View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repository@Hull - CRIS

1 **TITLE:**
2 How to study basement membrane stiffness as a biophysical trigger in prostate cancer and
3 other age-related pathologies or metabolic diseases
4
5 **AUTHORS:**
6 Mercedes Rodriguez-Teja
7 Departamento de Genética, Facultad de Medicina
8 Universidad de la República (UDELAR)
9 Montevideo, Uruguay
10 mercedesrodriguez@fmed.edu.uy
11
12 Claudia Breit
13 Department of Mechanistic Cell Biology
14 Max Planck Institute of Molecular Physiology
15 Dortmund, Germany
16 claudia.breit@mpi-dortmund.mpg.de
17
18 Mitchell Clarke
19 School of Biological, Biomedical & Environmental Sciences
20 University of Hull
21 Hull, United Kingdom
22 mitchell.clarke@2010.hull.ac.uk
23
24 Kamil Talar
25 School of Biological, Biomedical & Environmental Sciences
26 University of Hull
27 Hull, United Kingdom
28 k.talar@2012.hull.ac.uk
29
30 Kai Wang
31 School of Biological, Biomedical & Environmental Sciences
32 University of Hull
33 Hull, United Kingdom
34 k.wang@2013.hull.ac.uk
35
36 Mohammad A Mohammad
37 School of Biological, Biomedical & Environmental Sciences
38 University of Hull
39 Hull, United Kingdom
40 m.a.mohammad@2012.hull.ac.uk
41
42 Sage Pickwell
43 School of Biological, Biomedical & Environmental Sciences
44 University of Hull

Hull, United Kingdom
s.pickwell@hull.ac.uk

Guillermina Etchandy
Departamento de Genética, Facultad de Medicina
Universidad de la República (UDELAR)
Montevideo, Uruguay
getchandy@fmed.edu.uy

Graeme J Stasiuk
School of Biological, Biomedical & Environmental Sciences
University of Hull
Hull, United Kingdom
g.stasiuk@hull.ac.uk

Justin Sturge
School of Biological, Biomedical & Environmental Sciences
University of Hull
Hull, United Kingdom
j.sturge@hull.ac.uk

CORRESPONDING AUTHOR:
Justin Sturge, Ph.D.

KEYWORDS:
Advanced glycation endproducts, basement membrane, biophysical strain, cell migration, collagen IV, epithelial cells, extracellular matrix, laminin, non-enzymatic crosslinking, prostate cancer, stiffness

SHORT ABSTRACT:
Here we explain a protocol for modelling the biophysical microenvironment where crosslinking and increased stiffness of the basement membrane (BM) induced by advanced glycation endproducts (AGEs) has pathological relevance.

LONG ABSTRACT:
Here we describe a protocol that can be used to study the biophysical microenvironment related to increased thickness and stiffness of the basement membrane (BM) during age-related pathologies and metabolic disorders (e.g. cancer, diabetes, microvascular disease, retinopathy, nephropathy and neuropathy). The premise of the model is non-enzymatic crosslinking of reconstituted BM (rBM) matrix by treatment with glycolaldehyde (GLA) to promote advanced glycation endproduct (AGE) generation via the Maillard reaction. Examples of laboratory techniques that can be used to confirm AGE generation, non-enzymatic crosslinking and increased stiffness in GLA treated rBM are outlined. These include preparation of native rBM (treated with phosphate-buffered saline, PBS) and stiff rBM (treated with GLA)

for determination of: its AGE content by photometric analysis and immunofluorescent microscopy, its non-enzymatic crosslinking by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) as well as confocal microscopy, and its increased stiffness using rheometry. The procedure described here can be used to increase the rigidity (elastic moduli, E) of rBM up to 3.2-fold, consistent with measurements made in healthy versus diseased human prostate tissue. To recreate the biophysical microenvironment associated with the aging and diseased prostate gland three prostate cell types were introduced on to native rBM and stiff rBM: RWPE-1, prostate epithelial cells (PECs) derived from a normal prostate gland; BPH-1, PECs derived from a prostate gland affected by benign prostatic hyperplasia (BPH); and PC3, metastatic cells derived from a secondary bone tumor originating from prostate cancer. Multiple parameters can be measured, including the size, shape and invasive characteristics of the 3D glandular acini formed by RWPE-1 and BPH-1 on native versus stiff rBM, and average cell length, migratory velocity and persistence of cell movement of 3D spheroids formed by PC3 cells under the same conditions. Cell signaling pathways and the subcellular localization of proteins can also be assessed.

INTRODUCTION:

The basement membrane (BM) is a sheet of specialized extracellular matrix (ECM) that maintains stable tissue borders by separating layers of epithelial cells from the stroma[1]. Covalent crosslinking between adjacent triple helices of collagen IV in the BM stabilizes their lateral association by establishing an irregular network of super-twisted helices[2]. These collagen IV lattices act as a scaffold for its interaction with laminin and other BM components[1]. The structural arrangement of the BM provides it with the mechanical strength and rigidity necessary for the normal development of glandular epithelia[3].

During aging and disease the BM progressively thickens and stiffens[3,4]. For example, a 3-fold increase in the elastic modulus (E) of the ocular BM occurs between the ages of 50 and 80 in the normal population, and this stiffening is further exacerbated in metabolic disorders like diabetes[5]. The structural and biomechanical changes in the BM that result in its increased stiffness occur when its ECM components, collagen IV and laminin, become non-enzymatically crosslinked following their exposure to advanced glycation endproducts (AGEs).

