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1 **MI192 induced epigenetic reprogramming enhances the therapeutic efficacy of human bone**
2 **marrows stromal cells for bone regeneration**

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16 **Abstract**

17 Human bone marrow stromal cells (hBMSCs) have been extensively utilised for bone tissue
18 engineering applications. However, they are associated with limitations that hinder their clinical utility
19 for bone regeneration. Cell fate can be modulated via altering their epigenetic functionality. Inhibiting
20 histone deacetylase (HDAC) enzymes have been reported to promote osteogenic differentiation, with
21 HDAC3 activity shown to be causatively associated with osteogenesis. Therefore, this study aimed to
22 investigate the potential of using an HDAC2 & 3 selective inhibitor - MI192 to induce epigenetic
23 reprogramming of hBMSCs and enhance its therapeutic efficacy for bone formation. Treatment with
24 MI192 caused a time-dose dependant reduction in hBMSCs viability. MI192 was also found to
25 substantially alter hBMSCs epigenetic function through reduced HDAC activity and increased histone
26 acetylation. hBMSCs were pre-treated with MI192 (50 µM) for 48 hrs prior to osteogenic induction.
27 MI192 pre-treatment significantly upregulated osteoblast-related gene/protein expression (Runx2,
28 ALP, Col1a and OCN) and enhanced alkaline phosphatase specific activity (ALPSA) (1.43-fold) ($P \leq$
29 0.001). Moreover, MI192 substantially increased hBMSCs extracellular matrix calcium deposition (1.4-
30 fold) ($P \leq 0.001$) and mineralisation when compared to the untreated control. In 3D microtissue
31 culture, MI192 significantly promoted hBMSCs osteoblast-related gene expression and ALPSA (> 2.41-
32 fold) ($P \leq 0.001$). Importantly, MI192 substantially enhanced extracellular matrix deposition (ALP,
33 Col1a, OCN) and mineralisation (1.67-fold) ($P \leq 0.001$) within the bioassembled-microtissue (BMT)
34 construct. Following 8-week intraperitoneal implantation within nude mice, MI192 treated hBMSCs
35 exhibited enhanced extracellular matrix deposition and mineralisation (2.39-fold) ($P \leq 0.001$) within
36 the BMT when compared to the untreated BMT construct. Taken together, these results demonstrate
37 that MI192 effectively altered hBMSCs epigenetic functionality and is capable of promoting hBMSCs
38 osteogenic differentiation *in vitro* and *in vivo*, indicating the potential of using epigenetic
39 reprogramming to enhance the therapeutic efficacy of hBMSCs for bone augmentation strategies.

40 **Keywords:** HDAC inhibitor, histone deacetylase, osteogenic differentiation, MI192, bone marrow stromal cells,
41 bone tissue engineering, epigenetics, additive manufacturing

42 Introduction

43 There is a tremendous need for clinically relevant bone tissue for the treatment of critical-sized bone
44 defects caused by numerous situations such as traumatic injury and age-associated disorders,
45 including osteoporosis [1]. This clinical need is expected to increase in the future due to the growing
46 ageing population. Current therapies such as autografts are associated with drawbacks such as their
47 limited availability and donor site morbidity [2, 3]. As such, there is great precedence for new
48 approaches to regenerate damaged/lost bone. Hence, extensive research has been conducted within
49 the tissue engineering field to meet the rising demand for bone tissue. For cell-based therapies,
50 controlling mesenchymal stromal cells (MSCs) osteogenic differentiation is a key factor for the
51 development of functional engineered bone [4]. Although extensively utilised, there has been limited
52 clinical success with the use of MSCs for bone repair due to their low procurement yield, inherent
53 heterogeneity, and the need for extensive *in vitro* expansion [5]. While methods such as gene therapy
54 have been used to enhance MSCs clinical efficacy, this approach is cost-intensive and poses a risk of
55 tumourigenesis [6].

