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Extracellular Matrix Production by Mesenchymal Stromal Cells in Hydrogels Facilitates Cell Spreading and Is Inhibited by FGF-2

Horton, Edward R ; Vallmajo-Martin, Queralto ; Martin, Ivan ; Snedeker, Jess G ; Ehrbar, Martin ;
Blache, Ulrich

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Title: Extracellular Matrix Production by Mesenchymal Stromal Cells in Hydrogels facilitates Cell Spreading and is Inhibited by FGF-2

Authors: Edward R Horton, Queralto Vallmajo-Martin, Ivan Martin, Jess G Snedeker, Martin Ehrbar*, Ulrich Blache*

Affiliation:

Dr. ER Horton

Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark

Dr. Q Vallmajo-Martin, Dr. M Ehrbar, Dr. U Blache

Department of Obstetrics, University and University Hospital of Zurich, Zurich, Switzerland

Email: Martin.Ehrbar@usz.ch, Ulrich.Blache@hest.ethz.ch

Dr Q Vallmajo-Martin

Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Prof. I Martin

Department of Biomedicine, University Hospital Basel, University of Basel, Basel, Switzerland

Prof. JG Snedeker, Dr. U Blache

Institute for Biomechanics, ETH Zurich, Zurich, Switzerland

Prof. JG Snedeker

Balgrist University Hospital, University of Zurich, Zurich, Switzerland

Key words: hydrogel, extracellular matrix, FGF-2, mesenchymal stromal cell, biomaterial

Abstract:

In native tissues, the interaction between cells and the surrounding extracellular matrix (ECM) is reciprocal, as cells not only receive signals from the ECM but also actively remodel it through secretion of cell-derived ECM. However, very little is known about the reciprocal interaction between cells and their secreted ECM within synthetic biomaterials that mimic the ECM for use in engineering of tissues for regenerative medicine or as tissue models. Here we use poly(ethylene glycol) (PEG) hydrogels with fully defined biomaterial properties to investigate the emerging role of cell-derived ECM on culture outcomes. We show that human mesenchymal stromal cells (MSCs) secrete ECM proteins into the pericellular space early after encapsulation and that, even in the absence of material-presented cell adhesion motifs, cell-derived fibronectin enables cell spreading. We next investigate how different culture conditions influence MSC ECM expression in hydrogels. Most strikingly, we find by RNA sequencing that the fibroblast growth factor 2 (FGF-2) changes ECM gene expression and, in particular, decreases the expression of structural ECM components including fibrillar collagens. In summary, this work shows that cell-derived ECM is a guiding cue in 3D hydrogels and that FGF-2 is a potentially important ECM regulator within bioengineered cell and tissue systems.

Introduction:

The physiological material surrounding cells in tissues is the extracellular matrix (ECM). The ECM is a hydrated network of many structural and adhesive macromolecules that not only provides physical support to cells, but also is itself bioactive and serves as a reservoir for several growth factors ^[1]. Additionally, physical ECM properties activate intracellular signaling pathways, thereby determining cell fate and organizing cells into specific tissues ^[2]. To engineer tissues *in vitro* or to study cells in 3D tissue and disease models, researchers have commonly used hydrogels generated from natural ECM polymers. However, despite important biological insights obtained from natural ECM hydrogels, their application is often limited as they are isolated from animal tissue and cell cultures. To address this issue, bioengineers have developed synthetic hydrogels that mimic the natural ECM but are synthesized with defined chemical properties to provide reproducible and tunable 3D tissue culture conditions ^[3]. Therefore, synthetic hydrogels are an ideal platform to study basic biological behavior, such as tissue morphogenesis, in the absence of ill-defined ECM ^[4]. Similarly, they enable regulation of stem cell differentiation in response to tightly controlled extracellular stimuli ^[5].

Cells in tissues remodel their microenvironment by both degrading and depositing ECM molecules, which also occurs when cells are in biomaterials. While biomaterial degradation is a well-characterized property, astonishingly cell-secreted ECM and its related biological implications have often been overlooked in the biomaterials field. However, cells encapsulated within synthetic hydrogels do secrete and assemble ECM proteins into the pericellular space, which influences cell behavior and cell fate ^[6]. These recent findings clearly suggest that a better understanding of cell-derived ECM within synthetic hydrogels is required, which might then also provide an opportunity to harness secreted ECM for the engineering of tissues ^[7].

In the present study, we investigate how cell culture conditions influence ECM expression of mesenchymal stromal cells (MSCs) in synthetic poly(ethylene glycol) (PEG) hydrogels. MSCs are widely used progenitor cells in regenerative medicine, have been employed in myriads of clinical trials, and secrete high amounts of ECM when activated ^[8]. We show that MSCs secrete and assemble ECM proteins into hydrogels quickly after encapsulation, which then influences their own behavior. We further show that 3D culture itself influences ECM gene expression while the RGD-motif used for cell attachment does not. Moreover, supplementing cultures with fibroblast growth factor 2 (FGF-2), a critical component in MSC culture, strongly reorganized and downregulated the expression of ECM genes of MSCs in hydrogels. In summary, we show that culture conditions govern the ECM fingerprint of MSCs in hydrogels, which demonstrates that cell-derived ECM must be considered and could be harnessed, for example, to engineer stem cell niches.

Results

ECM deposition by MSCs in hydrogels

To form synthetic hydrogels, Lys and Gln PEG molecules (8-arm) are crosslinked by the transglutaminase factor XIIIa (FXIIIa), as we have previously described ^[9]. In addition, a matrix metalloproteinase (MMP)-sensitive degradation domain (to enable cell-mediated hydrogel degradation) and, if required, an RGD peptide (to enable integrin-mediated cell adhesion) are incorporated (**Figure 1a**). We confirmed that RGD incorporation maintains the same stiffness of the formed hydrogels as shown by rheology (**Figure 1b**). To culture human bone marrow-derived MSCs in soft PEG hydrogels (500 Pa), cells were mixed with the gel precursor solution during polymerization. It has previously been shown that MSCs are able to spread, migrate, and build cellular networks in this artificial ECM microenvironment ^[6a, 10]. Similarly to when cells are grown as 2D monolayers (tissue culture glass, TCG), MSCs in hydrogels deposited extracellular proteins in their vicinity within 24 h, as visualized by AHA labeling of nascent protein deposition prior to fixation and without membrane permeabilization (**Figure 1c**). However, whereas cells in 2D monolayers produce a fibrillar protein network, cells in 3D rather start to deposit a dense matrix shell surrounding themselves. This cell-secreted ECM contains various matrix proteins, as by immunofluorescence we found fibronectin (FN), collagen type III and perlecan already present after 24 h of culture (**Figure 1d**). More specifically, cell-secreted ECM lines the cell's encapsulation cavities in a spherical manner, indicating that ECM deposition into the pericellular space starts even before, or in parallel, to cellular spreading and migration (**Figure 1e**).

