twofold stiffer than brain spots; by comparison, there is an estimated ~20-fold difference in stiffness between fresh heart and brain tissues.

Reproducibility. Masson’s trichrome stain was used to contrast the collagen composition between tissue spots. Spots derived from connective tissues, cartilage, bone and bladder contained qualitatively more collagen than did spots from solid organs such as liver.
Supplementary Fig. 4). The variability in the processing of lung tissue yield arrays can be used with multiple tissue-types (Fig. 2d). Lung and cardiac tissues spots per tissue type) for 10 of the 11 tissue types analyzed with staining intensity of the most prominent color in tissue spot imaging pathogenesis (Supplementary Fig. 4). The variability in the staining intensity of the most prominent color in tissue spot replicates remained below 25% (coefficient of variation; n = 3 spots per tissue type) for 10 of the 11 tissue types analyzed with ImageJ (Supplementary Fig. 5a,b). We confirmed the reproducibility of tissue particle composition using mass spectrometry and proteomic analysis, comparing replicates of small intestine (Supplementary Fig. 5c) with similar peptide abundances in triplicate runs. Approximately 95% of proteins identified by at least two peptide-spectrum matches (PSMs) were found in all small intestine samples, whereas only 42% of less abundant proteins (one PSM) were identified across all three runs (Supplementary Fig. 5d). Taken together, the results showed low tissue interspot variability and high tissue specificity in tissue array chips.

We demonstrated broad utility of our tissue array with a range of tissue-preparation methods by exposing cardiac and lung tissues to a range of chemical processing conditions that produced ECMs of various molecular weights (Supplementary Fig. 6a). Spots from the same tissues processed with different methods were clearly differentiated by their collagen content (Supplementary Fig. 6b). Using an automated algorithm, we also looked for any changes in the morphology of primary mouse bone marrow–derived macrophages (BMDMs) seeded on the variably processed tissue ECMs (Supplementary Fig. 7a). Lung and cardiac tissues (and combinations of the two) that underwent different types of preparation produced distinct cell morphologies (Supplementary Fig. 7b,c), with changes in the processing of lung tissue yielding the greatest effect on cell morphology (Supplementary Fig. 7d). Thus, the arrays can be used with multiple tissue-processing methods, and, importantly, variable cell responses to the arrays can capture differences in tissue processing and thus in the tissues’ resulting compositions.

Proteomic analysis. The tissue particles used to fabricate the 2D and 3D arrays retained a complex protein composition that was representative of the native tissues from which they were derived. Nearly 4,000 unique proteins were identified across the 11 processed porcine tissue types, of which 111 were ECM and 98 were ECM associated. To understand the effect of processing on tissue composition in the primary arrays, we compared several processed tissues from this study to native tissues from a recently published draft map of the human proteome6. Processed tissue particles showed strong similarities in ECM-specific proteins compared to the native tissues; however, processing reduced the protein diversity of non-ECM proteins (Fig. 2a). The total abundance of ECM and non-ECM proteins varied by tissue type, although at similar ratios, with the exception of bone and cartilage, which had lower amounts of non-ECM proteins (Fig. 2b).
Subclassification of ECM and ECM-associated proteins in tissue particles revealed tissue-specific differences. Proteoglycans were enriched in adipose, cartilage and brain tissues, whereas amounts of secreted factors (including growth factors) were miniscule in most tissues except for brain, where they accounted for 8% of ECM total protein (Fig. 2c). Collagen subtypes accounted for the largest ECM fraction in all tissues except for brain and heart and represented more than 50% of the ECM in bladder, bone and cartilage (Fig. 2c). Detailed analysis of collagen composition showed the expected tissue specificity (Fig. 2d); for example, type X collagen is associated with endochondral ossification and was identified only in bone and cartilage.

**Human stem cell response to 2D and 3D tissue ECM arrays**

We used both 2D and 3D tissue arrays to study stem cell interactions with different ECMS. We seeded hASCs on 2D tissue ECM arrays or cultured them in 3D cell-ECM spheroids, in both instances in either control or osteogenic medium. The percent area of calcified matrix on each spot or microtissue represented the expected tissue specificity (Fig. 2d); for example, type X collagen is associated with endochondral ossification and was identified only in bone and cartilage.

**Figure 3** Stem cell–tissue ECM interactions in 2D and 3D arrays. (a) hASCs stained with calcine AM (green, left and right) or Alizarin Red (middle) were cultured on 2D tissue microarray substrates for 6 d in osteogenic media (OM) or control or growth media (CM). Images are representative of n = 3 microarrays. (b) Osteogenic differentiation, as quantified by the percent area of each spot that stained positively with Alizarin Red. Data are mean and s.d. (n = 9 spots from three microarrays). (c) Chips incubated in OM and CM. Images are representative of n = 3. (d,e) hASCs were cultured with the indicated types of tissue particles in 3D microtissue spheroids for 11 d in OM or CM. (d) Histological sections of individual spheroids (images representative of n = 5 spheroids) stained with Alizarin Red. (e) Quantification of the percent area stained by Alizarin Red. Data are mean and s.d. (n = 5 spheroids). (f) Normalized staining comparison between tissue types in 2D and 3D culture conditions. Spearman’s rank correlation coefficient: 0.79 (P < 0.05). One-way analysis of variance with a post hoc Tukey test was used for statistical analysis (b,e).

*P < 0.05*, Bl, bladder; Bo, bone; Br, brain; Ct, cartilage; Col, purified collagen I particles; Ad, adipose; Cdc, cardiac; Kd, kidney; Lv, liver; Lg, lung; Sp, spleen; SI, small intestine; Ct, control soluble collagen only; All, all other tissues.

Overall osteogenic differentiation was similar in the 2D and 3D tissue arrays (Spearman’s rank coefficient, 0.79 (P < 0.05)), but the amount of calcified matrix present in the 3D cellular microtissues was less than that in the 2D arrays (Fig. 3f). As expected, bone tissue matrix supported and stimulated osteogenesis. However, newly formed calcified matrix was also abundant on lung ECM in both array systems, so differentiation was confirmed in bone and lung spheroids by increased expression of the gene osteocalcin (Bglap), a late-stage marker of osteogenesis (Supplementary Fig. 8a).

**Cancer and immune cell behavior on tissue ECM arrays**

We then demonstrated that the arrays are broadly applicable to cancer and immune cell types and their relevant biological outputs. For proof of concept, we screened human cancer cell lines in 2D and 3D to measure cell adhesion and proliferation, and we screened mouse primary macrophages on the 2D tissue ECM arrays to quantify cell morphology and phenotype changes.