The purpose of the method described here was to establish a model for the investigation of how BM stiffness, due to AGE exposure, promotes prostate epithelial cell (PEC) and prostate tumour cell (PTC) invasiveness in the context of the switch to metastatic prostate cancer (PCa). To do this a previous method used for generating 3D glandular acini from mammary epithelial cells (MECs) in reconstituted rBM gels[6] was adapted to include an additional step where the rBM gels are pre-treated with glycolaldehyde (GLA). Several techniques for assessing GLA induced crosslinking and stiffening of pre-treated rBM gels are described, including photometric analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), confocal microscopy and rheometric analysis. The prostate cell types selected for culture on the pre-stiffened rBM include: RWPE-1, PECs derived from a normal prostate gland[7]; BPH-1, PECs derived from a prostate gland affected by BPH[8]; and PC3, metastatic PTCs derived from a secondary tumor located in the vertebral bone of a prostate cancer (PCa) patient[9].

133
134 In addition to advancing the study of prostate gland pathology, the protocol for stiffening of
135 rBM gels by their treatment with GLA can be adapted to investigate how BM stiffness
136 contributes to other age-related pathologies and metabolic disorders. For example, the model
137 can be directly applied to investigate how metastatic cancer is induced by BM stiffness in
138 organs such as the breast, colon, ovary and pancreas by the incorporation of appropriate cell
139 types. Furthermore, the protocol can be adapted to investigate how stiff BM promotes
140 biomechanical mechanisms of disease progression in diabetes-related microvascular disease,
141 retinopathy, nephropathy and neuropathy.
142
143 **PROTOCOL:**
144
145 **1. Induction of BM stiffness induced by GLA treatment (non-enzymatic crosslinking)**

146 1.1) Thaw a frozen vial of BM matrix (10 mL) by incubating at 4 °C (standing on ice in a cold
147 room or refrigerator) until the contents of the vial have become liquid (8-16 hr).

148 Caution: If a cold room/refrigerator is not used, cover the entire bottle with ice. This will
149 prevent the stock solution of BM from solidifying.

150 1.2) For future experiments and to avoid repeated freeze thaw cycles, prepare 25 X 0.4 mL
151 aliquots from each new 10 mL vial of BM matrix. Store vials at -80 °C until the expiry date
152 indicated by the manufacturer. When needed, thaw vials at 4 °C standing on ice for 2 hr.

153 1.3) Prepare an even surface of ice. Place an 8-well chamber glass slide on top of the ice to
154 maintain a temperature of 4 °C during the coating procedure. Thaw a vial of BM matrix at 4 °C.

155 Note: One 0.4 mL vial of BM matrix is sufficient to coat an entire 1 X 8-well chamber slide. Keep
156 the vial covered in ice while handling to prevent the BM matrix from solidifying.

157 1.4) Cut off the dispensing end of a 200 µL pipette tip using scissors. Cool the blunt-ended
158 200 µL pipette tip to 4 °C and place it on to a 200 µL capacity pipetting aid. Take up 40 µL of the
159 cold BM matrix solution into the pipette tip and transfer it into a well on the chilled 8-well
160 chamber glass slide.

161 Note: 40 µL of BM solution is enough to cover a surface area of 0.8 cm^2. Keep pipette tips
162 chilled during the coating procedure to avoid solidification of the BM solution. Do not introduce
163 air bubbles into the BM matrix solution and ensure the well is evenly coated without the
164 formation of a visible meniscus at the edges.

165 1.5) Repeat step 1.4 according to the number of wells and chambers required.

166 1.6) After coating, place the 8-well chamber slide at 37 °C for 30 min to promote
167 polymerization of the BM. Close the incubator door very carefully to avoid unwanted
168 disturbance of the liquid rBM. Do not exceed the 30 min incubation time to avoid dehydration

169 of the rBM gel.

170 Note: The resulting gel is the native reconstituted BM (rBM). The 37 °C incubation step does not
171 require 5 % CO_2. However, for convenience perform this step in a tissue culture incubator set at
172 37 °C and 5 % CO_2 (with humidification).

173 1.7) Prepare 50 mM glycolaldehyde (GLA) diluted in 0.2 M phosphate buffer (pH 7.8).
174 Sterilize the solution by passing it through a 0.22 micron syringe filter using a 50 mL syringe.

175 1.7.1) For a crosslinking reaction in a final volume of 250 µL of 50 mM GLA, add 25 µL of 0.5 M
176 sodium cyanoborohydride or 2.5 M aminoguanidine to 125 µL of 100 mM GLA (2X stock) and
177 100 µL of 0.2 M phosphate buffer (pH 7.8). Sterilize the stock solutions by passing them through
178 a 0.22 micron syringe filter using a 50 mL syringe.
179
180 Caution: Handle sodium cyanoborohydride wearing a lab coat, gloves, faceshield and respirator
181 while working in a fume hood.

182 1.8) Add 250 µL of GLA solution to cover the polymerized rBM gel and incubate at 37 °C for 6
183 hr to produce a semi-stiff rBM gel or 14 hr to produce a stiff rBM gel.

184 Note: The volume of GLA added must cover the polymerized rBM gel and should be adjusted
185 accordingly. If different GLA incubation times are used, the rBM gels should be analyzed to
186 determine the fold-increase in rBM stiffness (see Step 2.4).

187 1.8.1) Prepare a negative control by incubating a native rBM gel in 250 µL of sterile phosphate
188 buffered saline (PBS) for 14 hr at 37 °C.

189 1.8.2) Prepare two additional controls where the formation of Schiff base or Amadori adduct
190 rearrangement during the crosslinking reaction are inhibited, by the addition of 50 mM sodium
191 cyanoborohydride or 250 mM aminoguanidine.

192 1.9) Prepare 1 M glycine ethyl ester (GEE) diluted in PBS. Sterilize the solution by passing it
193 through a 0.22 micron syringe filter using a 50 mL syringe.

194 1.10) After the indicated incubation time, carefully remove the GLA solution from the
195 crosslinked rBM gels, GLA solution containing inhibitors from the control rBM gels and PBS from
196 the control native rBM gels. Add 250 µL of GEE solution to all of the rBM gels and incubate at 37
197 °C for 1 hr.

