Mechanically stimulated osteocytes promote the proliferation and migration of breast cancer cells via a potential CXCL1/2 mechanism

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Keywords

Bone; Breast Cancer; Fluid shear; Mechanobiology; CXCL

Abstract

Bone represents the most common site for breast cancer metastasis. Bone is a highly dynamic organ that is constantly adapting to its biophysical environment, orchestrated largely by the resident osteocyte network. Osteocytes subjected to physiologically relevant biophysical conditions may therefore represent a source of key factors mediating breast cancer cell metastasis to bone. Therefore, we investigated the potential proliferative and migratory capacity of soluble factors released by mechanically stimulated osteocytes on breast cancer cell behaviour. Interestingly the secretome of mechanically stimulated osteocytes enhanced both the proliferation and migration of cancer cells when compared to the secretome of statically cultured osteocytes, demonstrating that mechanical stimuli is an important physiological stimulus that should be considered when identifying potential targets. Using a cytokine array, we further identified a group of mechanically activated cytokines in the osteocyte secretome, which potentially drive breast cancer metastasis. In particular, CXCL1 and CXCL2 cytokines are highly expressed, mechanically regulated, and are known to interact with one another. Lastly, we demonstrate that these specific factors enhance breast cancer cell migration independently and in a synergistic manner, identifying potential osteocyte derived factors mediating breast cancer metastasis to bone.

1 **1.0 Introduction**

2 At the time of diagnosis, up to 10% of breast cancer patients present with metastatic cancer
3 [1]. Around 70% of metastatic breast cancer patients develop bone metastasis, which makes
4 it the most common site amongst the various target tissues [2]. The bone microenvironment
5 provides a unique site for breast cancer cells to metastasize by offering favourable conditions
6 for growth and development of breast cancer as it is enriched with several essential cytokines,
7 chemokines and growth factors that are released from resident cells and the matrix following
8 bone resorption/remodelling [3]. Although several factors have been suggested to contribute
9 to the metastatic process, due to off target effects, very few of these are treatment targets [4].
10 Therefore, there is a need to identify new targets that may help to control advancement of
11 cancer within bone.
12 Bone tissue is a reservoir of matrix proteins, growth factors, and cells which can potentially
13 coordinate cancer cell metastasis. While approximately 90% of bone matrix protein consists
14 of type 1 collagen, the remaining 10% include fibroblast growth factors (FGF), insulin-like
15 growth factor (IGF), transforming growth factor- β (TGF-β) and bone morphogenetic
16 proteins (BMP) [5]. These factors are released during the remodelling process and can
17 contribute to breast cancer metastasis. Moreover, tumour cells that metastasise to the bone
18 can also alter bone remodelling. Breast cancer cells within bone release PTHrP, which
19 inhibits osteoprotegerin (OPG) released from the stromal/osteoblast cells by allowing PTHrP
20 binding to receptor activator of RANKL, which in turn activates osteoclastogenesis. This
21 creates a cycle of bone resorption which releases factors to sustain survival and progression
22 of cancer [6]. In addition to these embedded factors, resident osteocytes, which account for

23 90% of cells in bone tissue, are known to release a plethora of factors which can regulate
24 bone remodelling and potentially breast cancer cell behaviour. Therefore, the osteocyte may
25 represent an alternative target for treatment development [7].

26 Bone is a highly dynamic organ and it is constantly adapting to meet the demands of its
27 biochemical and biophysical environment. Osteocytes are believed to be the master
28 orchestrator of bone adaptation, coordinating osteogenesis and osteoclastogenesis [8]. In
29 particular, due to habitual loading, osteocytes experience mechanical stimuli in the form of
30 dynamic fluid flow which is reported to between 0.8-3 Pa *in vivo* [9]. Mechanically stimulated
31 bone cells have been shown to release numerous growth factors which influence bone
32 physiology and could also play significant role in bone metastasis [8, 10]. For example, under
33 physiological levels of fluid stress, osteocytes enhance the production of prostaglandin E2,
34 ATP, Ca^{2+}, OPN, nitric oxide, OPG and TGF-β and decrease expression of RANKL and
35 sclerostin, many of which have been linked to cancer cell metastasis. Therefore, osteocytes
36 under physiological environments, may represent a source of key factors mediating breast
37 cancer metastasis to bone.