Figure 1: Cellular matrix deposition in synthetic hydrogels. (a) Schematic of ECM-mimicking PEG hydrogels containing the MMP-sensitive sites and optionally the integrin adhesion ligand RGD. (b) Storage moduli of PEG hydrogels with and without RGD as measured by rheology over time (left, representative data from one experiment) and after 30 mins (right, data from 3 independent experiments, two-tailed t test, no significant difference). (c) Stromal cells encapsulated in hydrogels remodel their microenvironment by assembling secreted proteins around themselves. Representative fluorescence images (Z-projections) of MSC cytoskeleton (F-actin, magenta) and nascent cell-secreted proteins (labeling of the methionine analog AHA, green) in 2D and 3D (50 μ M RGD) after 24 h. Scale bar 20 μ m. (d) MSCs (F-actin, magenta) deposited cell-derived ECM components (green) to sites of their initial embedment in hydrogels (50 μ M RGD) after 24 h of culture. Images show Z-projections. Scale bar 20 μ m. (e) Stromal cells protrude (left) and move out of their secreted ECM cavity (right) in hydrogels (50 μ M RGD). Representative immunofluorescence images and 3D reconstruction of MSC cytoskeleton (F-actin, magenta), nucleus (blue) and cell-secreted, extracellular fibronectin (green) in 3D after 24 h. Scale bars 10 μ m.

Next, we addressed the role of cell-secreted ECM in hydrogels. One of the early morphological events stromal cells undertake when placed in a new environment is to establish cell adhesions and to spread. In synthetic hydrogels, adhesion peptides derived from the ECM such as the RGD motif of fibronectin are used to facilitate cell adhesion and spreading^[11]. Indeed, MSCs spread more in PEG hydrogels functionalized with RGD compared to non-RGD conditions, although MSCs still spread under non-RGD conditions (**Figure 2a, b, Figure S1**). To investigate the contribution of cell-secreted ECM to MSC spreading, we chose to deplete FN in MSCs by siRNA knockdown, as FN is secreted very early upon cell encapsulation and acts as a template for many other ECM proteins^[12]. FN knockdown was confirmed at the protein level (**Figure S3**). Cell spreading in hydrogels was

reduced in FN-depleted cells under both RGD and non-RGD conditions (**Figure 2b, Figure S2**). In particular, cell spreading was almost completely abolished in the absence of cell-secreted FN in hydrogels without RGD. However, addition of human plasma FN to hydrogels restored spreading of FN-depleted cells (**Figure 2b**). These data show that when MSCs are encapsulated in hydrogels, secreted FN enables cell spreading. Next, we aimed to test whether cell-secreted FN also supports spreading in 2D on top of hydrogels, where cells in contrast to 3D are not held in place by physical entrapment. However, when MSCs were plated in 2D on top of hydrogels, cells spread only on RGD hydrogels. In contrast, under non-RGD conditions cells could not spread and formed spherical multicellular clusters, indicating that they did not adhere to the hydrogel surface (**Figure 2c**). FN depletion strongly reduced spreading of MSCs on RGD hydrogels (**Figure 2d, e**), whereas cell spreading was unaffected in FN-depleted cells when grown on tissue culture glass or plastic (TCP) in 2D (**Figure 2d, S3**).

Figure 2: Cellular fibronectin secretion reinforces cell spreading in hydrogels. (a) The adhesion ligand RGD and cell-secreted fibronectin mutually support cell spreading. Individual cells with intact or fibronectin knockdown (green) after 72 h of culture in hydrogels with and without RGD. Upper scale bar: 20 μ m. Representative F-actin images that were used for quantification of cell spreading are shown. Lower scale bar: 200 μ m. (b) Additionally, human plasma FN (50 μ g mL⁻¹) was added to hydrogels without RGD. Quantification of cell spreading measured by the spreading index: Box plot (25th and 75th percentiles) with whiskers (5th and 95th percentiles), median

(line), $n \geq 9$ (3 independent experiments were performed using 3 donor MSCs in each replicate), ANOVA with Bonferroni's post hoc test shows significances among conditions without exogenous FN protein (black stars) and the significance of FN rescue conditions compared to their corresponding FN-free conditions (green stars): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. (c) Representative fluorescence images (actin,) of MSCs on top of hydrogels with and without RGD after 72 h. Scale bar: 200 μm . (d) Cell-secreted fibronectin reinforces cell spreading on RGD hydrogels but not on tissue culture glass (TCG). Representative images of MSCs (F-actin, magenta) with intact or siRNA fibronectin (green) knockdown on RGD hydrogels and TCG. Scale bar: 200 μm . (e) Quantification of cell spreading for MSCs on top of hydrogels containing RGD. Plot shows $n=3$ individual technical replicates of one experiment using one donor MSC, mean (line), ANOVA with Bonferroni's post hoc test. All images show Z-projections.

In summary, these data suggest that cell-derived ECM is required for initial cell attachment and subsequent spreading atop hydrogels, while MSCs encapsulated in 3D PEG hydrogels are physically constrained and assemble their own pericellular ECM, which supports cell spreading even in absence of material-presented cell adhesion motifs.

Culture conditions influence ECM expression of MSCs in hydrogels

Since deposited ECM appears soon after MSC encapsulation in hydrogels, we next wanted to investigate if and how 3D culture conditions influence ECM gene expression. Initially, we cultured MSCs in two different cell densities in hydrogels and found no differences in ECM gene expression between the two cell densities (**Figure S4**). Next, we cultured MSCs on 2D TCP or encapsulated them in RGD hydrogels and analyzed the gene expression of fibronectin (FN1), fibrillar collagens (COL1A1, COL1A2, COL3A1) and collagen type 4 (COL4A1) (**Figure 3a**). In both 2D TCP and 3D hydrogel conditions, the expression of all ECM genes increased after 7 days compared to one day of culture, except for FN1 and COL4A1 in 3D. When comparing between 2D and 3D conditions, we found very similar gene expression of fibrillar collagens. In contrast, FN1 and COL4A1 were ~2-fold and ~25-fold reduced, respectively, in 3D compared to 2D at day 7.