198 Note: This step quenches the crosslinking reaction.

199 1.11) Wash all rBM gels 10 times in 500 µL PBS to remove all traces of GLA and GEE. Incubate
200 the rBM gels overnight at 37 °C in 400 µL of PBS to prevent their dehydration.

201 1.11.1) Analyze the rBM gels for AGE accumulation, non-enzymatic crosslinking and viscoelastic
202 properties (Steps 2.1 – 2.4). For rheometric analysis of their viscoelastic properties prepare the

203 rBM gels in cloning rings (Step 2.4).

204 1.11.2) For cell culture, rinse rBM gels 2 times with 500 µL culture media before seeding the
205 cells (Steps 5 and 6). Perform washes gently without the pipette tip touching the gel surface.

206 **2. Quantification of non-enzymatic crosslinking and stiffness of rBM treated with GLA**

207 2.1) Photometric analysis

208 2.1.1) Measure AGE accumulation in GLA-treated and control rBM gels using photometric
209 analysis to determine the extent of the Maillard reaction.

210 2.1.1.1) After Step 1.11, remove the PBS from rBM gels in the 8-wells chamber slides and add
211 250 µL ice-cold double distilled water. Incubate at 4 °C for 16-24 hr to ensure that the matrix is
212 completely liquefied.

213 Note: rBM peptides in this solution contain AGEs with auto-fluorescent properties.

214 2.1.1.2) Transfer the liquefied BM solution to a 1.5 mL tube and measure the fluorescent
215 emission of the solution using a spectrophotometer (excitation wavelength = 370 nm; emission
216 wavelength = 440 nm).

217 2.2) SDS-PAGE analysis of cyanogen bromide peptides

218 2.2.1) Resolve the GLA-treated and control rBM gels on a polyacrylamide gel to confirm that
219 GLA has induced crosslinking and the formation of macro-fibres.

220 2.2.1.1) Centrifuge the liquefied BM solution collected at Step 2.1.1.2 at 10,000 x g for 5 min at
221 room temperature.
222
223 2.2.1.2) Prepare a stock solution containing 2 g/mL of cyanogen bromide diluted in acetonitrile.
224
225 Caution: Always handle cyanogen bromide in a fume hood while wearing a lab coat, gloves,
226 faceshield and respirator.
227
228 2.2.1.3) Remove the supernatant, re-suspend the BM gel pellet in 500 µL of 20 mg/mL
229 cyanogen bromide + 70 % v/v formic acid and incubate overnight at room temperature.

230 2.2.1.4) Use a 1 mL disposable syringe to transfer the resuspended BM gel pellet into a dialysis
231 cassette with a molecular weight cut off 3.5 kDa.

232 2.2.1.5) Submerge the cassette into a 500 mL glass beaker containing 500 mL of double distilled
233 water and a magnetic stir bar. Place this onto a magnetic stirrer and dialyze overnight (16 hr) at
234 4 °C (in a cold room) to remove all traces of cyanogen bromide and formic acid.

235 2.2.1.6) Use a 1 mL disposable syringe to transfer the dialyzed BM solution from the cassette

236 into a 1.5 mL tube.

237 2.2.1.7) Analyse 25 µL of each BM sample on a 12% v/v polyacrylamide gel[10,11]. Following SDS-
238 PAGE, carry out silver staining of the polyacrylamide gel[12] to visualize the electrophoretic
239 pattern of cyanogen bromide-matrix peptides[13].

240 2.3 Immunofluorescent microscopy analysis

241 2.3.1) Perform immunofluorescent staining of GLA treated and control rBM gels with anti-
242 AGE/pentosidine, anti-collagen IV and anti-laminin antibodies followed by confocal microscopy
243 to visualize accumulated AGEs and collagen IV/laminin fibre structural rearrangements in the
244 crosslinked rBM gels[13].

245 Note: Always use a sufficient volume to cover the entire rBM gel during incubations and washes
246 without touching the rBM surface with the pipette tip. For details of analyzing 3D acini cultures
247 by immunofluorescence see [6] and for confocal microscopy of 3D acini see [14].

248 2.3.1.1) Wash GLA treated and control rBM gels in 8-wells chamber slides 2 times with 300 µL
249 of PBS+ (PBS containing 0.1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) for 5 min at room temperature.

250 2.3.1.2) Remove the PBS+ then add 300 µL of 4 % w/v paraformaldehyde (PFA) diluted in PBS+
251 to cover each rBM gel. Incubate for 30 min at room temperature to fix the rBM components.

252 2.3.1.3) Remove the 4 % w/v PFA solution. Add add 300 µL of 75 mM NH_4Cl + 0.5 mM $MgCl_2$
253 solution and incubate for 5 min at room temperature (repeat 5X) to quench the fixation.

254 2.3.1.4) Prepare Immunofluorescence buffer (IF buffer) by making the following solution in
255 sterile water: 130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% w/v bovine
256 serum albumin, 0.5% v/v polyethylene glycol *tert*-octylphenyl ether and 0.05% v/v polyethylene
257 glycol sorbitan monolaurate.

258 2.3.1.5) Prepare IF blocking buffer by supplementing IF buffer with 20 % v/v goat serum.

259 2.3.1.6) Remove the quenching solution and add 300 µL of IF blocking buffer to the rBM gels to
260 prevent nonspecific reactions. Incubate 2 hr at room temperature on a shaking platform.

261 2.3.1.7) Remove the IF blocking buffer and incubate the rBM gels for 16 hr at 4 ^oC with 300 µL
262 of primary antibody diluted in IF blocking buffer (1: 500 mouse anti-pentosidine mAb; 1/250
263 rabbit anti-collagen IV pAb; 1/250 rabbit anti-laminin A/C pAb).