38 Our hypothesis is that osteocytes are releasing essential cytokines when they are
39 mechanically stimulated, which is potentially providing a favourable metastatic
40 microenvironment for breast cancer cells. Therefore, in this study, we set out to mechanically
41 stimulate osteocytes to mimic physiological relevant conditions in the bone
42 microenvironment. We harnessed conditioned media from mechanically stimulated and
43 unstimulated cells to study the effect of this secretome on proliferation and migration of
44 MCF-7 breast cancer cells. To establish which factors may be present in the osteocyte

45 secretome that are potentially modulating the activity of the breast cancer cells, we utilized
46 a cytokine array. From this, we further selected CXCL1 and CXCL2 cytokines and
47 highlighted their role in enhancing the migration of breast cancer cells, thus identifying
48 potential novel targets in osteocyte mediated breast cancer cell metastasis to bone.

49

50 **2.0 Materials and Methods**

51 ***2.1 General chemicals and reagents***

52 E-plates for RTCA xCELLigence system were purchased from ACEA Biosciences,
53 (Cambridge, U.K.). Cytokine CXCL1 and CXCL2 were purchased from Peprotech (London,
54 U.K.). All other chemicals were purchased from Sigma Aldrich Ltd (Dublin, Ireland).

55 ***2.2 Cell culture***

56 MCF-7 cells (epithelial like cells from human breast cancer) and MLO-Y4 cells (murine
57 osteocyte-like cell line) were utilized in this study. All cells were maintained at 37°C and 5%
58 CO_2. MCF-7 cells were cultured in DMEM with 10%FBS and 1% Penicillin/Streptomycin
59 and 1%L-glutamine unless otherwise mentioned. Before seeding MLOY-4 cells, the flask was
60 coated with 0.15mg/ml rat tail collagen-1 for 1 hour. MLOY-4 cells were seeded in MEM-
61 alpha with 5%CS, 5%FBS, 1%Penicillin/Streptomycin and 1%L-glutamine. MLOY-4 cells
62 were seeded in T-75 flasks at $4x10^3$ cells/cm^2 to collect conditioned media.

63 ***2.3 Mechanical stimulation***

64 To mechanically stimulate osteocytes, dynamic fluid flow was generated through the 65 utilisation of a rocking platform which oscillated at a frequency of 0.5 Hz and with a tilt angle 66 of 6.5° consistent with previously published work [11, 12]. This system is used in order to 67 generate large amounts of conditioned media when compared to other experimental apparatus 68 such as the parallel flow plate chamber [13]. MLO-Y4 cells were seeded at a density of 4000 69 cells/cm^2 in a T-75 cm^2 flask. 24 hrs post-seeding media was replaced with 6ml of fresh media, 70 followed by application of mechanical stimulation for 24hrs. Controls included MLO-Y4 71 cells cultured as above but under static conditions, and a collagen coated flask with no cells 72 subjected to 24hrs mechanical stimulation.

73 ***2.4 Real time proliferation assay***

74 Cell proliferation was examined in real time using the xCELLigence platform. 20x10^3 MCF-75 7 cells were seeded in each well with desired media. As cells adhere and proliferate in the 76 bottom of the well, changes in electrical impedance is recorded as cell index at 5 minute 77 intervals. To assess effect of CXCL1 and CXCL2 on proliferation of MCF-7 cells. These cells 78 were allowed to adhere overnight and the cytokines were added 20hours after seeding. Hence 79 to monitor the effect of these cytokines specifically the data was normalized at 20-hour time 80 point to assess its effect on the proliferation of the MCF-7 cells.

81 ***2.5 Migration assay***

82 To test cell migration, we used the scratch and wound assay. MCF-7 cells were seeded in a 83 24 well plate to achieve 95-100% confluency 24hours after seeding. The scratch was created 84 using a P200 tip and two scratches were made in each well. The cells were then washed with

85 PBS and appropriate test media was added to the wells. Images were taken at 4 different
86 locations along the gap at 24hour and 48hour after introducing the gap. To analyse wound
87 gap closure, images were processed in ImageJ software.

88 ***2.6 Cytokine array***

89 The Cytokine Array kit (AAM-CYT-6-8, C series) was purchased from RayBiotech
90 (Norcross, USA). All the steps were performed according to the supplier's instructions. Post-
91 incubation the membranes were scanned and protein expression quantified using the Licor
92 C-Digit scanner and Licor Image studio Ver 2.0 software respectively. The intensity of each
93 spot on the membrane was recorded and the data was analysed as per the manufacturer's
94 instructions. The positive control spots were used to normalize and orientate the array. We
95 selected the 'control' membrane, as a reference array and the expression of the cytokines in
96 'mechanically stimulated' and 'static' media test membrane were normalized to the
97 corresponding cytokine in the control membrane. The relative expression was reported as
98 fold change.