**Cancer cell adhesion and proliferation.** The tissue microenvironment has a major role in tumor progression and metastasis\(^27\), and tissue-specific ECMS could provide insight into how the local environment affects tumor growth or metastatic preferences. To this end, we screened the adhesion of three different human cancer cell lines (lung, breast and skin) after 2 h on 2D ECM microarrays. Cell adhesion on different tissue matrices varied by up to tenfold for all cell types (Fig. 4a,b and Supplementary Table 1), but notably the cancer cells exhibited minimal adhesion to kidney- and liver-derived ECMS, whereas they adhered to all of the mesenchymal tissues (adipose, bone, cardiac and cartilage) except spleen.

We next compared human breast cancer cell proliferation on 2D and 3D tissue ECMS. In 2D, bone ECM suppressed cell proliferation,
Immune-cell morphology and polarization. Macrophages have a key role in normal tissue repair and response to disease. For example, the tissue regenerative response has been linked to macrophage skewing toward a remodeling phenotype called M2; conversely, the M2 phenotype is associated with negative outcomes in a tumor environment. Recent studies have also demonstrated the importance of the local tissue environment on macrophage phenotype.

We used our 2D tissue array method to probe M2 and M1 (proinflammatory) macrophage responses to specific tissue microenvironments. We skewed mouse BMDMs toward an M1 phenotype (interferon-γ + Escherichia coli lipopolysaccharide) or an M2 phenotype (interleukin 4 (IL-4)) and then quantified cell morphology, as a surrogate marker of immune polarization, on tissue ECMs. Macrophage shape (convex hull area ratio) varied by up to 40% across the tissue matrix types in both cytokine environments, suggesting that tissue-specific matrix environments can modulate cell response. Macrophages changed morphology dramatically when cultured on bone tissue ECM, with M1-stimulated cells elongated and spread compared with the more compact M2-stimulated cells.

Figure 4 | Cancer cell and macrophage interactions with tissue microarrays. (a) Adhesion of three different human cancer cell lines and a control mouse fibroblast line to different tissue ECM types. Cells (black) were stained with calcein AM and counted with ImageJ software (representative of n = 6 array spots). (b) Cell number normalized to the average number of cells on soluble collagen control spots located on the same microarray. Data are mean and s.d. (n = 6 array spots). (c) The breast cancer cell doubling rate calculated from the cell number after 1, 2 and 7 d of culture on each tissue microarray spot. Data are mean and s.d. (n = 9 array spots). (d) Heat map and hierarchical clustering of normalized (0–1) B16 mouse melanoma cell adhesion between F0 (parent) and metastatic KY8 (liver) and F10 (lung) cell lines seeded on a tissue array (n = 6 tissue array spots and n = 4 soluble collagen control spots). (e,f) The immunological properties of different tissue array spots as screened by mouse BMDMs chemically polarized toward M1 or M2 phenotypes. (e) Cell morphology visualized after 24 h with actin-phalloidin staining (n = 3 array spots; scale bar, 200 μm). (f) Cell morphology quantified as the convex hull area ratio. LPS, lipopolysaccharide; IFN-γ, interferon-γ. Data are mean and s.d. (n = 3 array spots). *P < 0.05; abbreviations for tissue types defined as in Figure 3.

whereas cardiac tissue amplified proliferation (Fig. 4c and Supplementary Fig. 8b). However, proliferation in 3D cell-ECM spheroids over 7 d was similar in bone, kidney and cardiac ECMs (Supplementary Fig. 8c). We also compared the adhesion of three types of mouse B16 melanoma cells to evaluate tissue preferences of primary and metastatic tumor lines: the B16-F0 parent cell line; the B16-F10 line, associated with lung metastasis; and the B16-KY8 line, associated with liver metastasis. F0 cells demonstrated the highest overall attachment, and KY8 the lowest, as expected from their known in vivo behavior. Cartilage and spleen tissues promoted adhesion of all three melanoma cell types; conversely, liver, kidney and collagen control did not support the attachment of melanoma cells, which was similar to the response of human breast cancer cells. The most substantial difference between cell lines was observed for attachment to adipose tissue. Cluster analysis showed similarities in tissue adhesion between the two metastatic melanoma lines (F10 and KY8) in comparison to the primary cells, despite the fact that the metastatic lines have distinct metastatic profiles (Fig. 4d and Supplementary Table 1).
We further investigated macrophage polarization on the 2D tissue arrays by immunostaining M1 and M2 cytokine-stimulated BMDMs for the M1 marker inducible nitric oxide synthase (iNOS, also known as NOS2) and the M2 marker arginase 1 (Arg-1) on both bone ECM and control collagen (insoluble particles) spots. Macrophages on the bone ECM spots showed increased Arg-1 staining in M2 conditions and increased iNOS staining in M1 conditions compared with collagen, suggesting enhanced polarization and responsiveness to either M1 or M2 conditions (Supplementary Fig. 10a). To provide a correlation between in vitro and in vivo results and demonstrate the predictive value of in vitro results for in vivo responses, we implanted bone ECM and control collagen matrices subcutaneously in C57BL/6 mice. Both scaffolds showed substantial mononuclear cell infiltration after implantation, and many of these cells were F4/80+ macrophages expressing iNOS (Supplementary Fig. 10b). Similar to the increased macrophage polarization of the bone ECM observed in vitro, more macrophages expressed iNOS in the bone ECM implants than in collagen in vivo.

Lastly, we evaluated the effect of 2D tissue substrates on macrophage polarization by examining the expression of M1- and M2-associated genes (Supplementary Fig. 10c). Macrophage gene expression was dependent on the source tissue and cytokine stimulation, and we noted a number of statistically significant differences between tissue ECM sources (Supplementary Table 1). Bone ECM induced extensive expression of M1-associated genes in M0 cytokine conditions (Illib, \( P = 0.0193 \); Nos2, \( P = 0.0009 \)), whereas liver did not alter phenotype relative to uncoated wells. In contrast, all tissues resulted in decreased expression of M2-associated genes in M0 cytokine conditions (\( P < 0.05 \)) and did not substantially alter the expression of M1-associated genes in M1 conditions, although cardiac tissue increased the expression of Ill10 in M2 conditions (\( P < 0.0001 \)).

**Systems analysis of tissue composition and outcomes**

An advantage of the tissue arrays is that they enable the user to perform multiplexed assays with a variety of cell types and matrix compositions. For example, to identify potential mediators of in vitro biologic function, we integrated the proteomic composition of tissue array spots with the outputs of the various in vitro assays. A heat map representation of correlation coefficients showed that different ECM and ECM-related proteins clustered with in vitro osteogenesis, cancer cell adhesion and macrophage polarization—the three outputs we investigated in this study (Fig. 5a). We selected the most statistically significant correlations (\( n = 28 \) proteins with \( P < 0.05 \) for both original and
rank-transformed data; Supplementary Table 2) for network analysis to further probe common proteins that might influence cell behavior. Several ECM components, including S100A9, SERPINB10, CTSB, HAPLN3 and PRSS2, were strongly linked with multiple in vitro assay outcomes (Fig. 5b). The S100A family, for instance, correlated with both osteogenic and immunomodulatory assay outcomes. Some of the more abundant ECM proteins, such as collagens, could not be used to distinguish different in vitro outcomes between tissues. Conversely, ECM glycoproteins showed correlation with several biological assay outputs in the network analysis—specifically, positive correlations to cancer cell adhesion and osteogenesis and negative correlations to macrophage morphology.