264 Note: Incubations for longer than 20 hr at 4 ^oC can liquidize the rBM.

265 2.3.1.8) Remove the primary antibody and wash 3 times (10 min each) with 300 µL of IF
266 buffer at room temperature on a shaking platform.

267 2.3.1.9) Remove the IF buffer and add 300 µL of the secondary antibody (goat anti-rabbit

or anti-mouse IgG [H+L]) conjugated with a fluorochrome diluted 1: 500 in IF blocking buffer. Incubate for 2 hr at room temperature on a shaking platform.

2.3.1.10) Remove the secondary antibody and incubate in 300 µL of IF buffer for 10 min at room temperature. Remove the IF buffer and wash 3 X 10 min in 300 µL of PBS+ at room temperature.

2.3.1.11) Fix and quench a second time, as described above (Steps 2.3.1.2 and 2.3.1.3).

2.3.1.12) Mount stained rBM gels in mounting media and analyze the formation of dense bundles of major components using epifluorescent or confocal microscopy.

Note: For details of analyzing 3D acini cultures by immunofluorescence see [6] and for epifluorescent and confocal microscopy of 3D acini see [14].

2.4) Rheological analysis

2.4.1) Perform rheometric analysis of GLA treated and control rBM gels to measure their viscoelasticity (stiffness).

2.4.1.1) Set up rBM gels that are 1 mm thick in a circular mold with a diameter of 8 mm. To do this, place a cloning ring (8 mm diameter) inside a well of a 24-well culture plate and add BM matrix solution prepared as described in Steps 1.3-1.6.

Note: For accurate recapitulation of the rBM gels used for experiments, the rBM gels prepared for rheometric analysis need to have the same surface area and thickness as the rBM gels set up in the 8-well chambers. The rBM gels analyzed in Figure 3 were 1 mm thick and 8 mm in diameter.

2.4.1.2) Treat the rBM gels set up in the cloning rings with PBS, GLA for 6 h and GLA for 14 h as described above (Steps 1.8 to 1.11).

2.4.1.3) Measure the elastic modulus (E) of the 8 mm diameter rBM gels on a rheometer with an 8 mm parallel plate serrated geometry, over a range of 1 - 3% strain, at a fixed frequency oscillation of 1Hz and temperature of 21 °C. For additional details about the rheometric analysis of ECM gels see references see [13,15,16].

Note: E is determined from the resulting shear storage modulus (G’) through the use of the following equation $E = 2 * G' * (1+v)$ where v is the Poisson’s ratio of 0.5, as described in [13,15,16].

3. Culture and handling of the normal PEC line, RWPE-1

3.1) Grow RWPE-1 cells in keratinocyte serum-free media (KSFM) supplemented with 5 ng/mL epidermal growth factor (EGF), 50 µg/mL bovine pituitary extract (BPE) and 50 U/mL penicillin with 50 µg/mL streptomycin (complete KSFM).

300 Note: To avoid induction of epithelial-to-mesenchymal (EMT)-like transition do not expose
301 RWPE-1 cells to serum. Allow complete KSFM to reach room temperature for 30 min after
302 removing from storage at 4 °C and do not warm in a 37 °C water bath as this will inactivate the
303 EGF and BPE.

304 3.2) Aspirate the complete KSFM from a confluent 10 cm^2 plate of RWPE-1 cells, rinse with 5
305 mL of pre-warmed PBS and add 5 mL of 0.05 % v/v trypsin ensuring that all cells are covered
306 with the solution.

307 3.2.1) Place the cells in a tissue culture incubator set at standard conditions of 37 °C and 5% CO_2
308 (with humidification) for 5 to 10 min. Check the extent of trypsinization after 5 min and gently
309 tap the culture plate to detach the cells.

310 Note: RWPE-1 cells do not tolerate long periods of trypsinization so it is advised not to handle
311 more than two plates at the same time. It is also important to dissociate all cells from the plate
312 to avoid clonal selection.

313 3.3) When all RWPE-1 cells have disassociated, add 5 mL of warm PBS containing 2 % v/v
314 fetal calf serum (FCS) to quench the trypsin. Gently pipette up and down to break up the cell
315 aggregates before transferring the cells to a centrifuge tube.

316 3.4) Centrifuge the disassociated cells at 125 - 150 x g for 5 min at 25 °C, discard the
317 supernatant and re-suspend the pellet of cells in 5 mL of complete KSFM until a suspension of
318 single cells is obtained.

319 3.5) Transfer 1 mL of the re-suspended cells into a new tube and add 9 mL of complete KSFM
320 to propagate the cells at a 1:5 passage dilution for subsequent experimental use. Count the rest
321 of the cells using a hemocytometer for setting up acini (see Section 5.1).

322 Note: Do not culture RWPE-1 cells for more than 10 passages since after prolonged periods of
323 culture they do not form acini with the correct architecture.

324 3.6) Change the culture media every 48 hr to ensure the EGF and BPE remain active.

325 Note: Include this medium change for any treatments that extend beyond 48 hr.

326 **4. Culture and handling of the BPH cell line, BPH-1**

327 4.1) Culture BPH-1 cells in RPMI 1640 media complemented with 5 % v/v FCS, 50 U/mL
328 penicillin and 50 µg/mL streptomycin. Warm the culture media, PBS and 0.25% w/v trypsin-0.53
329 M EDTA solution to 37 °C before use. Note: Cells can also be cultured in media with 2.5 % v/v
330 FCS[8].

331 4.2) Aspirate the culture media from a confluent 10 cm^2 plate of BPH-1 cells and wash the
332 cells 2 X with 3 mL of PBS to remove all the traces of culture media with serum that may quench
333 the trypsin reaction.

334 4.3) Aspirate the PBS and add 3 mL of trypsin-EDTA solution to cover the cells. Place the
335 plate in an incubator set at 37 °C and 5% CO_2 (with humidification) for 5 min. Remove the
336 trypsin-EDTA solution when the cells are round but remain attached to the dish. Wash cells
337 with 5 mL of PBS.