As integrins are important receptors for outside-in signaling^[13], the RGD motif is used to enable integrin-mediated cell-material adhesions in synthetic hydrogels. Therefore, we tested if cell adhesion to the RGD motif alters ECM gene expression of MSCs in hydrogels. We found that RGD did not influence the expression of any ECM genes tested here, independently of whether we examined early (day 1) or late (day 7) time points (**Figure 3b**).

Finally, as MSCs are often cultured in the presence of the cytokine FGF-2, we investigated whether FGF-2 affects ECM gene expression of MSCs in hydrogels. Here, we observed a strong decrease in fibrillar collagen gene expression over time when MSCs were cultured with FGF-2 in hydrogels (**Figure 3c**), whereas the expression of FN1 and COL4A1 genes was only partially decreased by FGF-2. In summary, we show that ECM gene expression of MSCs cultured in hydrogels is strongly reduced by FGF-2 treatment.

Figure 3: Influence of culture conditions on ECM gene expression of MSCs in synthetic hydrogels. (a) Gene expression of MSCs cultured on 2D plastic or in 3D hydrogels (RGD-modified, 50 μ M) after 1 or 7 days. (b) Gene expression of MSCs cultured in 3D hydrogels functionalized with or without the adhesion peptide RGD (50 μ M) after 1 or 7 days. (c) Gene expression of MSCs cultured in 3D hydrogels (RGD-modified) supplemented +/- FGF-2 after 1 or 7 days. Gene expression was measured by qRT-PCR and normalized to GAPDH and YWHAZ expression. Data are shown as fold-change over the control condition (light grey bars). Bars represent mean values + SD, individual replicate values are shown, $n = 4$ (4 independent experiments were performed using different donor MSCs in each replicate)). ANOVA with Bonferroni's post hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

FGF-2 determines MSC ECM gene expression in hydrogels

We next investigated the global changes MSCs undergo in hydrogels in response to FGF-2 by transcriptome analysis using RNA sequencing (**Figure 4, Supplementary Table 1**). In deep sequenced mRNA of MSCs, we found 678 genes to be strongly and significantly upregulated ($\log_2$ -fold > 1 , FDR adjusted p -value < 0.01), and 733 genes downregulated by FGF-2 (**Figure 4a**). To reveal pathways associated with FGF-2 signaling in MSCs, we analyzed the data set for overrepresented Gene Ontology (GO) terms (**Figure 4b, c**). It has previously been reported that in MSCs treated with FGF-2 in 2D, FGF-2 treatment upregulates genes associated with cell proliferation and downregulates genes associated with collagen-related processes in MSCs^[14]. Among the 20 most overrepresented GO terms associated with MSCs treated with FGF-2 here, we found many terms related to mitosis and cell division,

clearly showing the proliferative effect of FGF-2 (**Figure 4b**). On the other hand, GO terms that were overrepresented in the absence of FGF-2 almost all relate to the ECM (**Figure 4c**). We compared our dataset representing 3D-cultured MSCs to the published RNAseq dataset of MSCs treated with FGF-2 in 2D ^[14], and found a positive correlation between both datasets (**Figure S5a**). However, 3D culture induced larger gene expression changes than when MSCs were cultured in 2D, which were mainly related to cell proliferation (induced by FGF-2) and ECM (reduced by FGF-2) GO terms (**Figure S5b, c**). These data indicate that 3D culture may amplify the effects of FGF-2 on MSCs compared to 2D.

To further investigate the functional relationship of differentially expressed genes and to facilitate the visualization and understanding of the 3D data set, we enriched all genes within the GO domain Cellular Component and displayed the data as an enrichment map (**Figure 4d**). In this visualization map, Cellular Component terms that are gene-enriched appear as clusters, which capture related functional cell changes to facilitate data interpretation (**Figure 4e**). Strikingly, the most prominent clusters show a fundamental rearrangement of MSC ECM gene expression in response to FGF-2 (**Figure 4e**; cluster 1 and 2). Specifically, suppressed ECM genes by FGF-2 comprised many collagens including fibrillar collagens (COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL11A1, COL11A2, COL27A1), COL4A1 and fibril-associated collagens with interrupted triple helices (FACIT; COL9A2, COL9A3, COL12A1, COL16A1, COL16A1), which is in agreement with PCR analysis (**Figure 3c**). Furthermore, gene expression of collagen fibril maturation enzymes known for connective tissue organization ^[15] from the A disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) and lysyl oxidase (LOX) families were decreased by FGF-2. In addition, genes encoding several non-collagenous ECM proteins such as tenascin X, elastin, aggrecan, decorin and periostin were all reduced in MSCs when cultured in the presence of FGF-2. Conversely, we observed a smaller group of ECM genes that were upregulated by FGF-2 (cluster 2). These genes encode proteins including membrane-associated collagens with interrupted triple helices (MACIT; COL13A1, COL17A1), “rare collagens” (COL26A1, COL28A1), the hypertrophic chondrocytes collagen (COL10A1) and some non-collagenous ECM proteins (NNTG1, DMP1, LAMA3). These data show that FGF-2 is a potent inhibitor of MSC ECM gene expression in hydrogels.

Furthermore, we found that a gene cluster positively associated with FGF-2 (**Figure 4e**; cluster 3) related to chromosome organization, suggesting a mitotic effect of FGF-2 on MSCs in hydrogels. Finally, we detected a large cluster (**Figure 4e**; 4) that shows cellular components responsible for skeletal muscle organization (sarcomere, contractile fiber, myofibril, actin cytoskeleton). Interestingly, here we found that FGF-2 differentially

regulates genes within this cluster, indicating that it might control a reorganization of the contractile cell phenotype.

Figure 4: Transcriptome profile of MSCs cultured in synthetic hydrogels in response to FGF-2 (a) A volcano plot shows the differentially expressed genes (DEG) between MSCs cultured in hydrogels with or without FGF-2.