56 It has become increasingly apparent that epigenetics plays a significant role in regulating cell fate.
57 Specifically, researchers have demonstrated that post-translational modifications such as the process
58 of acetylation result in alterations in the chromatin structure, regulated by two histone-modifying
59 enzymes [7]. Histone acetyltransferase (HAT) is responsible for acetylation, resulting in the opening of
60 the chromatin structure and enhanced transcriptional activity, while deacetylation activity from
61 histone deacetylase (HDAC) causes chromatin condensation leading to transcriptional repression [7-
62 9]. Several studies have induced hyperacetylation through the use of HDAC inhibitors (HDACis) which
63 has been reported to enhance MSCs osteogenic capacity [10-12]. Although epigenetic approaches
64 have shown promise, non-selective panHDACis have been primarily utilised, which inhibits a broad
65 range of HDAC isoforms. This may result in off-target effects such as inducing apoptosis and inhibiting
66 the cells' differentiation efficacy [13]. Hence, there is great precedence to shift towards a selective
67 approach. Studies have reported that HDAC3 is causatively associated with osteogenic differentiation
68 due to its role in repressing the activity of the osteogenic transcription factor, Runx2 [14, 15]. MI192,
69 an HDAC2 & 3 selective inhibitor, has demonstrated its efficacy in the fields of rheumatoid arthritis,
70 leukaemia and neuroregeneration research [16-18]. The increased selectivity in which MI192 targets
71 HDAC3 may provide a novel approach to enhance MSCs efficacy for bone formation. Recently, it has
72 been reported that MI192 significantly promoted the mineralisation capacity of human dental pulp
73 stromal cells (hDPSCs) in 2D *in vitro* culture[19], indicating the efficacy of this engineering approach
74 for bone augmentation strategies.

75 While HDACis efficacy in promoting MSCs differentiation has been reported, these investigations have
76 primarily been conducted in 2D *in vitro* culture. 2D culture limits the cell-cell and cell-extracellular
77 matrix interactions, which do not replicate *in-situ* conditions mechanically or biologically [20, 21].
78 Several studies have demonstrated the increased osteoinduction of MSCs when cultured in 3D
79 compared to conventional 2D culture [22, 23]. Moreover, it has been reported that cells exhibit
80 altered receptiveness to bioactive signals when cultured in 3D [21]. Hence, there is a tremendous need
81 to investigate the osteoinductive efficacy of selective HDACis in more physiological 3D culture
82 environments. Studies have shown that spheroid culture (microtissues) enhances MSCs mineralisation
83 [24, 25], due to facilitating cell-cell/cell-extracellular matrix interactions [22]. Although the use of
84 microtissues has shown promise for bone repair [22], their inherent lack of mechanical strength limits

85 their clinical application for load-bearing tissues. Advances in additive manufacturing and
86 biofabrication have allowed for increasing control for engineering functional tissue [26]. In particular,
87 modular assembly approaches where cellular components are combined with structural scaffolds has
88 created functional constructs that possess huge potential for bone tissue engineering. An example of
89 this modular assembly strategy is the bioassembled-microtissue (BMT) construct [27]. This model
90 consists of assembling pre-cultured microtissues with a 3D printed porous scaffold, therefore
91 harnessing the advantages of spheroid culture for bone formation, while the 3D printed scaffold
92 provides both spatial control of microtissues and structural support for load-bearing defects [27, 28].
93 Hence, the BMT model offers a platform to assess the efficacy of MI192 in stimulating bone-like tissue
94 formation in a more physiologically relevant model.

95 Therefore, this study aimed to examine the effects of MI192 epigenetic reprogramming on the
96 behaviour and osteogenic differentiation of human bone marrow stromal cells (hBMSCs) in 2D and 3D
97 *in vitro* and *in vivo*. The effects of MI192 on hBMSCs viability and epigenetic functionality was
98 evaluated. MI192 optimum treatment regimen (50 μ M for 48 hours) was determined to effectively
99 promote hBMSCs osteogenic differentiation and mineralisation capacity. Within the BMT construct,
100 the efficacy of MI192 treatment on stimulating hBMSCs bone formation was evaluated *in vitro* and *in*
101 *vivo*.