338 4.4) After removal of the PBS, add 5 mL of culture media and gently pipette up and down to
339 produce a suspension of single cells. Transfer the cells to a 15 mL tube.

340 4.5) Take 2 mL of the cell suspension into a new centrifuge tube with 8 mL of complete
341 media and plate the BPH-1 cells onto a 10 cm^2 culture plate at a 1:5 passage dilution for
342 subsequent experimental use. Count the rest of the cells using a hemocytometer for setting up
343 acini (see Section 5.2).

344 Note: Keep a record of the passage number, as older BPH-1 cells do not form acini with a
345 proper architecture. A passage number more than 10 is not desired.

346 4.6) Change the culture media every 72 hr.

347 **5. 3D culture of prostate gland acini on native and stiff rBM**

348 5.1) If RWPE-1 cells are being used to form acini, dilute 5,000 cells prepared in step 3.5 in
349 300 µL of complete KSFM supplemented with 2 % v/v of BM solution.

350 5.2) If BPH-1 cells are being used to form acini, dilute 2,500 cells prepared in step 4.5 in 300
351 µL of RPMI 1640 culture media supplemented with 2 % v/v of BM solution.

352 Note: BPH-1 cells are larger than RWPE-1 cells so lower numbers of BPH-1 cells are used to
353 obtain a similar distribution of acini after 6 days of culture.

354 5.3) Gently seed the cells onto the native and AGE-stiffened rBM and carefully place the
355 cultures in an incubator set at 37 °C and 5% CO_2 (with humidification) to ensure an even
356 distribution of growing acini in the well and that each cell divides to produce one acina.

357 5.4) Every 2 days replace the culture media with fresh culture media containing 2 % v/v BM
358 solution to ensure that cells have the growth factors required for normal acina homeostasis.

359 5.5) Monitor acinar morphology in growing cultures using brightfield microscopy[13].

360 Note: After 3 days in culture individual cells will form a cluster of >3 cells and after 1 week
361 prostate gland acini with a diameter of ~50 µm will be observed.

362 5.6) Follow protocol described in 2.3 to perform immunofluorescence using antibodies
363 specific for markers of cell-matrix adhesions, cell-cell adhesions, apico-basal polarity and
364 invasiveness[13].

365 5.6.1) Use a mounting media with 4',6-diamidino-2-phenylindole (DAPI) or include an extra step

366 (after 2.3.12) to stain cell nuclei by incubating with DAPI for 5 min and wash 2 X 5 min with
367 PBS+.

368 **6. 3D culture of prostate tumor cell aggregates on native and stiff rBM**

369 6.1) Culture PC3 cells in RPMI 1640 medium containing 10 % v/v FCS and 50 U/mL penicillin
370 with 50 µg/mL streptomycin. Warm the culture media, PBS and 0.25 % w/v trypsin-0.53 M
371 EDTA solution to 37 °C before use.

372 6.2) Aspirate the culture media from a confluent 10 cm^2 culture dish of PC3 cells and wash
373 the cells 2 X with 3 mL of PBS to remove all traces of FCS that can quench the trypsin reaction.

374 6.3) Aspirate the PBS and add 3 mL of trypsin-EDTA solution to cover the cells and incubate
375 for 1 min.

376 6.4) When the cells become rounded, but remain attached to the dish, carefully aspirate the
377 trypsin-EDTA solution and wash with 3 mL of PBS to remove all traces of trypsin.

378 6.5) After removal of the PBS, add 5 mL of culture media and gently pipette up and down to
379 produce a suspension of single cells. Transfer the cells to a 15 mL tube.

380 6.6) Take 1 mL of the PC3 cell suspension into a new centrifuge tube and add 9 mL of culture
381 media. Plate the cells on a 10 cm^2 culture dish (1:10 dilution) for subsequent experimental use.
382 Count the remaining cells using a hemocytometer.

383 6.7) Dilute 2,500 PC3 cells prepared in step 6.6 in 300 µL of RPMI 1640 culture media
384 supplemented with 2 % v/v of BM solution to allow for the formation of a gradient gel in the
385 culture.

386 6.8) Gently seed the cells onto the native and AGE-stiffened rBM and carefully place the
387 culture into the incubator set at 37 °C and 5% CO_2 (with humidification) to ensure even
388 distribution of growing spheroids in the well.

389 6.9) Change the culture media every 72 hr.

390 6.10) To study the effect of stiff (AGE-rich) rBM on prostate tumor cell migration, image PC3
391 cells using brightfield video time-lapse microscopy using temperature/CO_2 control and a
392 humidified chamber[17].

393 Note: PC3 cells grow in strands on native rBM and do not form acini with a lumen, but if left to
394 grow more than 72 hr on native rBM they will form 3D spheroids.

395 6.11) Following data acquisition, manually track PC3 cells and calculate their migration speed,
396 shape (elongation ratio) and persistence of migration[17-19].

397 Note: Persistence = ratio D/T, D = distance from start to end of cell trajectory, T= total length of

398 cell trajectory.