To further investigate the unexpected stem cell osteogenesis that we observed on lung tissue in 2D and 3D cultures (Fig. 3), we probed the ECM and ECM-associated proteomic composition using gene ontology (GO) enrichment, specifically, terms related to skeletal development or bone formation. Lung ECM components showed significant enrichment for these terms in lung compared to spleen (Fig. 5c), including ossification (GO:0001503) and bone morphogenesis (GO:0060349), suggesting that factors were retained in lung after processing that individually or in combination might have promoted osteogenesis (Supplementary Table 3). Cartilage was also enriched in these pro-osteogenic factors to an extent similar to that of bone, although it did not show any in vitro osteogenic potential, indicating that GO enrichment analysis alone is not sufficient to explain this behavior, or that there are inhibitors of osteogenesis present in processed cartilage. Correlation networks and GO analysis revealed individual molecules and groups of molecules, respectively, in the complex tissue ECMs.

DISCUSSION

Microarrays designed to recreate the ECM use a reductionist approach that incorporates select components to modulate cell behavior. Strategies for studying single or paired protein combinations have been unable to mimic the complex composition of healthy and diseased tissues. Tissue-derived scaffolds that maintain natural tissue complexity have been under investigation for more than 25 years and, during that time, have been implanted in more than 1 million patients. However, the biochemical diversity of tissue- and organ-derived matrices renders it difficult to fully understand the material composition, specific interactions with cells and therapeutic action. Our mechanically processed ECMs retain many of the inherent physical and biochemical cues. Although tissues are processed with a wide range of mechanical, chemical and/or enzymatic treatments, it may not be possible to completely remove all cell-associated components from a tissue matrix. These cell-associated proteins, both intracellular and secreted, may be relevant for the biological and even therapeutic outcomes of these materials, and should therefore be preserved in in vitro models. Tissue ECMs from different sources exhibited relatively minor differences in mechanical and structural properties but large differences in composition, which is likely to be the primary influence in cell response.

The effects of specific tissues on stem cell osteogenesis were consistent in both 2D and 3D cultures, although the 2D assays showed an estimated 50-fold higher ratio of tissue matrix to cells. Bone tissue ECM enhanced osteogenesis, which was expected because demineralized bone materials are used clinically to stimulate bone healing. However, lung tissue ECM unexpectedly enhanced osteogenesis compared with most other tissues. These unexpected results may lead to further study and understanding of lung tumor calcification. GO analysis showed that processed lung ECM was enriched for components associated with osteogenesis and chondrogenesis relative to other tissues, including ECM proteins such as type II and type XII collagen, matrilins, thrombospondin 1 and asporin (Supplementary Table 3).

Correlating in vitro assay outcomes with in vivo behavior will be an important link between our method and biology, with the hope that specific components of the tissue matrix can be linked to cell-signaling pathways. The extensive network analysis we performed to correlate proteomics and in vitro functional outcomes is ideal for identifying individual ECM candidates and for discovering potentially overlapping mechanisms across different cell processes. Conversely, GO enrichment is well suited for deriving complex combinations of ECM components for further investigation using previously defined annotations. The two analysis methods are complementary, and both are easily applied to data sets generated by these in vitro arrays to discover drivers of the in vivo biological responses. Of particular interest was the connection between immune phenotype and osteogenic potential based on shared correlation of these outcomes to the S100A family of proteins. Consistent with our findings, S100A8 and S100A9 are damage-associated molecular pattern proteins that exert immunoregulatory functions via interaction with the receptor for advanced glycation end products, in addition to activating osteogenic gene programs. Whereas the dual immunoregulatory and osteogenic functions of S100A proteins have been partially characterized, other protein-function correlations are not as obvious. Application of ECM correlation networks to the cancer cells showed that both metastatic melanoma lines, but not the parent, were positively correlated with PRSS2, a serine protease that was enriched in bladder, spleen and brain tissue arrays. PRSS2 and other enzymes have long been associated with cancer and promote metastasis by cleaving cell-adhesion molecules and ECM barriers. However, our data suggest that PRSS2 has a direct effect on metastatic cancer adhesion and might promote initial metastasis to these tissues through mechanisms such as shedding of other membrane-bound growth factors or enzymes. Although these data do not define mechanisms of tissue ECM modulation of processes such as osteogenic differentiation, cancer metastasis and immune modulation, the array screening and resulting analysis were able to establish correlations and provide promising candidate molecules for further investigation—either isolated components with novel, noncanonical functions or complex combinations of proteins that are not otherwise apparent.

The 2D and 3D arrays both present advantages and may complement each other; 2D microarrays are compatible with high-throughput microscope slide scanning and are simple to culture, whereas 3D microarrays better represent the natural microenvironment, do not have sample cross-talk, can be sectioned multiple times and can be used with varying ratios of cells to tissue matrix to measure dose dependence. Three-dimensional microtissues are also compatible with metabolic assays, single-cell analysis or sorting, and gene expression analysis. Each method,
however, offers the unprecedented ability to probe mechanisms of cell interactions with complex tissue microenvironments, support the discovery of new therapeutic targets and applications and ultimately transform the therapeutic potential of ECM-based therapeutics.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD002571.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

V.Z.B. and J.H.E. conceived the idea. V.Z.B. made 2D and 3D tissue arrays. V.Z.B. performed stem cell studies. M.R.B. and H.J. developed seeding chambers and performed cancer adhesion and proliferation studies. K.S. and M.T.W performed macrophage studies. K.S. performed gel electrophoresis work and collagen ECM classification. S.S.M., V.Z.B., M.T.W. and A.P. performed proteomic analysis. A.P. provided melanoma lines and guidance for cancer studies. K.S. and M.T.W. performed cancer adhesion and proliferation studies. K.S. and M.T.W. evaluated all data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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**ONLINE METHODS**