99 ***2.7 Data/Statistical Analysis***

100 Statistical analysis was performed using the GraphPad Prism 5 software. Each experiment
101 was carried out at least three times with the exception of the cytokine array experiment. To
102 test normality of the data Shapiro-Wilk test was used. To study significance between two
103 groups, two tailed Student t-test was performed for normally distributed data. For multiple
104 comparison the significance was calculated using one-way ANOVA with Bonferroni test if
105 the data was normally distributed or else the Kruskal-Wallis test with Dunn's multiple

106 comparison test was used. All the statistical analysis was considered significant when $p < 0.05$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

108

109 **3.0 Results**

110 ***3.1 Mechanically stimulated osteocytes secrete factors that promote the proliferation and*** 111 ***migration of breast cancer cells.***

112 We hypothesized that mechanically stimulated osteocytes release essential growth factors 113 and cytokines that may play a crucial role in breast cancer metastasis to bone. Therefore, we 114 wanted to study the effect of these secretomes on the growth of breast cancer cells, by 115 culturing MCF-7 cells (ER+ breast cancer cells) in the different test media. However, as the 116 conditioned media is depleted of serum by the osteocytes, it was necessary to supplement it 117 with fresh culture media. Hence, we cultured MCF-7 cells in different % v/V (test 118 media/fresh media) to determine the best concentration to use for further experimentation. 119 To determine which concentration shows maximum difference in proliferation, we tested 120 40% and 60% concentration of test media on the xCELLigence platform and recorded cell 121 activity every 5 minutes for duration of 80 hours (Fig.1A-B). A comparative analysis was 122 performed at 24hrs for each group (Fig.1C-D). While there was a trend towards decreased 123 proliferation in the osteocyte conditioned media in the 40% v/V media compared to control, 124 there was no significant differences identified. At 60% v/V media both mechanically 125 stimulated and static conditioned media demonstrated significantly reduced proliferation 126 compared to control. This is likely due to nutrient depletion in the CM groups and is

127 consistent with previous findings [11]. Interestingly, at 60% v/V the mechanically stimulated

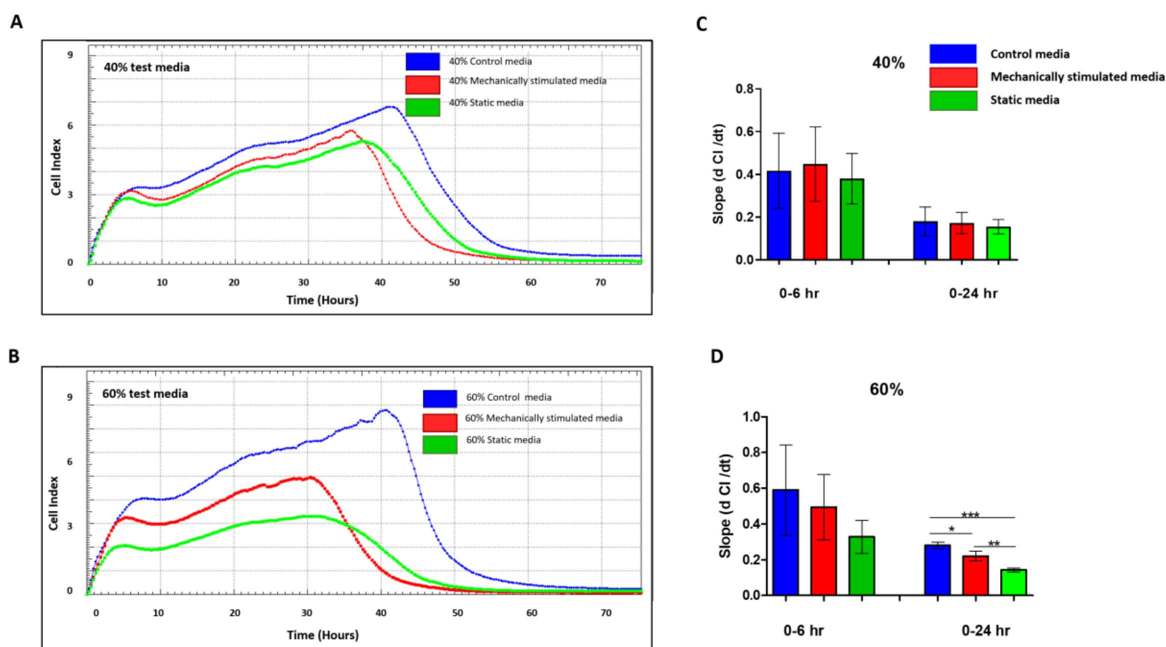
128 test media elicited a significant 56% increase (P<0.01) in proliferation of MCF-7 cells when

129 compared to static control demonstrating that mechanically stimulated osteocytes secrete

130 paracrine factors that regulate breast cancer cell proliferation (Fig.1D).