Horizontal line: FDR adjusted p-value = 0.01. Vertical lines: log₂-fold change of 1 in expression. (b, c) The 20 most significantly overrepresented gene ontology terms for genes that were significantly enriched in the control (blue) or with FGF-2 (red) (corrected P-value < 0.05). (d) Functional enrichment map of the transcriptome data. Genes identified as DEG following transcriptome analysis (RNA-seq) are enriched and clustered by the gene ontology domain GO *Cellular Component*. Up- and downregulated genes in FGF-2-treated MSCs are shown in red and blue, respectively. (e) Strongly represented GO *Cellular Component* clusters show a prominent rearrangement of the ECM (1,2), chromosome and mitotic activity (3) and sarcomere organization and skeletal muscle contraction/contractile apparatus (4). See **Supplementary Table 2** for a full list. Three independent experiments were performed using different donor MSCs. (f) Western blot confirmation of COL1A2 and LOX protein levels. Quantification of n = 4 (4 independent experiments were performed using different donor MSCs in each replicate) is shown in **Figure S6**.

In summary, groups of genes modulated by FGF-2 when MSCs are in hydrogels are related to the ECM, cell cycle and contractile cytoskeleton. These gene expression changes could influence the production and secretion of proteins involved in how MSCs remodel their microenvironment in hydrogels, which might interfere with outside-in cell signaling. To test whether the observed gene expression changes translate to altered protein levels, we selected two ECM genes whose expression was reduced by FGF2 (COL1A2 and LOX), and confirmed a reduction at the protein level by western blotting (**Figure 4f, S6**).

Conclusion

Dynamic reciprocity in biomaterials

Bidirectional interactions between cells and their microenvironment occur in every tissue. In vivo, such ‘dynamic reciprocity’^[16] between cells and the ECM drives cell fate decisions and ECM reshaping, which is crucial for tissue development, homeostasis and regeneration^[17]. Similarly, when placed in biomaterials, cells and their artificial microenvironment undergo bidirectional interactions. Indeed, the impact of biomaterial properties on cells is well understood and is harnessed in thousands of tissue engineering approaches. In contrast, the impact of cells on biomaterial remodeling is only characterized for catabolic remodeling (biomaterial degradation), whereas relatively little is known about anabolic remodeling (cell-secreted ECM). Recent literature has shown, however, that cell-secreted ECM is important for stem cell fate in synthetic hydrogels^[6a-c]. In the present study, we show ECM secretion into the pericellular space enables spreading and adhesion of MSCs in the absence of material-presented adhesion motifs, which extends our previous findings of the important role of fibronectin in adhesive materials^[6a]. These data indicate that cells may remodel their own microenvironment in biomaterials such that cell-derived ECM not only provides additional cues but also overrides the effects of material-presented RGD motifs, which is in agreement with the work by Loebel et al^[6c]. This proof of concept finding underlines that there

are likely many consequences downstream of cell-secreted ECM, as the pericellular space is an important master regulator of cell functions ^[18].

As cell-secreted ECM influences cell behavior, it is important to understand how experimental conditions govern cellular ECM production. Synthetic hydrogels are helpful in this regard, because they are free of ill-defined ECM and the influence of individual factors on cell-derived ECM secretion can be dissected ^[6e]. Here, we have investigated how culture conditions drive ECM gene expression in hydrogels. MSCs are tissue progenitor cells that are widely used in the biomaterials field for tissue engineering approaches as well as for basic cell-material interaction studies. We find that hydrogel culture itself impacts ECM gene expression of MSCs, which is in line with previous data showing gene expression and cell signaling differ between 2D and 3D culture ^[19]. In contrast, neither the adhesion peptide RGD nor cell density influenced ECM gene expression in hydrogels tested here. However, we have only tested some ECM genes and work by others, for instance, has shown that high cell density stimulates the secretion of a glycoprotein-rich ECM in synthetic hydrogels ^[20]. Furthermore, in addition to the culture conditions tested in our study, the effects of many other culture properties on ECM regulation could be examined in the future, such as modulation of material properties (e.g. stiffness, material density, pore size, other adhesion motifs, stress relaxation) or oxygen concentration.

FGF-2 as an MSC and ECM regulator in hydrogels: potential application in regenerative medicine

FGF-2 is a key component of MSC *ex vivo* cultures because it supports proliferation, maintains stemness, reinforces multi-lineage differentiation capacity and supports immunosuppressive conditions of MSCs ^[21]. In synthetic hydrogels, FGF-2 has been shown to support MSC viability and spreading ^[22]. Here, we identify a massive re-organization of ECM gene expression in 3D-cultured MSCs in response to FGF-2. Specifically, we find many ECM genes encoding, for example, fibrillar and fibril-associated collagens that decreased in response to FGF-2. This finding reinforces the idea of harnessing FGF-2 for regulation of cell-secreted ECM in regenerative medicine approaches, for example by using FGF-2 to suppress excessive ECM production and tissue fibrosis ^[23]. To engineer 3D biomaterials with anti-fibrotic effects targeting either endogenous or hydrogel-delivered MSCs, previously reported biotechnology strategies could be used to immobilize FGF-2 to hydrogels via non-covalent heparin affinity ^[24] or via covalently linked, engineered cytokine-binding proteins ^[25]. By combining such technologies with photo-cleavable chemical groups, FGF-2 could be patterned into and even released from hydrogels on demand using photochemistry, which would allow ECM regulation in a time- and user-dependent

manner ^[26]. For such ambitious biomedical application strategies to be successful, further studies are required to better understand the role of cell-derived ECM in hydrogels.

Materials & Methods

Cell culture

Bone marrow-derived mesenchymal stromal cells (MSCs) from human donors were isolated using previously described methods ^[27] following local regulations (University Hospital Basel; Prof. Dr. Kummer; approval date 26/03/2007 Ref Number 78/07). MSCs were cultured in MEM α containing nucleosides supplemented with FBS (10%, Gibco), penicillin (100 U mL⁻¹, Gibco) and streptomycin (100 μ g mL⁻¹, Gibco), plus FGF-2 (5 ng mL⁻¹, PeproTech) at 37°C in a humidified 5% CO₂ atmosphere.