**Tissue processing.** Porcine tissues were harvested from 6-month-old market-weight pigs weighing approximately 100 kg (Wagner’s Meats, Mt. Airy, Maryland, USA). Mouse tissues were harvested from normal mammary glands of 6–10-week-old female wild-type C57BL/6 mice and early-stage (diameter: ~9 mm) and late-stage (diameter: ~16 mm) tumors from MMTV-PyMT mutant mice (Jackson Laboratories). Skeletal muscle was harvested from the quadriceps of 26–32-week-old female Dmd

\[\text{Dmd}^{\text{ndx Cay}}\] mutant mice (Jackson Laboratories) and wild-type control mice. Whole tissues and organs were cut into pieces approximately 100 mm³ containing 10 mM MgCl₂ for 18 h at 37 °C; and digest—ECM particles were suspended in 4% SDS for 16 h at 37 °C, (2) 1% Triton X-100 containing 2 mM EDTA for 18 h at 37 °C, (3) 600 U/mL DNase (Sigma) containing 10 mM MgCl₂ for 18 h at 37 °C. After the final treatment, the tissue was washed thoroughly with phosphate-buffered saline (PBS). Bone tissue required an additional decalcification preparation in 10% formic acid for 18 h at room temperature, and fat was mechanically pressed to reduce the lipid content before processing. Unless otherwise noted, tissue was processed by incubation with three different solutions, with thorough washing in PBS between each step: (1) 3% peracetic acid for 3 h at 37 °C; (2) 1% Triton X-100 containing 2 mM EDTA for 18 h at 37 °C and (3) 600 U/mL DNase (Sigma) containing 10 mM MgCl₂ for 18 h at 37 °C. After the final treatment, the tissue was washed thoroughly with PBS followed by distilled water and then lyophilized.

For experiments comparing multiple processing protocols, the following modifications were used: fresh—tissues were not treated with acid, detergent or DNase; mild—tissues were incubated in (1) 3% peracetic acid for 10 min at 37 °C and (2) 600 U/mL DNase containing 10 mM MgCl₂ for 18 h at 37 °C; moderate—tissues were incubated in (1) 3% peracetic acid for 3 h at 37 °C, (2) 1% Triton X-100 containing 2 mM EDTA for 18 h at 37 °C and (3) 600 U/mL DNase containing 10 mM MgCl₂ for 18 h at 37 °C; harsh—tissues were incubated in (1) 3% peracetic acid for 3 h at 37 °C, (2) 1% Triton X-100 containing 2 mM EDTA for 18 h at 37 °C, (3) 4% SDS for 16 h at 37 °C and (4) 600 U/mL DNase containing 10 mM MgCl₂ for 18 h at 37 °C; and digest—ECM particles were digested in 1 mg/mL porcine pepsin in 0.01 NHCl for 72 h.

**Tissue particle fabrication.** Lyophilized processed tissue was cryogenically pulverized in a cryomill (SPEX 6770, SPEX SamplePrep) at −195 °C under liquid nitrogen. Approximately 300–500 mg of sample was processed in each batch. Cryomill settings were 8–15 1-min cycles at 10 cycles s⁻¹ with 3-min cooling periods between runs. The resulting powder was suspended in distilled water or DMEM at 10 mg/mL and sonicated with a probe sonicator (GE 130PB, Cole-Parmer) at an output power of 10–15 W two times for 30 s in an ice bath. Water suspensions were centrifuged at 4,000 r.p.m. for 10 min and resuspended in fresh deionized (DI) water. Sonication was repeated, and the suspension was filtered through a 40-µm cell sieve. The final concentrations of solutions were determined from the mass of lyophilized aliquots.

**Microarray-chip fabrication.** Glass coverslips (22 × 60 mm) were cleaned and functionalized with methacrylate groups using a silane reaction, as previously described.

Acrylamide was mixed with bis-acrylamide and dissolved in DI water at concentrations of 10.55% and 0.55% (wt/vol). A photoinitiator solution of Irgacure (12959) dissolved in methanol at 200 mg/mL was added to the acrylamide solution at a concentration of 10% (vol/vol). A 20-µL drop of working solution was pipetted onto the functionalized 22 × 60–mm coverslip, and an untreated 22 × 50–mm glass slide was carefully placed on top of the liquid to form a thin layer estimated to be 36 µm thick. The solution was polymerized with UV light (~2 mW/cm²) for 10 min, and the 22 × 50–mm coverslip was removed after incubation in DI water for 30 min. Gel-coated slides were soaked in DI water overnight and dried on a hot plate at 40 °C for 45 min.

Silicon gaskets with arrays of 3-mm-diameter wells (GWCS-50R, Grace Bio-Labs) were placed on the dry gel-coated slides with 40 wells in full contact. Collagen (C7661, Sigma-Aldrich) dissolved at 0.25 mg/mL in 0.1 M acetic acid was pipetted (9 µL) in each chamber and allowed to dry overnight. Next, 10 µL of tissue particle suspension was spotted in each of the collagen-coated wells. The working tissue particle concentration was 2 mg/mL for suspensions of liver, lung, spleen and small intestine and 3 mg/mL for all other tissue types. The selected concentration was determined visually as the amount required to form a complete monolayer spot without visualization of the underlying acrylamide gel surface. Mixed cardiac and lung ECM solutions were combined at ratios of 1:3, 3:1 and 1:1 (vol/vol) and spotted at a total concentration of 3 mg/mL to form composite ECM substrates. Spotted chips were left to dry overnight in a cell-culture hood at room temperature, and the gaskets were removed. Chips were sterilized in a culture hood with UV light for 30 min on each side.

**Tissue spot variability.** The reproducibility of the spotting techniques was validated by histochemical staining and proteomic analysis. Dried substrates spotted from ECM particle suspensions of 11 different tissues and collagen controls were stained using Masson’s trichrome. Three replicates of each tissue type were visually compared for color content and distribution. We quantitatively compared the overall staining variation between tissues using a hue filter in ImageJ to threshold the percentage of each total spot area contained in the hue ranges 151–159, 209–229, 237–255, 188–238 and 227–234. The s.d. and coefficient of variation were calculated for each tissue (n = 3 spots) at each hue range.

**Protein-size characterization.** We compared the effects of different processing methods on protein size for lung and cardiac tissues by boiling samples in Laemmli buffer and running them on an SDS-PAGE gel at 120 V for 1.5 h. Changes in protein size after pepsin digestion were compared for tissue ECM particles from bladder, cartilage, lung, spleen and purified collagen. Undigested tissue was either prepared in Laemmli buffer as described above or digested in 1 mg/mL porcine pepsin (Sigma) in 0.01 M HCl. After 72 h of agitation in pepsin solution, the pH was neutralized and a sample was run on an SDS-PAGE gel to allow comparison of changes in protein size distribution relative to that of control undigested tissue particles.