131

132



133

134 **Figure 1: Mechanically stimulated osteocytes secrete factors that enhance breast cancer**

135 **cell proliferation.** Sample graphs showing MCF-7 breast cancer cells cultured in real time

136 on the xCELLigence platform in **(A)**40% or **(B)** 60% v/V of osteocyte conditioned media

137 diluted in standard growth media for MLO-Y4 cells. Cell adhesion and growth was recorded

138 as cell index every 5 minutes for duration of 80 hours in real time as shown in the

139 representative graphs from one experiment. **(C-D)** Average data taken from 3 different
140 experiments for 40% and 60% v/V dilutions. One-way ANOVA was used with Bonferroni
141 test to analyse statistical significance. N = 3.

142

143 After observing increased proliferation of MCF-7 cells in 60% v/V mechanically stimulated
144 osteocyte media we next wanted to test the effect of these media on the migration of breast
145 cancer cells using a traditional scratch and wound assay. At both 24hr and 48hr incubation,
146 the static osteocyte conditioned media demonstrated significantly reduced migration when
147 compared to control media. However, similar to that seen with proliferation, mechanically
148 stimulated osteocyte conditioned media significantly increased migration of breast cancer
149 cells at 24-hours and 48-hours ($P<0.01$) by 33.3% and 53.3% respectively (Fig.2). Together,
150 these results suggest that mechanically stimulated osteocytes secrete growth factors and
151 chemokines which are potentially driving growth and migration of breast cancer cells.

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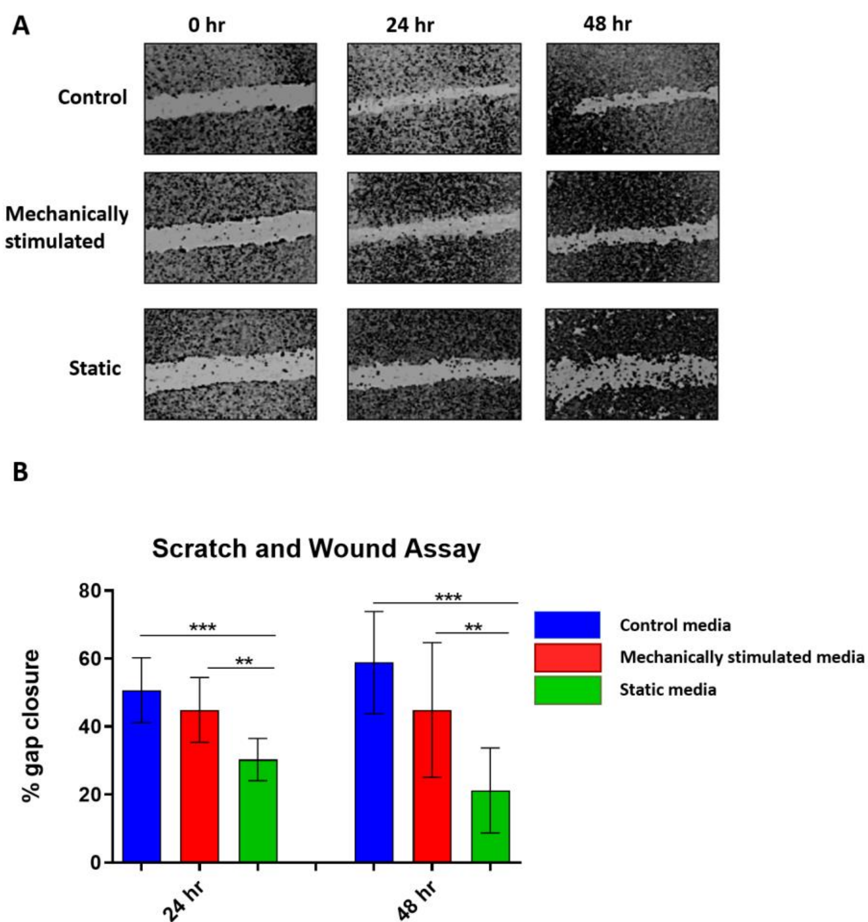


153

154 **Figure 2: Mechanically stimulated osteocytes secrete factors that enhance breast cancer**

155 **cell migration. (A)** Scratch and wound assay was performed using MCF-7 cells seeded in

156 60% v/V test media. **(B)** % gap closure depicting speed to which cells migrated to fill the

157 gap. The gap closure was calculated using ImageJ software. Data represents mean with SD.