399 **REPRESENTATIVE RESULTS:**
400 3D prostate acini cultured on stiff rBM:
401 After 6 days in culture, PECs derived from normal prostate tissue (RWPE-1) (Figure 1A) and BPH
402 tissue (BPH-1) (Figure 1B) form acini on native (PBS treated) rBM that are organized into
403 uniform spheroids of epithelial cells. These acini also have the characteristics of highly
404 organized PECs with apical-to-basal polarity and a visible luminal space[13,20].
405
406 The acini formed by PECs derived from normal prostate tissue (RWPE-1) (Figure 1A) and BPH
407 tissue (BPH-1) (Figure 1B) on stiffened (AGE-rich) rBM (treated with GLA) have a disrupted
408 architecture (shifting from spheroidal to polygonal in shape and cells protruding/migrating from
409 the acini into the AGE-rich rBM) (Figure 1A). These acini are also characterized by highly
410 disorganized PECs that have lost their apical-to-basal polarity with a small or non-existent
411 luminal space[13].
412
413 [Place Figure 1 here]
414
415 [Place Table 1 here]
416
417 AGE dependent increased rBM stiffness promotes PC3 prostate tumor cell migration:
418 PC3 cells grown on native rBM migrate by maintaining continuous cell-cell contact, whereas
419 PC3 cells grown on AGE-rich (stiff) rBM move independently from each other (Figure 2A). After
420 72 hr in culture PC3 cells form foci (spheroids) on native (PBS treated) rBM, whereas PC3 cells
421 on stiff (AGE-rich) rBM do not from spheroids and migrate independently (Figure 2B). PC3 cells
422 on stiff (AGE-rich) rBM are more elongated than PC3 cells grown on native rBM (Figure 2C). PC3
423 cells on stiff rBM migrate faster than PC3 cells grown on native rBM (Figure 2D). PC3 cells on
424 stiff rBM display a decrease in persistence compared to PC3 cells grown on native rBM (Figure
425 2E).
426
427 [Place Figure 2 here]
428
429 **Figure 1: Prostate epithelial cells grown as 3D glandular acini on native and stiff reconstituted**
430 **basement membrane (rBM).** A. Brightfield images of RWPE-1 cells grown for 12 hr up to 6 days
431 on rBM gels treated with PBS (native) or 50 mM glycolaldehyde for 14 hr (AGE-rich; stiff); scale
432 bar = 50 µm. B. BPH-1 cells, grown as described in panel A; scale bars = 50 µm; data is
433 representative of 3 independent experiments.
434
435 **Figure 2: Prostate tumor cell migration on native and stiff reconstituted basement membrane**
436 **(rBM**). A. Brightfield images of PC3 cells grown on rBM gels treated with PBS (native) or 50 mM
437 glycolaldehyde for 14 hr (AGE-rich, stiff). Cells were imaged using a brightfield microscope (10 X
438 objective) and an acquisition rate of 1 image per h for 12 hr followed by cell tracking to
439 generate trajectories. Images shown correspond to the time points after 0, 3, 6, 9 and 12 hr.
440 Trajectories of single cells are shown for the 12 hr time point. Scale bar = 100 µm. B. PC3 cells

441 cultured on native or stiff rBM for 72 hr, and imaged as described in panel A. Scale bar = 100
442 µm. Detail shows selected area at 2 X magnification. C. Mean ± S.D. cell length (µm); significant
443 difference between native rBM and stiff rBM ($p = 1.2 \times 10^{-23}$). D. Mean ± S.D. velocity (µm/sec)
444 calculated from cell trajectories; significant difference between native rBM and stiff rBM (p =
445 0.004). E. Mean ± S.D. persistence of cell movement (ratio D/T, where D = distance from start to
446 end of cell trajectory, T = total length of cell trajectory); significant difference between native
447 rBM and stiff rBM (p = 0.0007). For panels C-E > 10 cells were analyzed, data is representative
448 of 3 independent experiments.
449
450 **Figure 3: Overview of the different protocols presented here**. The diagram depicts how to
451 prepare and stiffen the reconstituted basement membrane (rBM) with glycolaldehyde (Maillard
452 reaction), how to seed cells on to the stiff rBM, how to analyze the stiff rBM (extent of Maillard
453 reaction) and procedures that can be used to analyze the cellular and molecular changes
454 induced by AGE-rich rBM. AGE, advanced glycation endproducts; BM, basement membrane;
455 DAPI, 4',6-diamidino-2-phenylindole; EEA1, early endosomal antigen 1; GAPDH, glyceraldehyde-
456 3-phosphate dehydrogenase; GLA, glycolaldehyde; GEE, glycine ethyl ester; GM130, 130 kDa
457 cis-Golgi marker; p-MLC2 (Thr18/Ser19), myosin light chain-2 phosphorylated at sites threonine
458 18 and serine 19; rBM, reconstituted basement membrane; SDS-PAGE, sodium dodecyl sulfate
459 polyacrylamide gel electrophoresis. For RWPE1 acini scale bar = 10 µm; for PC3 tumor cell
460 spheroids scale bar = 100 µm. This figure has been modified from[13].
461
462 **Figure 4: Simple overview of the protocol with critical steps and timings indicated**. The flow
463 diagram depicts how to prepare and stiffen the reconstituted basement membrane (rBM) with
464 glycolaldehyde (Maillard reaction) with critical steps and timings indicated. Points where the
465 protocol can be stopped, and rBM gels stored, are also indicated. rBM, reconstituted basement
466 membrane; GLA, glycolaldehyde; GEE, glycine ethyl ester.
467
468 **Table 1: Characteristics of prostate epithelial RWPE-1 acini grown on native, semi-stiff and**
469 **stiff reconstituted basement membrane (rBM).** RWPE-1 acini were grown on rBM pre-treated
470 with PBS for 14 h (native), glycolaldehyde (GLA) for 6 hr (semi-stiff) or GLA for 14 hr (stiff). For
471 acinar shape, the percentage (%) ± standard deviation (SD) of round, semi-polygonal and
472 polygonal acini were calculated from 5 independent experiments (50 acini quantified per
473 condition). Relative acinar size was calculated (native rBM = 100 %) from 3 independent
474 experiments. For invasiveness, % ± SD acini with one or more protruding cells were calculated
475 from 3 independent experiments. Fold change is calculated by dividing the average value
476 obtained under semi-stiff or stiff conditions by the corresponding value for native conditions. P
477 values calculated using Student's t-test ($\alpha = 0.05$).
478
479 **DISCUSSION:**
480 A protocol for the generation of 3D glandular acini from MECs in pure rBM gels[6] was modified
481 in a previous study by the addition of 4 mg/mL type I collagen to the rBM matrix. The addition
482 of collagen resulted in the elastic modulus of the rBM gel increasing from 175 ± 37 to 1589 ±
483 380 Pascals. This 9.1-fold increase in stiffness modulated the growth, survival, migration and
484 differentiation of MECs[21]. The protocol was modified again by including a treatment step with