**Proteomics analysis.** Tissue samples were reduced with dithiothreitol, alkylated with iodoacetamide and digested in solution with trypsin (Promega) at 37 °C overnight. Digested peptides were acidified and dried. The peptides were reconstituted in 40 µL of 2% acetonitrile, 0.1% formic acid, and 6 µL were injected (15% of the total volume). Protein identification by
liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of peptides was performed using a Q-Exactive interfaced with a Thermo Easy-nLC 1000 system (Thermo Scientific) or a Velos Orbitrap (Thermo Scientific) interfaced with a NanoACQUITY UPLC system (Waters). Peptides were fractionated by reversed-phase HPLC on a 75 µm x 12 cm column with a 15-µm emitter tip (New Objective, Woburn, MA) packed in-house with Magic C18AQ (5 µm, 120 Å, Michrom Bioresources) using a 0–90% acetonitrile, 0.1% formic acid gradient over 90 min at 300 nL/min. Eluting peptides were sprayed directly into the Q-Exactive or Velos at 2.0 kV. Q-Exactive survey scans were acquired from 350–1800 m/z with up to 15 peptide masses (precursor ions) individually isolated with a 2.0-Da window and fragmented (MS/MS) using a collision energy of 27 and 30-s dynamic exclusion. Precursor and fragment ions were analyzed at 70,000 and 17,500 resolution, respectively. Velos survey scans were acquired at 350–1800 m/z with up to eight peptide masses (precursor ions) individually isolated with a 1.9-Da window and fragmented (MS/MS) using a collision energy of 35 and 30-s dynamic exclusion. Precursor and fragment ions were analyzed at 30,000 and 15,000 resolution, respectively. The mass spectrometry–derived data were searched against a combined human and porcine RefSeq protein database (version 65 with common contaminants added) using the SEQUEST HT search algorithm through Proteome Discoverer (version 1.4.1.14, Thermo Scientific). Search parameters included a maximum of one missed trypsin cleavage, cysteine carbamidomethylation as a fixed modification, and methionine oxidation as a variable modification. The precursor mass tolerance was 20 ppm, the fragment mass tolerance was 0.05 Da, and the maximum peptide length was specified as seven amino acids. Peptides that passed the 1% false discovery rate threshold were used for protein identification. Protein inference was based on rules of parsimony as employed by Proteome Discoverer software. The mass spectrometry–proteomics data have been deposited to the ProteomeXchange Consortium37 via the PRIDE partner repository with the data set identifier PXD002571. We obtained gene-level measurements by summing the PSMs for all proteins corresponding to a given gene. We normalized the summed spectral counts in each category. The identified genes were then categorized as ECM (subclassified as collagen, proteoglycan or glycoprotein), ECM-associated (subclassified as ECM-affiliated protein, ECM regulator or secreted factor) or non-ECM as previously described38. The proteomic composition of processed tissues was compared to that of the native tissues from a recent draft map of the human proteome online resource available at http://www.humanproteonomemap.org/). Proteins found in both processed tissues and the corresponding native tissues were normalized between 0 and 1 and summarized in separate heat maps for ECM, ECM-associated and non-ECM components using Gene-E software (http://www.broadinstitute.org/cancer/software/GENE-E/). Hierarchical clustering for each protein was performed across all processed and native tissues in each heat map. The total abundance of ECM and non-ECM components in each tissue was determined from the sum of the normalized PSMs for all proteins in each category.

The variation in proteomic composition between replicate spots in a fabricated microarray was evaluated using three separate aliquots of a representative 3 mg/ml small intestine particle suspension. The total normalized PSMs and the corresponding number of identified proteins in ECM and non-ECM categories were compared for each run. Protein-identification consistency was evaluated for rare proteins (identified by one PSM) and more abundant proteins (identified by two or more PSMs) across the three runs and defined as follows: No Overlap, identified in one of three samples; 2 of 3, identified in two of three samples; or 3 of 3, identified in all samples.

Cell-seeding methods. The uniformity of cell distribution on 2D culture plates can be enhanced with a confined chamber. To limit variation in the initial cell-seeding density on each tissue array spot, we designed custom confined flow chambers. The flow chambers were made from polydimethylsiloxane (PDMS) such that the depth of media over the microarray was approximately 1 mm. The tissue microarrays were placed on a tissue culture plate (one well, rectangular; Nunc), and their edges were sealed with Parafilm to stabilize them and prevent media from flowing under the tissue microarray chip. The PDMS flow chamber was pressed over the array to seal the chamber, and cell suspension was slowly injected through the inlet. The locations of tissue spot replicates were randomized in the microarrays so that each type was evenly distributed across rows and columns to minimize systematic error associated with flow patterns during cell seeding (Supplementary Fig. 2a).

We performed a cell adhesion assay on control microarrays to quantitatively investigate the experimental error associated with cell seeding and washing procedures. Control microarrays were fabricated in which all spots were collagen controls. Breast cancer cells were seeded at 15,000 cells/cm² in serum-free media and allowed 1.5 h to attach. The PDMS flow chambers were removed, and the microarrays were washed four times in PBS, incubated in media with 10% FBS for 30 min, stained with calcein AM (3 µg/ml) for 30 min, and imaged. The total number of cells per spot was counted with ImageJ. The assay was run in duplicate, and the total error was quantified as the coefficient of variation for all spots in the microarray. We analyzed the systematic error by quantifying the average number of cells per spot in each row and each column. The coefficient of variation between rows or columns was calculated for each experiment. We calculated the final coefficient of variation in rows or columns by averaging the row and column coefficients of variation of duplicate tissue microarray chips.

3D microtissue optimization. We screened different ratios of cells to tissue ECM weight to optimize parameters for the formation of breast cancer tissue ECM 3D spheroids. Cells were suspended at 500,000 cells/ml and added to tissue ECM suspended at 4–0.25 mg/ml concentrations at a 1:1 (vol/vol) ratio. 40 µL of the cell–tissue suspension was pipetted into a 96-well hanging drop culture plate (InSphero) to form a hanging drop. Seven different tissue particle types (bone, cardiac, kidney, liver, lung, spleen and collagen) were mixed at final concentrations of 0, 0.125, 0.25, 0.5, 1 and 2 mg/ml with a final concentration of 250,000 breast cancer cells/ml in hanging drops. We imaged spheroids with a 2.5x objective on a Zeiss inverted microscope in
bright field on days 1, 2, 4 and 6 to capture spheroid-formation kinetics over time. The relative cell viability and/or proliferation at day 6 was compared between all groups with alamarBlue assay (Life Technologies).