158 Normality test was done using the Shapiro wilk test. One-way ANOVA test was done for

159 statistical analysis. N=3.

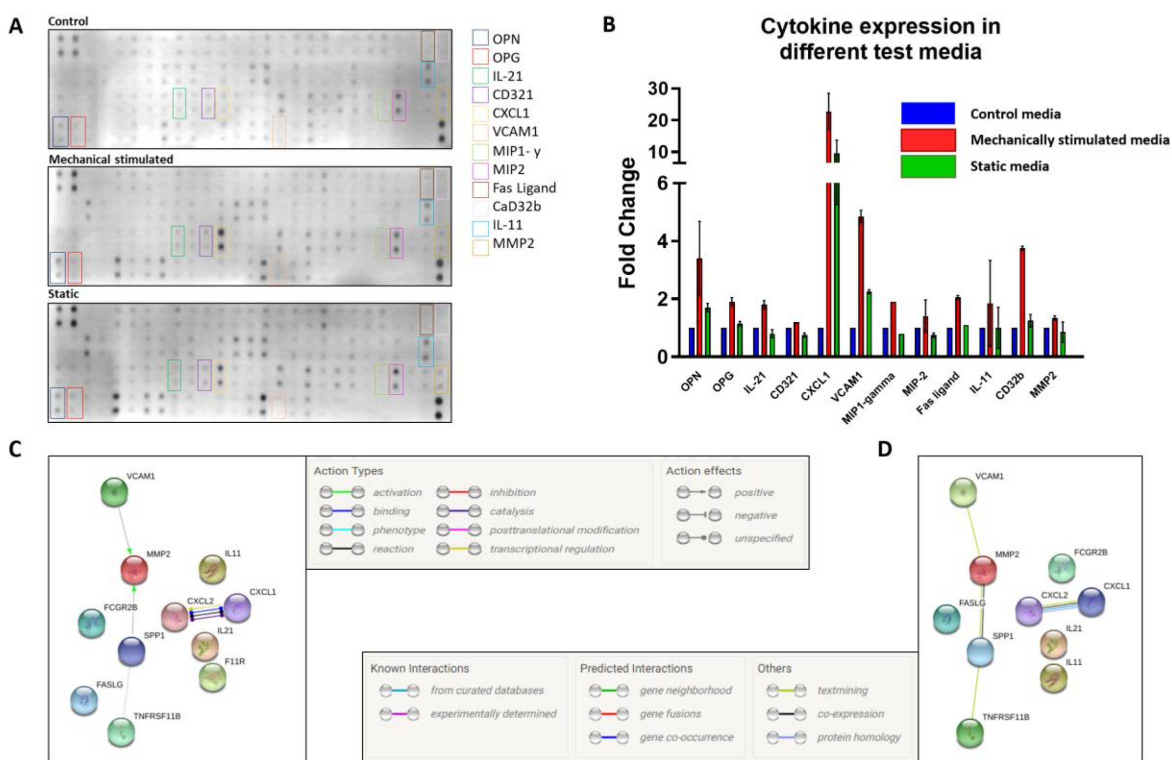
160

161 **Osteocytes release a number of mechanically regulated cytokines associated with cell**
162 **migration, including CXCL1.**

163 Having established that the secretome of the mechanically stimulated osteocyte enhances
164 proliferation and migration of MCF-7 cells, we wanted to decipher what growth factors and
165 chemokines are present in the media which may be potentially driving these processes.
166 Therefore, the expression level of 97 cytokines were determined simultaneously using a
167 cytokine array (Fig.S1). The cytokine array was imaged and analysed as per the
168 manufacturer's instruction. The results demonstrated that numerous factors had differential
169 expression in the three different test media groups (Fig.3A). To narrow down potential candidate
170 factors, only the cytokines which demonstrated greater than a 1.5-fold difference in expression
171 between the test medias was brought forward. 12 cytokines were identified as being
172 upregulated in mechanically stimulated media when compared to static controls (Fig.3B). These
173 consisted of OPN, OPG, IL-21, Cluster of Differentiation 321 (CD321) or F11R or Junctional
174 adhesion molecule A (JAM-A), chemokine (C-X-C motif) ligand 1 (CXCL1) or GROα, Vascular
175 cell adhesion protein 1 (VCAM1), macrophage inflammatory protein-1 gamma (MIP1-γ) or
176 CCL9, Macrophage Inflammatory Protein 2 (MIP-2 or CXCL2), Fas ligand, Immunotherapy
177 Targeting Inhibitory Fcγ Receptor IIB (CD32b), interleukin 11 (IL-11) and Matrix
178 MetalloProteinase-2 (MMP2).