Hydrogel formation

3D hydrogels (1.7% (w/v) PEG) were prepared as previously described ^[9a, 9b]. Briefly, equal amounts of n-PEG-Gln and n-PEG-MMP-sensitive-Lys, such that [Lys]/[Gln] = 1, were mixed in 50 mM Tris buffer (pH 7.6, plus 50 mM CaCl₂). MSCs at 1.5x10⁶ cells mL⁻¹, Lys-RGD (50 μ M, if required) and activated FXIII (10 U mL⁻¹) were subsequently added. To activate FXIII, thrombin (100 μ L, 20 U mL⁻¹, Sigma-Aldrich) and FXIII (1 mL, 200 U mL⁻¹, Fibrogammin, CSL Behring) were incubated (30 min, 37°C) and stored at -80°C until needed. Hydrogels were pipetted dropwise onto a hydrophobic glass slide (pre-treated with SigmaCote, Sigma-Aldrich), and an additional slide was placed on top. MSCs in hydrogels were cultured in MEM α containing nucleosides supplemented with FBS (10%), penicillin (100 U mL⁻¹) and streptomycin (100 μ g mL⁻¹), plus FGF-2 (50 ng mL⁻¹, if required), at 37°C in a humidified 5% CO₂ atmosphere. After 4 days, medium was removed and fresh medium was added. For 2D experiments, hydrogels were prepared as described above but instead using Tris buffer (10 mM, pH 7.6) plus CaCl₂ (50 mM). Hydrogel solutions (22 μ L) were cast into hydrophobic PTFE rings (4.0 mm of inner diameter) placed at the glass bottom of the 96 well plate. To make the gel surface flat a metallic cylindrical bar (base diameter approx. 5 mm) covered the gels for 5 min and was then removed. Hydrogels were rinsed (5 min) with Tris buffer (50 mM, pH 7.6) and 10,000 cells were seeded immediately on the hydrogel surfaces.

Stiffness measurements by in situ rheology

To measure hydrogel stiffness, cell-free hydrogels (1.7% (w/v), 80 μ L, 37 °C in a humidified atmosphere) were analysed using a MCR 301 rheometer combined with the 20 mm plate-plate PP20 measuring system (Anton Paar) and 0.2 mm working gap. Measurements of the storage modulus (G') were taken for 30 min with the following parameters: angular frequency = 1 Hz and constant shear strain = 4%.

AHA labeling of nascent protein secretion

3D Hydrogels (10,000 cells, 10 μ L) were prepared as described above. For 2D culture, cells (10,000) were seeded into glass bottom 96-well plates. For cell seeding and culture, the following medium was used: Methionine-free DMEM (Thermo Fisher Scientific, 21013024) supplemented with FBS (10%), L-glutamine (2 mM), L-cysteine (0.2 mM), ascorbic acid (0.1 mM), and L-Azidohomoalanine (AHA, Jena Bioscience 1066-25, 0.1 mM). After 24 h, medium was removed and cultures were washed twice with PBS. Next, AHA was identified using Click-iT™ Alexa Fluor™ 555 sDIBO Alkyne (10 μ M, 60 min, Thermo Fisher Scientific, C20021) diluted in DMEM supplemented with FBS (10%). Afterwards, cultures were washed twice with PBS, fixed in PFA (4%, 30 min, RT), and then washed once in PBS (1 h). Actin filaments were visualized by Alexa Fluor 488 phalloidin (1:200, Molecular Probes). Confocal images were acquired using an inverted confocal laser scanning microscope (A1R Nikon) equipped with a 60x objective.

Fibronectin knockdown experiments

To knockdown fibronectin (FN1) in MSCs, the lipofectamine RNAiMAX protocol (Invitrogen, 13778030) was used as previously described ^[6a]. We used one negative control siRNA (SilencerSelect Negative control No 1 siRNA, 4390843, Ambion) and three siRNAs against human FN1 (A: s223585, B: s223586, C: s5321; 4427037; Ambion). Briefly, siRNA (20 pmol) and lipofectamine (3 μ L) were mixed in OptiMEM (100 μ L, Invitrogen, 31985062) for 5 min, which was then added to MSCs in culture at 20 nM for two days. This resulted in at least 80% intracellular and complete extracellular depletion of fibronectin protein levels (see Figure S3 and ref ^[6a]). Transfected cells (20,000) were embedded in a hydrogel droplet (20 μ L) and cultured for 3 days, followed by FN and F-actin immunostaining (see below). For fibronectin rescue experiments, human plasma fibronectin (50 μ g mL⁻¹, Sigma-Aldrich) was added to the gel precursor mix before hydrogel polymerization was initiated.

Immunofluorescence microscopy

To ensure staining of extracellular proteins, hydrogels were incubated with primary antibodies (4 h, 37°C in a humidified 5% CO₂ atmosphere) spiked directly into the culture medium prior to fixation. MSCs in hydrogels were washed twice in PBS, fixed in PFA (4%, 30 min, RT), and then washed once in PBS (1 h). Hydrogels were incubated with appropriate secondary antibodies (4 h, RT in the dark) diluted in BSA (1%, Albumin Fraction V, AppliChem in PBS). Primary antibodies used were mouse anti-fibronectin (IST-9, 1:300, sc59826, Santa Cruz Biotechnology), rabbit anti-collagen III (1:150, ab7778, Abcam) and mouse anti-perlecan (1:200, ab23418, Abcam). Alexa Flour 488-conjugated goat anti-mouse (1:200, ab150113, Abcam) and goat anti-rabbit (1:200, ab150077, Abcam) secondary antibodies were used. Actin filaments were visualized by Rhodamine phalloidin or Alexa Flour 546 phalloidin (1:500, Molecular Probes). Nuclei were visualized with NucBlue (1 drop mL⁻¹, Thermo Fisher Scientific). Confocal images were taken using an inverted confocal laser scanning microscope (TCS SP5, Leica) and either a 10x or 63x objective. When imaging cells for quantification (F-actin staining), Z stacks were taken (Figure 2b, Figure S2: 100 µm depth, 10x 10 µm increments; Figure 2e: 75 µm depth, 15x 5 µm increments). Raw images were processed using ImageJ (Fiji version 2.0.0-rc-59/1.51n). 3D reconstructions (Figure 1e) were performed using the surface rendering tool in Imaris Software (version 8.4.1).

Quantification of cell spreading experiments

Maximum Z projections of F-actin image stacks were binarized and individual cells (events > 30 µm²) were quantified using the analyze particles tool in ImageJ (Fiji version 2.0.0-rc-59/1.51n). The area (A) and perimeter (P) values of cells were used to calculate the spreading index according to the formula $1-4\pi(A/P^2)$. A perfectly round cell has a spreading index of 0 whereas a stretched, elongated cell has a spreading index approaching 1.