**Spheroid microarray fabrication.** We formed the microtissue array by first preparing an array of 1-mm wells in a plastic mold. The wells were filled with water and cleared of air bubbles. Cell-tissue particle spheroids were transferred from a GravityTRAP (InSphero AG) culture dish into the wells of the plastic mold. After tissues had settled to the bottom of the wells, the mold was infiltrated with a 2% agarose solution in water at 70 °C and allowed to cool and form a gel. The agarose diffused into the water-filled chambers and surrounded the microtissues. After cooling, the agarose block was removed, dehydrated and infiltrated with paraffin similarly to the previously described procedure39. Dehydration was performed with graded ethanol solutions (100 mL: 30%, 50%, 70%, 80%, 95% × 2, 100% × 2) applied for 3 h each, after which 100% ethanol was applied overnight. Ethanol solutions were cleared with HistoClear II (100 mL) three times for 2 h and once overnight, and the samples were infiltrated with paraffin (100 mL, 60 °C, four times for 2 h) and cast in paraffin.

**Macrophage cell culture and morphological analysis.** BMDM progenitors were isolated from the femurs of wild-type C57BL/6 mice and differentiated toward a macrophage phenotype in a mixture of 80% DMEM-F12 (supplemented with 10% FBS and 1% penicillin-streptomycin) and 20% fibroblast (L929) conditioned media (10% FBS, 100 mM L-glutamine and 1% penicillin-streptomycin). Macrophage progenitors were differentiated for 7 d, with media changed at day 4. The resulting BMDMs were suspended in M1 or M2 macrophage polarization media (2.5 × 10^5 cells in 1.5 mL) on the tissue microarrays and the surrounding acrylamide. The M1 polarization medium contained 200 ng/mL lipopolysaccharide (055:B5, Sigma) and 20 ng/mL interferon-γ (Peprotech), and M2 polarization medium contained 20 ng/mL IL-4 (Peprotech). Cells were incubated for 24 h in their respective cytokine environments before being fixed, stained for actin and imaged and analyzed with the Cellomics platform. BMDMs were suspended in unsupplemented macrophage-differentiation media for seeding on tissue spots with different degrees of processing.

**Cancer cell culture and adhesion analysis.** Human skin cancer cell line A375 (ATCC, CRL-1619) and human lung cancer cell line A549 (ATCC, CCL-185) were cultured in growth media. Human breast cancer cell line Hs 578T (ATCC, HTB-126) was cultured in growth media supplemented with 0.01 mg/mL bovine insulin. Melanoma-derived mouse cancer cell lines (B16-F0 (ATCC, CRL-6322), B16-F10 (ATCC, CRL-6475) and B16-KY8 (ref. 40)) and L929 fibroblasts were cultured in growth media. We verified cell line identity by referencing the International Cell Line Authentication Committee database of cross-contaminated or misidentified cell lines. Cell lines were not tested for mycoplasma contamination.

Cancer cells and L929 fibroblasts were seeded in serum-free media at a final concentration of 15,000 cells/cm^2 and allowed to attach for 2 h. Cells were washed gently four times with PBS, incubated in media with 10% serum for 45 min and then washed again with PBS. Cells were stained with calcine AM at 3 μL/mL from 1 mg/mL stock for 30 min at 37 °C and imaged at 50× magnification. The total number of cells per spot was counted with ImageJ. Two chips with different tissue spot patterns (Supplementary Fig. 2a) were analyzed for each cell type for a total of six spots of each tissue type (n = 6). We quantified cell adhesion for each tissue by normalizing to the solubilized collagen–coated control spots.

**Cancer cell proliferation analysis.** Chips were placed in one-well rectangular culture dishes (Nunc) surrounded by Parafilm and seeded with labeled breast cancer cells via the PDMS gasket method. Cells were pre-stained in a T-75 flask with CellTracker Green dye CMFDA (5-chloromethylfluorescein diacetate, Invitrogen) according to the manufacturer’s instructions. A suspension of dyed cells was seeded onto the chips at a concentration of 5,000 cells/cm^2 in 10% FBS media in a confined flow chamber. Cells were allowed to attach overnight. The next day the confined flow chamber was removed and the media was changed. Cells were imaged at 50× magnification on day 1 and day 2 after seeding. By day 3 the tracker dye's signal had become too weak for segmentation imaging. At day 7 cells were incubated with calcine AM at 3 μg/mL for 30 min at 37 °C and imaged, and the number of viable cells per spot was quantified using ImageJ. Autofluorescence of the liver ECM made it impossible to resolve cell staining at day 2, so these data were not available. Cells formed an overconfluent monolayer and peeled off of the soluble collagen control spot at day 7, so these data also were not available. The cell number over time was used in an exponential growth model to estimate the doubling rate when cells were seeded on different tissue spots.

**Final cell number = Initial cell number × 2^kt**

Cell attachment was confirmed at day 1, and a growth-lag phase was assumed between day 1 and 2. Thus t represents a 5-d time span, the day 2 and day 7 cell counts represent the initial and final cell counts, respectively, and the doubling rate is calculated as k.

Viability analysis of 3D cancer cell and tissue ECM microtissues was conducted with the alamarBlue assay. Relative cell viability was compared between all groups by repeated sampling from wells incubated with alamarBlue reagent (Life Technologies) at days 1, 3 and 6 (n = 3).

**hASC culture and analysis.** hASCs were isolated as previously described41 and passaged at 90% confluence in growth media (GM) with media changes every 3 d. hASC differentiation was induced with osteogenic differentiation media (DMEM, 10% FBS, 1% penicillin-streptomycin, 100 nM dexamethasone, 50 μM ascorbic-acid-2-phosphate, 10 mM β-glycerophosphate).

**2D microarrays.** For culture on microarray chips, hASCs were seeded on tissue microarrays (Nunc) at 6,000 cells/cm^2 as described above. hASCs were cultured on the microarrays for 5 d in GM to reach confluence, after which the media was replaced with either osteogenic media or GM and the cells were cultured for an additional 6 d before being fixed and stained with Alizarin Red.

**3D microtissues.** Microtissues were formed by self-assembly of cells and tissue particles into spheroids in hanging drop culture42. Tissue particle suspensions were diluted to 0.8 mg/mL in serum-free DMEM culture media and sterilized with UV light (~1.5 mW/cm^2 for 20 min). hASCs were suspended in GM at 850,000 cells/mL with a 1:1 mixture of ECM particle suspension.
for microtissue formation as described above. We changed the microtissue medium with GM every 2 d by replacing half of the hanging drop volume with fresh media twice. Microtissues were cultured for 6 d in GM and then transferred to 96-well GravityTRAP (InSphero) plates with control GM or osteogenic media with media changes every 3 d. After 11 d of culture, microtissues were fixed, embedded in well molds as described above, sectioned and stained with Masson’s trichrome or Alizarin Red.

Macrophage and hASC PCR. Changes in mouse macrophage and hASC gene expression after culture with tissue ECM were determined in 2D and 3D microenvironments, respectively.