485 D-(-)-ribose to promote non-enzymatic crosslinking of the type I collagen that had been added
486 to the rBM gel. The resultant 15-fold increase in stiffness was found to cooperate with
487 oncogenic transformation of MECs to promote their invasive behavior[22]. The experimental
488 approach of adding type I collagen to rBM gels facilitates the direct interaction of MECs with
489 collagen fibres, which only occurs in human tissue after the physical barrier between the
490 stroma and epithelium provided by the BM undergoes proteolytic degradation. By generating
491 3D glandular acini from PECs in pure rBM gels pre-treated with GLA, the current protocol opens
492 the way to study how BM stiffness *per se* can trigger their invasive behaviour (Figure 3). The
493 levels of BM stiffness induced in this protocol have physiological relevance. Incubation with 50
494 mM GLA for 6 hr and 14 hr respectively increased the elastic moduli of the pure rBM gel to 175
495 ± 90 and 322 ± 160 compared to 122 ± 55 Pascals in rBM gels treated with PBS (Table 1). This
496 1.7 to 3.2-fold increase in rBM stiffness recapitulates the 2.5- to 3.4-fold increase in stiffness
497 observed in malignant compared to normal prostate or BPH tissue[23-26]. As outlined in a recent
498 publication[13] the morphological changes induced by the accumulation of AGE and rBM stiffness
499 in PEC acini can be quantified for a statistically significant shift from a rounded to polygonal
500 shape, decreased luminal/total acinar area, and protruding cells migrating from the *acina* into
501 the AGE-rich rBM (Figure 3). Immunoblotting can also be used to assess markers of EMT (e.g.
502 loss of E-cadherin[13]) and the contractile behavior (e.g. phosphorylated myosin light chain-2,
503 pMLC2[13]) in PECs grown in normal versus stiff rBM (Figure 3). Further evaluation using
504 immunofluorescent staining and confocal microscopy can be applied to visualize the BM (e.g.
505 laminin, collagen IV and AGE accumulation[13]), cellular apical-to-basal polarity (e.g. apical
506 localization of EEA1: early endosomal antigen 1; and GM130: 130 kDa cis-Golgi marker[13]) and
507 cellular patterns of adhesion molecules (e.g. E-cadherin localization to cell-cell junctions[13])
508 (Figure 3).
509
510 [Place Figure 3 here]
511
512 Troubleshooting steps will be necessary if D-(-)-ribose is chosen as the crosslinking agent for
513 rBM. During protocol development it was found that treatment with 1 M D-(-)-ribose for 72 hr,
514 as previously described for rBM/collagen gels[22], resulted in the dehydration and shrinkage of
515 rBM gels. The evaluation of lower concentrations of D-(-)-ribose and shorter treatment times
516 may help to overcome this limitation. If longer incubation times and higher concentrations of
517 GLA are used to induce higher levels of rBM gel stiffness it will be necessary to assess whether
518 these treatment conditions have an impact on cell survival and proliferation, as previously
519 described[13].
520
521 When RWPE-1 cells are exposed to serum or serum-containing materials they adopt an EMT-
522 like phenotype. For this reason short interfering RNA (siRNA) oligonucleotide treatment needs
523 to be optimized in KSFM. If gene-silencing efficiency is compromised inducible shRNA vectors
524 should be employed to overcome this limitation.
525
526 This protocol will facilitate the future study of pro-invasive mechanisms triggered by AGE-
527 dependent BM stiffness in PECs (RWPE-1, BPH-1) and evaluation of anti-metastatic targets in
528 invasive PTCs (PC3). Given that BPH is considered to be a metabolic disorder[27], this protocol

also paves the way towards our improved understanding of the link between metabolic disorders and increased prostate cancer risk. Given that BM stiffness induced by its exposure to AGEs may be a trigger for invasiveness in other cancer types, it will be of interest to use the protocol to set up similar models that incorporate normal epithelial cells and tumor cells from other organs (e.g. breast, colon, ovaries, pancreas).

Critical steps within the protocol, together with their timings, are summarized in Figure 4.

[Place Figure 4 here]

ACKNOWLEDGMENTS:

We thank Simon Hayward (Vanderbilt University Medical Center) for the BPH-1 cells; and Thomas Cox and Janine Erler (Biotech Research & Innovation Centre, University of Copenhagen) for their assistance with rheological measurements. MR-T was funded by Worldwide Cancer Research, formerly The Association of International Cancer Research (Grant 08-0803 to JS), The British Embassy Montevideo and Agencia Nacional de Investigacion e Innovacion (UK_RH_2015_1_2 to MR-T). MC was supported by Prostate Cancer UK (Grant S14-017 to JS and GS). KW was funded by The China Scholarship Council. MAM was funded by The Saudi Arabian Cultural Bureau.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Timpl, R. & Brown, J. C. Supramolecular assembly of basement membranes. *Bioessays.* **18** (2), 123-132, doi:10.1002/bies.950180208 (1996).
2. Yurchenco, P. D. & Ruben, G. C. Type IV collagen lateral associations in the EHS tumor matrix. Comparison with amniotic and in vitro networks. *Am J Pathol.* **132** (2), 278-291, doi:amjpathol00131-0100.pdf (1988).
3. Halfter, W. *et al.* Protein composition and biomechanical properties of in vivo-derived basement membranes. *Cell Adh Migr.* **7** (1), 64-71, doi:10.4161/cam.22479 (2013).
4. Candiello, J., Cole, G. J. & Halfter, W. Age-dependent changes in the structure, composition and biophysical properties of a human basement membrane. *Matrix Biol.* **29** (5), 402-410, doi:10.1016/j.matbio.2010.03.004 (2010).
5. To, M. *et al.* Diabetes-induced morphological, biomechanical, and compositional changes in ocular basement membranes. *Exp Eye Res.* doi:10.1016/j.exer.2013.09.011 (2013).
6. Debnath, J., Muthuswamy, S. K. & Brugge, J. S. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods.* **30** (3), 256-268, doi:10.1016/S1046-2023(03)00032-X (2003).
7. Bello, D., Webber, M. M., Kleinman, H. K., Wartinger, D. D. & Rhim, J. S. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. *Carcinogenesis.* **18** (6), 1215-1223, doi:10.1093/carcin/18.6.1215 (1997).