179 To further narrow down potential candidate factors, we next performed a bioinformatics
180 analysis using the STRING tool to assess whether the shortlisted mechanically activated
181 cytokines share similar action effects/functionality (Fig.3C) or interact with one another
182 (Fig.3D) to potentially promote breast cancer proliferation and/or migration [14]. Different

183 colors of the connecting nodes represent discrete interactions and action effects between
184 proteins. Both VCAM1 and OPN show a positive action effect on MMP2 while there is a
185 strong association between CXCL1 and CXCL2 with unspecified action effects. Taken
186 together, this data demonstrates that osteocyte cells increase secretion of certain essential growth
187 factors and chemokines when they are mechanically stimulated which may interact with one
188 another to regulate breast cancer cell behavior.



189

190 **Figure 3: Mechanically stimulated osteocytes secrete numerous potential interacting**
191 **soluble factors in response to mechanical stimulation. (A)** Cytokine array membrane
192 illustrating differential expression of 97 proteins across the three test medias. The experiment
193 was repeated twice. **(B)** Cytokine expression data normalised to control media in which there

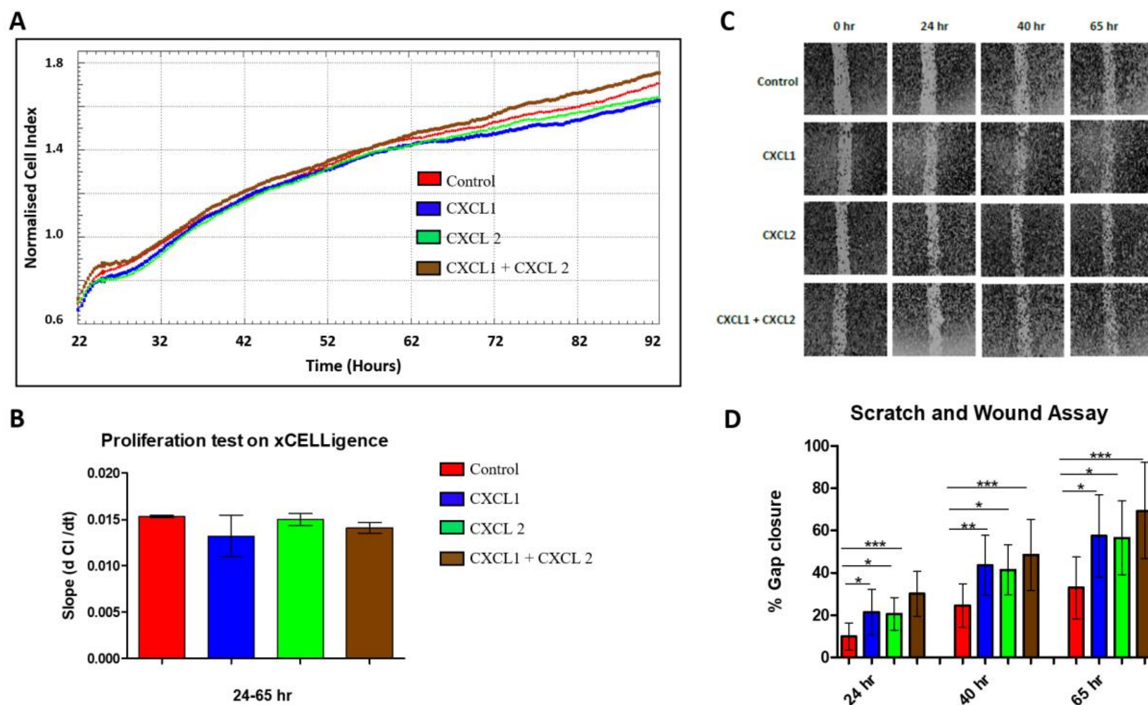
194 was at least a 1.5-fold increase in expression following mechanical stimulation when
195 compared to static control. **(C)** Action effect amongst cytokines of interest using String
196 software at a high confidence setting. **(D)** Associations amongst cytokines of interest using
197 STRING software at a high confidence setting.