102 **Materials and Methods**

103 **Cell culture and reagents**

104 hBMSCs were purchased from Lonza (Lonza, UK) (n = 2; 33 and 41 years of age), and the trilineage
105 potential verified (Supplementary figure 1). Cells were maintained in basal media, containing alpha
106 modified minimum essential medium (α -MEM, Lonza, UK) supplemented with 10% fetal calf serum
107 (FCS, Sigma-Aldrich, UK) and 100 units/ml penicillin with 100 μ g/ml streptomycin (Sigma-Aldrich, UK),
108 at 37°C in 5% CO₂ until 80% confluent. The cells at passage 4 were used for this study. 100 mM MI192
109 (Sigma-Aldrich, UK) stock solution was prepared fresh in sterile-filtered dimethyl sulfoxide (DMSO,
110 Sigma-Aldrich, UK) and this stock was further added to the culture medium to make the desired
111 concentration. The DMSO percentage in the 50 μ M MI192 working solution was 0.05%, a percentage
112 reported to not induced cytotoxicity [29, 30].

113 **Cell viability and morphology assessment**

114 Cells (1×10^4 cells/well) were seeded within a 96-well plate and incubated in basal medium for 24
115 hours. The medium was replaced with fresh basal medium supplemented with/without MI192 (1, 10,
116 20, 50 μ M) and incubated for 24, 48 and 72 hours. AlamarBlue reagent (Thermo Scientific, UK) was
117 added to each well and incubated for 4 hours at 37°C. Fluorescence readings were acquired using a
118 Varioskan Flash Multimode Microplate Reader (Thermo Scientific, UK) at 540/590 nm wavelength. For
119 morphological assessment, cells were seeded (1×10^5 cells/well) in a 24-well plate and incubated for
120 24 hours, after which the same treatment regime was utilised. Cell morphology was evaluated using
121 a Zeiss Axio Vert.A1 (Carl Zeiss, Oberkochen, Germany).

122

123

124 **DNA quantification**

125 DNA quantification was determined by Quant-iT PicoGreen DNA assay (Invitrogen, Life Technologies,
126 UK). Briefly, 10 µl of cell lysate (in 0.1% Triton™ X-100) was added to 90 µl of TE (10 mM Tris-HCl, 1
127 mM EDTA) buffer into a 96-well plate (Corning, UK). 100 µl of PicoGreen reagent was added to all
128 samples and incubated for 5 minutes. The fluorescence was then measured in a Varioskan Flash
129 Multimode Microplate Reader at 480/520 nm wavelength.

130 **HDAC activity and H3K9 histone acetylation**

131 Cells were cultured in 96-well plates (1 x 10⁴ cells/well) in basal medium for 24 hours. The medium
132 was replaced with fresh basal medium supplemented with/without MI192 (1, 5, 10, 20, 50 µM). At 24
133 and 48 hours, the medium was replaced with 100 µl of reaction mix (*in situ* HDAC activity fluorometric
134 assay kit: Biovision, UK) and incubated for 3 hours at 37°C. 100 µl of lysine developer was added then
135 incubated for a further 30 minutes at 37°C. The fluorescence was measured in a Varioskan Flash
136 Multimode Microplate Reader (Thermo Scientific, UK) at 368/442 nm wavelength. HDAC activity was
137 normalised with total DNA content.

138 EpiQuik™ *In Situ* Histone H3-K9 Acetylation Assay Kit (Epigentek, USA) was used for detecting H3K9
139 acetylation according to the manufacturer's protocol. The absorbance was read in a Varioskan Flash
140 Multimode Microplate Reader (Thermo Scientific, UK) at 450 nm. Histone acetylation was normalised
141 with the total DNA content.