2D macrophage microarrays. Bone, cardiac, liver, lung and spleen tissues were processed using a modified procedure. Tissues were mechanically fragmented using a knife-mill processor (Retsch, Germany) into particles with sizes no larger than 10 mm3 and rinsed with thoroughly distilled water until blood was fully cleared from the samples. Bone samples were decalcified by incubation in 10% formic acid (Sigma) for 3 d and verified by a colorimetric calcium test (STANBIO Laboratory). All tissues were then incubated in 3.0% peracetic acid (Sigma) with agitation at 37 °C for 4 h and moved to fresh solution 1 h after the end of the incubation period. The pH was adjusted to 7 by thorough, extensive rinsing with water and PBS. Samples were transferred to a 1% Triton X-100 (Sigma) + 2 mM sodium EDTA (Sigma) solution and incubated with agitation at room temperature for 3 d, with the solution replaced by fresh solution daily. Tissues were rinsed thoroughly with distilled water and incubated in 600 U/ml DNase I (Roche Diagnostics) + 10 mM MgCl2 (Sigma) + 10% antifungal-antimycotic (Gibco) at 37 °C for 24 h. Tissues were rinsed thoroughly with distilled water, frozen at −80 °C and lyophilized. Tissue particles were prepared from lyophilized samples as described above. Tissue particle suspensions in distilled water were adjusted to 4–5 mg/ml for coating of six-well plates with 1 ml of solution followed by air-drying. After drying, plates were sterilized under UV light for 1 h and then rinsed to remove any nonadhered particles before being seeded with macrophages. Macrophages were cultured as described above in M1, M2 or nonpolarizing M0 media conditions for 24 h (n = 3).

Macrophage RNA was extracted for PCR analysis using TRIzol reagent (Life Technologies), and RNA was purified using RNeasy Mini columns (Qiagen). cDNA was synthesized through the use of SuperScript Reverse Transcriptase III (Life Technologies) per the manufacturer’s instructions. We carried out RT-PCR on an Applied Biosystems Real Time PCR Machine using SYBR Green (Life Technologies) as a reporter. Macrophage polarization was evaluated on the basis of the expression of genes associated with M1 polarization (Il1b, Nos2 and Tnf) and M2 polarization (Arg1, Retnla (Fizz1) and Il10). Expression was calculated relative to the reference gene via the 2 −ΔΔCt method and the following primers: B2m forward, CAGAAGAATGGAAGAGTCAG; Arg1 forward, CAGAAGATGGAAAGGTCAG; Retnla forward, CTTTCCTGTGAG; Nos2 forward, GATAGGCAGAG; Tnf forward, GTCATTCCTGATTCTCT; Tnf reverse, GAAAGGTCCTGAAGGT AGG; Il1b forward, GTATGGGCTGACTGTTTCT; Il1b reverse, GCTGTCTGTCATTACAG; Nos2 forward, GACGAGACG GATAGGGAGAG; Nos2 reverse, GTGGGGTTGCTGTGACCTT; Arg1 forward, CAGAAGATGGAAAGGTCAG; Arg1 reverse, CAG ATATGCAGGGAGTCACC; Retnla forward, CTTTCCTGTGAG TCTGCCCCAG; Retnla reverse, CACAAGCACACCCAGTACCA; Il10 forward, TCTCACCAGGGAAAATCTAAA; Il10 reverse, AAGTGTGCACGAGGCA (Integrated DNA Technologies). The fold change in expression for each polarizing media condition was used to create a heat map with hierarchical clustering by tissue ECM coating.

3D hASC microtissues. Osteogenic differentiation was determined in bone and lung microtissues using quantitative RT-PCR. Microtissues were formed as described above for 7 d in GM followed by an additional 14 d in osteogenic media. A total of three microspheres per treatment group were pooled and homogenized for RNA extraction using TRIzol reagent per the manufacturer’s instructions. RNA was then reverse-transcribed to cDNA using Superscript III reverse transcriptase (Life Technologies) according to the manufacturer’s instructions. Real-time PCR was carried out with a StepOnePlus Real-time PCR system (Applied Biosystems). We calculated Bglap gene expression at day 21 for each tissue relative to that at day 7 using Gapdh as the reference gene via the 2 −ΔΔCt method and the following primers: Bglap forward, CCTCACACTCCTG GCCCTAT; Bglap reverse, CTTGGACACAAAGGCTGCAC; Gapdh forward, AGGAGCGAGATCCCTCACAAG; Gapdh reverse, AATAGGCC CCCCAGCTTCTC.

Histology. Microarray chips were fixed in 4% paraformaldehyde before staining procedures. 3D microtissues were fixed in paraformaldehyde for 1 h before being embedded in microarrays as described above. Paraffin blocks containing microtissue arrays were sectioned at 5 µm and stained as described below.

Actin and nuclear staining. Cells were permeabilized in 1% Triton X-100 and stained for actin with Alexa Fluor 647–conjugated phallolidin (Life Technologies) at a 1:25 dilution in 0.1% BSA (Sigma) in PBS for 30 min at 37 °C. Nuclei were stained with DAPI (100 ng/mL, Life Technologies) for 30 min.

In vitro macrophage immunofluorescence. Macrophages seeded on tissue arrays in M1 or M2 polarizing cytokines as described above were evaluated for expression of the M2 and M1 markers Arg-1 and iNOS. After 24 h, macrophages were fixed for 10 min in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in Tris-buffered saline (TBS) for 10 min. Nonspecific protein interactions were blocked with 1% BSA, 2% horse serum in 0.05% Tween-20. Tissue microarrays were then incubated overnight at 4 °C with primary antibodies to Arg-1 (rabbit polyclonal, diluted 1:100; GTX109242, GeneTex) and iNOS (mouse monoclonal (4E5), diluted 1:300; ab129372, Abcam) diluted in 1% BSA in TBS. Arrays were washed and probed with secondary FITC-conjugated goat anti-rabbit IgG (diluted 1:250; 111-095-003, Jackson ImmunoResearch) and Alexa Fluor 568–conjugated goat anti-mouse IgG (diluted 1:250; A-11004, Invitrogen) in 1% BSA for 1 h. ECM autofluorescence was then blocked by treatment with 0.1% Sudan Black B in 70% ethanol for 20 min. Arrays were washed, coveredslipped and imaged. We corrected variations in background autofluorescence by subtracting the mean background intensity of acellular regions of each tissue array spot using ImageJ software.