198 **CXCL1 and CXCL2 are effective promoters of breast cancer cell migration**

199 From the results of the cytokine array, we identified a number of cytokines with increased
200 expression in the mechanically stimulated media in contrast to the static media. Amongst
201 these cytokines, CXCL1 showed the highest difference in the mean fold change expression
202 compared to control media and static media. Using the STRING tool, we found key
203 associations between CXCL1 and CXCL2, suggesting that, they may potentially work
204 together in modulating progression of breast cancer (Fig.3). Therefore, we examined how
205 CXCL1 and CXCL2 are potentially contributing towards proliferation and migration of
206 breast cancer cells.

207 Using the xCELLigence system we first investigated breast cancer cell proliferation in
208 response to 50ng/ml CXCL1, 50ng/ml CXCL2 or as a combination to investigate any
209 potential synergistic effect. No difference in proliferation was seen with each treatment at
210 either 24hrs, 40hrs, or 65hrs (Fig.4A-B). Next, we wanted to investigate if these cytokines
211 would affect migration of breast cancer cells using the scratch and wound assay. There was
212 a significant increase in migration of MCF-7 cells at 24 hours ($P<0.05$), 40 hours ($P<0.05$)
213 and 65 hours ($P<0.05$) after incubation with either CXCL1 or CXCL2. We also performed
214 a dose response study (10ng/ml-150ng/ml) and demonstrated that the effect of both CXCL1

215 or CXCL2 on MCF-7 migration is saturated at 50ng/ml (Fig.S2). Interestingly when these
216 cytokines were added together there was a further increase in cell migration although this
217 trend was not significant when compared to the response elicited from individual cytokines
218 (Fig. 4C-D).



219

220 **Figure 4: The effect of CXCL1 and CXCL2 on the proliferation and migration of MCF-**
221 **7 cells. (A)** MCF7 cells were seeded in normal 10% normal medium overnight. Thereafter
222 media was changed and replaced with serum free media and cells were treated with 50 ng/ml
223 of CXCL1 and CXCL2 individually and in combination. The figure shows a representative
224 graph in real time from the xCELLigence system. **(B)** Quantification was performed by
225 taking average cell index values from each of the three experiments at 24-hour, 40-hours and
226 65-hour time points. **(C)** Scratch and wound assay was performed using MCF-7 cells treated

227 with 50 ng/ml of CXCL1 and CXCL2 individually and in combination. **(D)** % gap closure
228 depicting the amount of gap filled due to the migration of the cells. The gap closure was
229 calculated using ImageJ software. Data represents mean with SD. Statistical analysis between
230 multiple groups comparing control to the other test conditions was done using one-way
231 ANOVA with Bonferroni test. N=3.

232

233 **Discussion**

234 Osteocytes subjected to physiologically relevant biophysical conditions may represent a
235 source of factors mediating breast cancer cell metastasis to bone. Therefore, we investigated
236 the potential proliferative and migratory capacity of factors released by mechanically
237 stimulated osteocytes on breast cancer cells. Interestingly the secretome of mechanically
238 stimulated osteocytes enhanced both the proliferation and migration of cancer cells when
239 compared to the secretome of statically cultured osteocytes, demonstrating the mechanical
240 stimuli is an important physiological stimulus that should be considered when identifying
241 potential targets. Using a cytokine array, we further identified a group of mechanically
242 activated cytokines in the osteocyte secretome, which are potentially driving breast cancer
243 metastasis. In particular, CXCL1 and CXCL2 cytokines are highly expressed, mechanically
244 regulated, and are known to interact with one another. Lastly, we demonstrate that these
245 specific factors enhance breast cancer cell migration independently and in a synergistic
246 manner, identifying potential osteocyte derived factors mediating breast cancer metastasis to
247 bone.

248 Mechanical stimulation of resident bone cells results in the increased secretion of multiple
249 cytokines that enhance breast cancer cell migration and proliferation. Previous studies
250 demonstrated an increase in proliferation and migration of breast cancer cells in presence of
251 conditioned media from osteocytes [15]. However, bone and subsequently resident cells are
252 routinely subjected to mechanical loads as a result of habitual movement. This loading is an
253 essential stimulus maintaining bone mass [16] and thus is an important environmental factor
254 to consider when investigating the bone microenvironment. In this study we stimulated the
255 osteocyte cells to mimic loading conditions in the bone microenvironment. Interestingly we
256 observed significant increase in proliferation of MCF-7 cells when they were cultured in
257 mechanically stimulated conditioned media in comparison to the static media, suggesting that
258 osteocytes are potentially secreting growth factors and chemokines that are enhancing the
259 growth of MCF-7 in contrast to the static media. The control media showed higher
260 proliferation in comparison to the mechanically stimulated osteocyte media, as it was not
261 cultured with cells and all the nutrients and FBS was still available in abundance for the cells
262 to proliferate [17]. Interestingly, we also demonstrated that mechanically stimulated media
263 from MLO-Y4 cells significantly increased migration of MCF-7 cells as compared to static
264 osteocyte media. This work contributes to the emerging picture of how bone
265 microenvironment potentially provides a favourable metastatic site for breast cancer.