573 8 Hayward, S. W. *et al.* Establishment and characterization of an immortalized but non-
574 transformed human prostate epithelial cell line: BPH-1. *In Vitro Cell Dev Biol Anim.* **31**
575 (1), 14-24, doi:10.1007/BF02631333 (1995).
576 9 Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F. & Jones, L. W. Establishment and
577 characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol.* **17** (1), 16-
578 23 (1979).
579 10 Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of
580 bacteriophage T4. *Nature.* **227** (5259), 680-685, doi:10.1038/227680a0 (1970).
581 11 Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from
582 polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl*
583 *Acad Sci U S A.* **76** (9), 4350-4354, doi:pnas00009-0198.pdf (1979).
584 12 Rabilloud, T. Mechanisms of protein silver staining in polyacrylamide gels: a 10-year
585 synthesis. *Electrophoresis.* **11** (10), 785-794, doi:10.1002/elps.1150111003 (1990).
586 13 Rodriguez-Teja, M. *et al.* AGE-modified basement membrane cooperates with Endo180
587 to promote epithelial cell invasiveness and decrease prostate cancer survival. *J Pathol.*
588 **235** (4), 581-592, doi:10.1002/path.4485 (2015).
589 14 Graf, B. W. & Boppart, S. A. Imaging and analysis of three-dimensional cell culture
590 models. *Methods Mol Biol.* **591** 211-227, doi:10.1007/978-1-60761-404-3_13 (2010).
591 15 Yao, N. Y., Larsen, R. J. & Weitz, D. A. Probing nonlinear rheology with inertio-elastic
592 oscillations. *J Rheology.* (52), 1013 - 1025, doi:10.1122/1.2933171 (2008).
593 16 Baker, A. M., Bird, D., Lang, G., Cox, T. R. & Erler, J. T. Lysyl oxidase enzymatic function
594 increases stiffness to drive colorectal cancer progression through FAK. *Oncogene.* **32**
595 (14), 1863-1868, doi:10.1038/onc.2012.202 (2013).
596 17 Caley, M. P. *et al.* Tumor-associated Endo180 requires stromal-derived LOX to promote
597 metastatic prostate cancer cell migration on human ECM surfaces. *Clin Exp Metastasis*
598 doi:10.1007/s10585-015-9765-7 (2015).
599 18 Sturge, J., Wienke, D., East, L., Jones, G. E. & Isacke, C. M. GPI-anchored uPAR requires
600 Endo180 for rapid directional sensing during chemotaxis. *J Cell Biol.* **162** (5), 789-794,
601 doi:10.1083/jcb.200302124 (2003).
602 19 Sturge, J., Wienke, D. & Isacke, C. M. Endosomes generate localized Rho-ROCK-MLC2-
603 based contractile signals via Endo180 to promote adhesion disassembly. *J Cell Biol.* **175**
604 (2), 337-347, doi:10.1083/jcb.200602125 (2006).
605 20 Rodriguez-Teja, M. *et al.* Survival Outcome and EMT Suppression Mediated by a Lectin
606 Domain Interaction of Endo180 and CD147. *Mol Cancer Res.* **13** (3), 538-547,
607 doi:10.1158/1541-7786.MCR-14-0344-T (2015).
608 21 Paszek, M. J. *et al.* Tensional homeostasis and the malignant phenotype. *Cancer Cell.* **8**
609 (3), 241-254, doi:10.1016/j.ccr.2005.08.010 (2005).
610 22 Levental, K. R. *et al.* Matrix crosslinking forces tumor progression by enhancing integrin
611 signaling. *Cell.* **139** (5), 891-906, doi:10.1016/j.cell.2009.10.027 (2009).
612 23 Carson, W. C. *et al.* Material characterization of ex vivo prostate tissue via spherical
613 indentation in the clinic. *Med Eng Phys.* **33** (3), 302-309,
614 doi:10.1016/j.medengphy.2010.10.013 (2011).
615 24 Hoyt, K. *et al.* Tissue elasticity properties as biomarkers for prostate cancer. *Cancer*
616 *Biomark.* **4** (4-5), 213-225, doi:nihms306807.pdf (2008).

617 25 Krouskop, T. A., Wheeler, T. M., Kallel, F., Garra, B. S. & Hall, T. Elastic moduli of breast
618 and prostate tissues under compression. *Ultrason Imaging.* **20** (4), 260-274,
619 doi:10.1177/016173469802000403 (1998).
620 26 Zhang, M. *et al.* Quantitative characterization of viscoelastic properties of human
621 prostate correlated with histology. *Ultrasound Med Biol.* **34** (7), 1033-1042,
622 doi:10.1016/j.ultrasmedbio.2007.11.024 (2008).
623 27 Corona, G. *et al.* Benign prostatic hyperplasia: a new metabolic disease of the aging
624 male and its correlation with sexual dysfunctions. *Int J Endocrinol.* Article ID:329456,
625 doi:10.1155/2014/329456 (2014).
626
627