Quantitative real-time PCR (qRT-PCR)

For 2D monolayer experiments, cells (60,000) were seeded in 12-well plates. For 3D experiments, cells (1.5 or 4 x10⁶ cells mL⁻¹) were encapsulated in hydrogels (3x 25 µL) and were cultured for 7 days as described above. After 7 days, hydrogels were degraded with collagenase A (0.5 mg mL⁻¹, 37°C, 60-90 min; 11088793001, Sigma-Aldrich) and cells were collected. Total RNA was isolated from cells using the RNeasy Micro Kit following manufacturer's instructions (74004, QIAGEN) and was used for RNA sequencing or qRT-PCR. For qRT-PCR analysis, RNA from each sample (150 ng) was converted into cDNA (30 µL) using a high-capacity cDNA reverse transcription kit (Applied Biosystems) and qRT-PCR was performed using cDNA template (1.5 µL per reaction), the TaqMan Universal PCR Master Mix (Applied Biosystems) and the Viia 7 Real-Time PCR System (Applied

Biosystems). To analyse gene expression, TaqMan primers Hs00266237_m1 (COL4A1), Hs00943809_m1 (COL3A1), Hs00164004_m1 (COL1A1), Hs00164099_m1 (COL1A2) and Hs01549976_m1 (FN1) were used. In addition, Hs02758991_g1 (GAPDH) and Hs03044281_g1 (YWHAZ) were used for normalization and the comparative Ct method was applied to give relative gene expression values.

RNA sequencing and differential gene expression analysis

RNA was collected, as described above, using MSCs from 3 different donors cultured in hydrogels. RNA sample quality control was performed using the Agilent 4200 TapeStation system and RNA concentration was determined using the RiboGreen RNA reagent (Life Technologies) measured on an Infinite M1000 Pro plate reader (Tecan). RNA (200 ng) was used for RNA library preparation using the TruSeq Stranded mRNA Library Prep Kit (Illumina). Libraries were pooled and sequenced SR81 with the High Output Kit v2 (Illumina) on an Illumina NextSeq 500 system.

Primary data analysis was performed using Illumina RTA version 2.4.11 and FASTQ format conversion was done using bcl2fastq v2.18.0.12. RNA seq reads were mapped to the human reference genome and simultaneously soft-clipped using STAR_2.4.0. Picard, RSeQC and QoRTs were used to perform quality control and featureCount (subread, version 1.5.0) was used to count reads. From 57825 genes, 40980 genes were removed as the average number of reads across all samples was calculated to be fewer than 5. HGNC symbols and ENTREZIDs were retrieved for the remaining 16845 transcripts using the org.Hs.eg.db package and differential gene expression was performed using DESeq2^[28] in R^[29], where a gene was considered differentially expressed provided FDR < 0.01 and fold change > 2 or < -2.

Differentially expressed genes (DEG) were analysed for significantly overrepresented biological process, cellular component and molecular function gene ontology terms compared with the *Homo Sapiens* genome using DAVID (version 6.8^[30]) with search parameters fold enrichment ≥ 1.5 , Bonferroni-corrected P value < 0.05 and EASE score (modified Fisher's exact test) < 0.05. Gene ontology terms containing less than two DEG genes were excluded, and up- and downregulated genes were analysed separately.

DEG genes were compared with expression changes in a published RNA sequencing dataset (GEO: GSE115240) of MSCs cultured in 2D and treated with or without FGF-2 (10 ng mL⁻¹, 24 hours)^[14]. Genes were classed as significantly differentially expressed in the 2D dataset provided the Bonferroni-corrected P value < 0.05. Genes that

were significantly differentially expressed in 3D but not 2D were analysed for significantly represented terms as described above.

Functional enrichment maps were generated using the combined list of up- and downregulated genes, as described previously ^[31], using High-Throughput GoMiner ^[32] with the following parameters: 1000 randomisations; FDR 0.05; minimum category size 5 and maximum category size 500. For genes belonging to each significantly overrepresented gene ontology term, relative RNA expression values were assigned and the gene:term table was clustered using Cluster 3.0 ^[33] (parameters: hierarchical clustering (complete linkage); cluster genes and arrays; correlation (uncentered)) and visualised using Java TreeView ^[34].

Data availability

RNA sequencing data have been deposited to the Annotare database <https://www.ebi.ac.uk/fg/annotare> and assigned the identifier E-MTAB-8510.

Western blotting

Cells were retrieved from hydrogels as described above, or from tissue culture plastic, and lysed in RIPA buffer (10 min, Sigma Aldrich) on ice. Cell lysates were centrifuged (20,000 g, 10 min, 4°C) and proteins (15 µg) were boiled in 6× Laemmli sample buffer including 2-mercaptoethanol (10 min, 95°C, Alfa Aesar, J61337). Proteins were separated on Mini-PROTEAN TGX stain-free protein gels (4–15%, Bio-Rad, 4568086) and transferred on to polyvinylidene fluoride (PVDF) membranes with the Trans-Blot Turbo System (Bio-Rad). Membranes were blocked in non-fat dry milk/TBS-T (5%, 30 min, RT). Primary antibodies were incubated overnight (4°C) in BSA/TBST-T (5%). Membranes were washed three times in TBS-T and incubated with the corresponding HRP-conjugated secondary antibodies in non-fat dry milk/TBS-T (2.5%, 60 min, RT). HRP was visualized using the UltraScence Pico Ultra Western Substrate (GeneDireX, CCH345-B) and the ChemiDoc MP imaging system (Bio-Rad). Antibodies used were rabbit anti-fibronectin (1:2000, Sigma-Aldrich, F3648), mouse anti- α -tubulin (B-5-1-2, 1:3500, Santa Cruz Biotechnology, sc 23948), rabbit anti-COL1A2 (1:2000, Abcam, ab96723), rabbit anti-LOX (1:2000, Thermo Fisher Scientific, MA532817), goat-anti-mouse-HRP (1:20,000, Sigma-Aldrich, SAB3701073-2) and goat-anti-rabbit-HRP (1:20,000 Sigma-Aldrich, SAB3700878-1). Quantitative results of protein levels were calculated using the *gels* tool of ImageJ (Fiji version 2.0.0-rc-59/1.51n) and normalized to tubulin signal intensities.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism (version 8.2.0). For comparisons between two groups, two-tailed t test was used. For comparisons between more than two groups, ANOVA with Bonferroni's post hoc test was used. In all cases, significance was defined as $P < 0.05$ with $*P < 0.01$, $**P < 0.001$, $***P < 0.001$, $****P < 0.0001$. Data are represented either as box and whisker plots (showing 25th and 75th percentiles (box), whiskers (5th and 95th percentiles), median (line) and/or mean (plus sign)), bar charts (showing mean + SD) or individual data points are displayed. RNA sequencing data was analysed as described above and details of number of replicates are provided in figure legends.