266 Mechanical stimulation enriches the cytokine profile within the osteocyte secretome,
267 significantly enhancing a number of factors known to be involved in cancer. Utilising a
268 cytokine array, we evaluated the expression of ninety-seven cytokines and identified twelve
269 factors that are mechanically regulated with loading enhancing the expression greater than

270 1.5-fold. A number of these factors are known to be mechanoresponsive and thus our data
271 is consistent with previous studies. For example, bone cells are known to secrete OPN and
272 OPG, the expression of which is enhanced in response to mechanical stimulus [18]. These
273 findings were consistent with our study, as we have observed increased OPN and OPG
274 expression in mechanically stimulated media compared to the static. Interestingly, IL-11
275 mRNA expression was observed to be significantly elevated in breast cancer bone metastasis
276 tissue compared to the normal breast tissue [19]. From our cytokine array results, we have
277 shown that IL-11 is highly expressed at protein level when osteocyte cells are mechanically
278 stimulated, suggesting its increased expression is favourable for bone metastasis. Another
279 comprehensive investigation performed by Govey *et al.* identified CXCL1 and CXCL2
280 mRNA to be the most sensitive to mechanical stimulation amongst 14,000 genes investigated
281 in osteocytes subjected to fluid shear [20]. Our work compliments this finding and confirms
282 that CXCL1 and CXCL2 are also upregulated at a protein level when osteocytes are
283 mechanically stimulated. While it is important to identify individual mechanically regulated
284 cytokines, it is well known that factors can act in combination to enhance the bone metastasis
285 potential of breast cancer cells [21]. Utilising the STRING database, we identified that
286 CXCL1 and CXCL2 displayed several interactions and action effect, while VCAM1 and
287 OPN/SPP1 showed positive action effect on MMP2. CXCL1 also showed interaction with
288 MMP2 and VCAM1. This highlighted several mechanically regulated cytokines which may
289 potentially work collectively in progression of cancer.

290 CXCL1 and CXCL2 cytokines are highly expressed within the osteocyte secretome,
291 mechanically regulated, and are known to interact with one another. Interestingly, studies

292 have shown that CXCL1 and CXCL2 together play a key role in inflammatory processes [22]
293 and CXCL1 is over expressed in prostate and breast cancer cells where it has been shown to
294 significantly enhance cellular migration [23]. While neither cytokine elicited a proliferative
295 response in breast cancer cells, both CXCL1 and CXCL2 induced a significant increase in
296 migration alone independently and this response was partially enhanced when both cytokines
297 were applied together. Breast cancer cells are known to highly express CXCR2 receptors,
298 which play a key role in angiogenesis and metastasis [24]. Both cytokines CXCL1 and
299 CXCL2 bind to CXCR2 receptor [25], hence we believe it is due to competitive binding of
300 these cytokines to the CXCR2 receptor, we didn't observe a significant increase in breast
301 cancer migration when these cytokines were added together.

302 In conclusion, our findings demonstrate that osteocytes subjected to physiologically relevant
303 biophysical conditions represent a source of factors that could mediate breast cancer cell
304 metastasis to bone. Furthermore, we identified a range of mechanically regulated osteocyte
305 derived secreted factors which may influence cancer cell behaviour and specifically
306 demonstrated that CXCL1 and CXCL2 are potent mediators of cancer cell migration. These
307 factors represent potential future therapeutic targets to treat breast cancer patients who are
308 prone to develop bone metastasis.

309 **Competing interests**

310 The authors declare no conflict of interest with the contents of this article.

311 **Data availability statement**

312 The data that support the findings of this study are available from the corresponding author
313 upon reasonable request.

314 **Funding**

315 The study was supported by an Irish Research Council Government of Ireland Postgraduate
316 Scholarship (GOIPG/2014/1378/; to A.D.) and a European Research Council (ERC) Starting
317 Grant (336882) and Science Foundation Ireland Frontiers for the Future Project grant
318 (19/FFP/6533) to D.A.H.

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