Subcutaneous tissue ECM implantation and immunofluorescence. Animal experiments were conducted in accordance with guidelines set by the Johns Hopkins University Animal Care and Use Committee. Bone and collagen tissue ECM particulate was
hydrated with saline (100 mg dry wt/0.2 ml) and injected subcutaneously on the dorsum of 6–8-week-old female C57BL/6 mice. The mice were killed after 1 week, and implants were explanted, fixed in formalin and embedded in paraffin for sectioning. Sections were then deparaffinized, rehydrated and immunolabeled for the pan-macrophage marker F4/80 and the M1 marker iNOS. Antigen retrieval was conducted in citrate (10 mM, pH 6) for 30 min in a vegetable steamer, and samples were then rinsed with TBS in 0.05% Tween-20. Sections were blocked with 1% BSA, 2% goat serum and 0.05% Tween-20 for 1 h. Sections were then incubated overnight at 4 °C with primary antibodies to F4/80 (rat monoclonal (BM8), diluted 1:100; ab16911, Abcam) and iNOS (mouse monoclonal (4E5), diluted 1:200; ab129372, Abcam) in blocking solution. Sections were washed and probed with FITC-conjugated goat anti-rabbit IgG (diluted 1:250; A-11007, Invitrogen) secondary antibodies in blocking solution for 1.5 h. ECM autofluorescence was then blocked by treatment with 0.1% Sudan Black B in 70% ethanol for 20 min. Sections were counterstained with DAPI for 5 min, washed, coverslipped and imaged.

**Alizarin Red staining.** Staining of calcified matrix was performed on microarray chips with Alizarin Red (40 mM, pH 4.1) for 25 min. Chips were rinsed with water briefly three times and then a fourth time for 5 min before rapid dehydration and clearing in acetone, xylene:xylene (50:50) and xylene, followed by coverslipping. Sectioned slides containing microtissue arrays were deparaffinized; rehydrated; stained with Alizarin Red for 5 min; washed; dehydrated and cleared rapidly with acetone, 1:1 acetone-xylene, xylene; and coverslipped.

**Electron microscopy.** Dry tissue microarrays were placed in a desiccator overnight to dry completely, attached to aluminum stubs by carbon sticky tabs (Ted Pella) and coated with 20 nm of AuPd with a Denton Vacuum Desk III sputter coater (Denton Vacuum). Stubs were viewed and digital images were captured at a 60° tilt on a LEO 1530 field emission scanning electron microscope operating at 1 kV.

**Slide scanning and analysis.** Cell counts and morphology on microarray chips were imaged and quantified using a Cellomics high-content scanner (Cellomics) with ArrayScan VTI software (Thermo Fisher). Microarray chips were adhered to four-well rectangular culture plates (Nunc). Cell counts were quantified from 50× magnified images (two fields per spot) with calcein AM staining. Morphological quantification was performed after phalloidin staining as described above. The object-identification parameters were adjusted to outline the perimeters of individual cells in each image that were subsequently counted and analyzed for morphology using the convex hull area ratio. 2D microarrays stained with Alizarin Red and histological slides containing 3D spheroid microarrays were scanned with a ScanScope AT (Aperio) at 200× magnification and viewed with ImageScope software (Aperio).

**Image processing.** Cell counts for adhesion studies were analyzed from 50× magnified images using ImageJ (US National Institutes of Health, Bethesda, MD). The cell number for each spot (n = 6) was normalized to that of control spots (soluble collagen) on the same microarray. We analyzed color images of 2D and 3D Alizarin Red–stained microarrays with ImageJ to quantify the percent area of Alizarin Red staining. Regions of interest (ROIs) were traced around the perimeter of each individual sample in the array for measurements of total area. Microarrays were threshold color-filtered in the RGB color space to allow selection of areas of positive red staining, and the filtered image was converted to binary. The percent area of positive staining was calculated using the “measure” command in the ROI manager.

**Mechanical testing.** The elastic modulus and hardness of dried tissue spots were measured using a Nano Indentor XP (MTS Systems) and Nanosuite software, which supports measurements of complex, thin materials. Samples were approached with a velocity of 10 nm/s, and samples were indented with a Berkovich tip to a depth of 400 nm at a strain rate of 0.05 with a peak hold time of 10 s and 90% unload. To minimize the influence of the underlying glass coverslip on array mechanics, we increased the sample thickness by double spotting. We spotted 10 μL of particle suspension and dried it as described previously, and then we spotted and dried an additional 10 μL of particle suspension on top of the original spot. Because each test took several hours, measurements were made on samples in a dried state to avoid confounding factors of evaporation. Spots of six different tissues (bladder, brain, cardiac, liver, lung and spleen) were indented in 8–10 different locations per sample. Outlier measurements were excluded from analysis as determined by Grubbs’ test (one measurement each was excluded for bladder, brain, lung and spleen) as described under “Statistical Analysis.”

**Proteomics network analysis.** The abundance of extracellular proteins (identified at the gene level) in each tissue was correlated with *in vitro* assay results. In some cases, assay results were not available for all tissues, resulting in fewer than 11 tissues for a given assay. We calculated correlation coefficients for each of the 3,879 identified proteins in the 15 *in vitro* assays by using the assay results directly and replacing each assay result with its rank-ordered value. The P value and t-statistic for each protein-assay correlation was calculated from the linear model for both the original and rank-transformed assay data. We visualized the assay and protein results as a heat map showing the correlation coefficients for all genes and assays (Cluster 3.0) and by creating an assay–protein network (Cytoscape v3.2.1). In images, proteins and assays are displayed as vertices, and edges connect proteins to assays when they are strongly correlated (P < 0.05 for both the original assay and the rank-transformed assay results). The full results of these analyses are provided in Supplementary Table 2.

We conducted gene-set enrichment analysis to determine whether defined groups of proteins improved the predictive value for each assay. This was done for bone, cartilage, lung and spleen tissues using the Biological Processes Gene Ontology database. ECM proteins from each of these tissues were evaluated for enrichment terms related to osteogenesis, and the corresponding P values for enrichment were compared. Terms related to osteogenesis included skeletal system development (GO:0001501), ossification (GO:0001503), bone morphogenesis (GO:0060349), osteoblast differentiation (GO:0001649) and chondrocyte...
differentiation (GO:0002062). Identified ECM proteins annotated to these processes are listed in Supplementary Table 3.

**Statistical analysis.** Statistical significance for quantitative in vitro cell assays was determined by a one-way analysis of variance with post hoc Tukey’s test (Graphpad Prism v6, GraphPad Software). The effect of different processing methods on macrophage morphology was determined by Student’s t-test with Bonferroni correction. Statistical significance was defined as $P < 0.05$. Differences between tissue spots are represented as tissue symbols above each bar in figures (representing the mean ± s.d.) or included in Supplementary Table 1. Statistical outliers for nano-indentation measurements were observed from Tukey box plots as values more than 1.5 times the interquartile range of the quartile borders and confirmed via Grubbs’ outlier test using $\alpha = 0.05$ (Graphpad Prism v6). These outlier measurements were excluded from analyses of tissue spot elastic modulus and hardness. Power analysis was not conducted to determine sample size, and investigators were not blinded.

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