Conflict of Interest

All authors declare no conflicts of interest

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Author contribution

ERH performed data and bioinformatics analyses. QV-M functionalized PEG and performed rheology experiments. IM isolated, characterized and provided primary human cells. JGS and ME supervised the work and provided funding. UB conducted all cell experiments. QV-M, ME and UB designed the project. ERH and UB wrote the manuscript. All authors contributed to manuscript editing.

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TOC text: Cell-derived extracellular matrix produced by mesenchymal stromal cells is investigated within 3D PEG-based hydrogels with and without the cell-adhesive peptide RGD and the growth factor FGF-2. Cell-derived ECM surrounds cells in hydrogels. Secreted fibronectin is required for cell spreading in these 3D systems, even in the absence of RGD motifs. FGF-2 results in a decrease in ECM gene expression.

TOC keyword: Cell-derived ECM

References

- [1] R. O. Hynes, *Science (New York, N.Y.)* **2009**, 326, 1216.
- [2] a) A. Kumar, J. K. Placone, A. J. Engler, *Development (Cambridge, England)* **2017**, 144, 4261; b) K. H. Vining, D. J. Mooney, *Nature reviews. Molecular cell biology* **2017**, 18, 728.
- [3] a) D. Seliktar, *Science (New York, N.Y.)* **2012**, 336, 1124; b) S. R. Caliari, J. A. Burdick, *Nature methods* **2016**, 13, 405.
- [4] a) N. O. Enemchukwu, R. Cruz-Acuna, T. Bongiorno, C. T. Johnson, J. R. Garcia, T. Sulchek, A. J. Garcia, *The Journal of cell biology* **2016**, 212, 113; b) A. Ranga, M. Girgin, A. Meinhardt, D. Eberle, M. Caiazzo, E. M. Tanaka, M. P. Lutolf, *Proceedings of the National Academy of Sciences of the United States of America* **2016**, 113, E6831; c) R. Cruz-Acuna, A. Mulero-Russe, A. Y. Clark, R. Zent, A. J. Garcia, *Journal of cell science* **2019**, DOI: 10.1242/jcs.226639.
- [5] a) S. Khetan, M. Guvendiren, W. R. Legant, D. M. Cohen, C. S. Chen, J. A. Burdick, *Nature materials* **2013**, 12, 458; b) O. Chaudhuri, L. Gu, D. Klumpers, M. Darnell, S. A. Bencherif, J. C. Weaver, N. Huebsch, H. P. Lee, E. Lippens, G. N. Duda, D. J. Mooney, *Nature materials* **2016**, 15, 326; c) N. Huebsch, P. R. Arany, A. S. Mao, D. Shvartsman, O. A. Ali, S. A. Bencherif, J. Rivera-Feliciano, D. J. Mooney, *Nature materials* **2010**, 9, 518.
- [6] a) U. Blache, Q. Vallmajo-Martin, E. R. Horton, J. Guerrero, V. Djonov, A. Scherberich, J. T. Erler, I. Martin, J. G. Snedeker, V. Milleret, M. Ehrbar, *EMBO reports* **2018**, 19; b) S. A. Ferreira, M. S. Motwani, P. A. Faull, A. J. Seymour, T. T. L. Yu, M. Enayati, D. K. Taheem, C. Salzlechner, T. Haghighi, E. M. Kania, O. P. Oommen, T. Ahmed, S. Loaiza, K. Parzych, F. Dazzi, O. P. Varghese, F. Festy, A. E. Grigoriadis, H. W. Auner, A. P. Snijders, L. Bozec, E. Gentleman, *Nature communications* **2018**, 9, 4049; c) C. Loebel, R. L. Mauck, J. A. Burdick, *Nature materials* **2019**, 18, 883; d) H. Hezaveh, S. Cosson, E. A. Otte, G. Su, B. D. Fairbanks, J. J. Cooper-White, *Biomacromolecules* **2018**, 19, 721; e) Y. R. Devaud, E. Avilla-Royo, C. Trachsel, J. Grossmann, I. Martin, M. P. Lutolf, M. Ehrbar, *Advanced healthcare materials* **2018**, 7, e1800534; f) L. A. Sawicki, L. H. Choe, K. L. Wiley, K. H. Lee, A. M. Kloxin, *ACS biomaterials science & engineering* **2018**, 4, 836.
- [7] U. Blache, M. M. Stevens, E. Gentleman, *Nature biomedical engineering* **2020**, DOI: 10.1038/s41551-019-0500-6.
- [8] a) J. Galipeau, L. Sensebe, *Cell stem cell* **2018**, 22, 824; b) H. Ragelle, A. Naba, B. L. Larson, F. Zhou, M. Prijic, C. A. Whittaker, A. Del Rosario, R. Langer, R. O. Hynes, D. G. Anderson, *Biomaterials* **2017**, 128, 147.

- [9] a) M. Ehrbar, S. C. Rizzi, R. Hlushchuk, V. Djonov, A. H. Zisch, J. A. Hubbell, F. E. Weber, M. P. Lutolf, *Biomaterials* **2007**, 28, 3856; b) M. Ehrbar, S. C. Rizzi, R. G. Schoenmakers, B. S. Miguel, J. A. Hubbell, F. E. Weber, M. P. Lutolf, *Biomacromolecules* **2007**, 8, 3000; c) U. Blache, E. R. Horton, T. Xia, E. M. Schoof, L. H. Blicher, A. Schonenberger, J. G. Snedeker, I. Martin, J. T. Erler, M. Ehrbar, *Life science alliance* **2019**, 2.
- [10] U. Blache, S. Metzger, Q. Vallmajo-Martin, I. Martin, V. Djonov, M. Ehrbar, *Advanced healthcare materials* **2016**, 5, 489.
- [11] F. Karimi, A. J. O'Connor, G. G. Qiao, D. E. Heath, *Advanced healthcare materials* **2018**, 7, e1701324.
- [12] a) K. E. Kadler, A. Hill, E. G. Canty-Laird, *Current opinion in cell biology* **2008**, 20, 495; b) K. E. Kubow, R. Vukmirovic, L. Zhe, E. Klotzsch, M. L. Smith, D. Gourdon, S. Luna, V. Vogel, *Nature communications* **2015**, 6, 8026.
- [13] J. D. Humphries, M. R. Chastney, J. A. Askari, M. J. Humphries, *Current opinion in cell biology* **2019**, 56, 14.
- [14] B. Awan, D. Turkov, C. Schumacher, A. Jacobo, A. McEnerney, A. Ramsey, G. Xu, D. Park, S. Kalomoiris, W. Yao, L. E. Jao, M. L. Allende, C. B. Lebrilla, F. A. Fierro, *Stem cell reports* **2018**, 11, 325.
- [15] J. K. Mouw, G. Ou, V. M. Weaver, *Nature reviews. Molecular cell biology* **2014**, 15, 771.
- [16] M. J. Bissell, H. G. Hall, G. Parry, *Journal of theoretical biology* **1982**, 99, 31.
- [17] R. Xu, A. Boudreau, M. J. Bissell, *Cancer metastasis reviews* **2009**, 28, 167.
- [18] K. E. Scott, K. Rychel, S. Ranamukhaarachchi, P. Rangamani, S. I. Fraley, *Acta biomaterialia* **2019**, 96, 81.
- [19] a) K. M. Mabry, S. Z. Payne, K. S. Anseth, *Biomaterials* **2016**, 74, 31; b) A. Riedl, M. Schleder, K. Pudielko, M. Stadler, S. Walter, D. Unterleuthner, C. Unger, N. Kramer, M. Hengstschlager, L. Kenner, D. Pfeiffer, G. Krupitza, H. Dolznig, *Journal of cell science* **2017**, 130, 203; c) A. I. Marusina, A. A. Merleev, J. I. Luna, L. Olney, N. E. Haigh, D. Yoon, C. Guo, E. M. Ovadia, M. Shimoda, G. Luxardi, S. Boddu, N. N. Lal, Y. Takada, K. S. Lam, R. Liu, R. R. Isseroff, S. Le, J. A. Nolte, A. M. Kloxin, E. Maverakis, *Stem cells (Dayton, Ohio)* **2019**, DOI: 10.1002/stem.3105.
- [20] S. A. Ferreira, P. A. Faull, A. J. Seymour, T. T. L. Yu, S. Loaiza, H. W. Auner, A. P. Snijders, E. Gentleman, *Biomaterials* **2018**, 176, 13.
- [21] a) G. Bianchi, A. Banfi, M. Mastrogiacomo, R. Notaro, L. Luzzatto, R. Cancedda, R. Quarto, *Experimental cell research* **2003**, 287, 98; b) D. L. Coutu, M. Francois, J. Galipeau, *Blood* **2011**, 117, 6801; c) P. A. Sotiropoulou, S. A. Perez, M. Salagianni, C. N. Baxevanis, M. Papamichail, *Stem cells (Dayton, Ohio)* **2006**, 24, 462; d) H. J. Ahn, W. J. Lee, K. Kwack, Y. D. Kwon, *FEBS letters* **2009**, 583, 2922; e) I. Martin, A. Muraglia, G. Campanile, R. Cancedda, R. Quarto, *Endocrinology* **1997**, 138, 4456.
- [22] W. J. King, L. Jongpaiboonkit, W. L. Murphy, *Journal of biomedical materials research. Part A* **2010**, 93, 1110.
- [23] D. M. Dolivo, S. A. Larson, T. Dominko, *Cytokine & growth factor reviews* **2017**, 38, 49.
- [24] A. Zieris, S. Prokoph, K. R. Levental, P. B. Welzel, M. Grimmer, U. Freudenberg, C. Werner, *Biomaterials* **2010**, 31, 7985.
- [25] a) M. M. Martino, P. S. Briquez, A. Ranga, M. P. Lutolf, J. A. Hubbell, *Proceedings of the National Academy of Sciences of the United States of America* **2013**, 110, 4563; b) S. Metzger, U. Blache, P. S. Lienemann, M. Karlsson, F. E. Weber, W. Weber, M. Ehrbar, *Macromolecular bioscience* **2016**, 16, 1703.
- [26] J. A. Shadish, G. M. Benuska, C. A. DeForest, *Nature materials* **2019**, 18, 1005.
- [27] A. Papadimitropoulos, E. Piccinini, S. Brachat, A. Braccini, D. Wendt, A. Barbero, C. Jacobi, I. Martin, *PloS one* **2014**, 9, e102359.
- [28] M. I. Love, W. Huber, S. Anders, *Genome biology* **2014**, 15, 550.
- [29] W. Huber, V. J. Carey, R. Gentleman, S. Anders, M. Carlson, B. S. Carvalho, H. C. Bravo, S. Davis, L. Gatto, T. Girke, R. Gottardo, F. Hahne, K. D. Hansen, R. A. Irizarry, M. Lawrence, M. I. Love, J. MacDonald, V. Obenchain, A. K. Oles, H. Pages, A. Reyes, P. Shannon, G. K. Smyth, D. Tenenbaum, L. Waldron, M. Morgan, *Nature methods* **2015**, 12, 115.
- [30] W. Huang da, B. T. Sherman, R. A. Lempicki, *Nature protocols* **2009**, 4, 44.
- [31] E. R. Horton, A. Byron, J. A. Askari, D. H. J. Ng, A. Millon-Fremillon, J. Robertson, E. J. Koper, N. R. Paul, S. Warwood, D. Knight, J. D. Humphries, M. J. Humphries, *Nature cell biology* **2015**, 17, 1577.
- [32] B. R. Zeeberg, H. Qin, S. Narasimhan, M. Sunshine, H. Cao, D. W. Kane, M. Reimers, R. M. Stephens, D. Bryant, S. K. Burt, E. Elnekave, D. M. Hari, T. A. Wynn, C. Cunningham-Rundles, D. M. Stewart, D. Nelson, J. N. Weinstein, *BMC bioinformatics* **2005**, 6, 168.

- [33] M. J. de Hoon, Y. Makita, S. Imoto, K. Kobayashi, N. Ogasawara, K. Nakai, S. Miyano, *Bioinformatics (Oxford, England)* **2004**, 20 Suppl 1, i101.
- [34] A. J. Saldanha, *Bioinformatics (Oxford, England)* **2004**, 20, 3246.