142 **Osteogenic induction culture**

143 Untreated or MI192 pre-treated cells were cultured in osteogenic medium, consisting of basal medium
144 supplemented with 50 µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 10 mM β-
145 glycerol phosphate and 100 nM dexamethasone (Sigma-Aldrich, UK). The medium was changed every
146 3 days.

147 **Alkaline phosphatase specific activity (ALPSA) quantification assay**

148 ALPSA was determined using the 4-nitrophenyl colourimetric phosphate liquid assay (pNPP, Sigma-
149 Aldrich, UK). 90 µl of pNPP was added to 10 µl of cell lysate and incubated for 60 minutes at 37°C. The
150 absorbance at 405 nm was read on a Varioskan Flash Multimode Microplate Reader (Thermo
151 Scientific, UK). ALP activity was normalised with total DNA content.

152 **Quantitative RT-qPCR analysis**

153 RNA was extracted using the RNase mini kit (Qiagen, UK), and then 200 ng of RNA was transcribed to
154 cDNA using the high capacity RNA to cDNA Kit (Applied Biosystems, UK) following the manufacturer's
155 instructions. Thereafter, the cDNA was amplified using TaqMan primers (supplementary table 1) in a
156 20 µl reaction within a 96-well PCR plate (Starlab, UK). Amplification occurred within a LightCycler 480
157 real-time qPCR system (Roche, UK). For each sample, the cycle threshold (Ct) value was acquired, and
158 the comparative Ct method (2^{-ΔΔCt}) was utilised to quantify the gene expression levels relative to the
159 housekeeping gene GAPDH.

160 **In-Cell Western (ICW) assay**

161 Following fixation in 10% (v/v) neutral buffered formalin (NBF, Cellpath, UK), cells were permeabilised
162 with 0.1% Triton™ X-100. The samples were incubated overnight at 4°C with primary antibodies
163 (supplementary table 2) against osteocalcin (1:400), Runx2 (1:500), alkaline phosphatase (1:300) and
164 collagen type I (1:200) (Abcam, UK) in Odyssey® buffer. Samples were incubated with the IRDye
165 800CW secondary antibody (1:800) with the CellTag™ 700 stains (1:500; Li-Cor Biosciences, UK) in the
166 Odyssey blocking buffer (Li-Cor Biosciences, UK) for 1 hour, prior to scanning on the Odyssey SA
167 Imaging System (Li-Cor Biosciences, UK) using both 700 and 800 nm detection channels. Quantitative
168 analysis was performed using the Image Studio (Li-Cor Biosciences: version 5).

169 **Scaffold fabrication**

170 Bio-degradable poly (ethylene glycol)-terephthalate-poly (butylene terephthalate) (PEGT/PBT) block
171 copolymers were defined by a PEG molecular weight of 300 g/mol and a PEGT:PBT weight per cent
172 (wt%) ratio of 55:45 as previously described [27]. Briefly, scaffolds were fabricated in a layer by layer
173 process using 3D BioPlotter (EnvisionTEC, Germany) with a 1 mm fibre spacing. Fibres were oriented
174 in a 0 - 90° pattern in a bi-layered fashion. Scaffolds with dimensions of approximately 3.3 × 2.1 × 2.1
175 mm³ were sterilised in 70% ethanol overnight and washed thoroughly with PBS prior to use.

176 **Bioassembled-microtissue culture**

177 250 µl hBMSCs suspension (untreated or 50 µM MI192 treated for 48 hours) (2.5×10^5 cells) was added
178 into a V-bottom 96-well plate (Greiner Bio-One, UK) and centrifuged at 300 g for 4 minutes. The
179 osteogenic medium was changed every two days for up to one week to allow microtissue formation.
180 Then, the microtissues were transferred into the pores of the 3D printed scaffolds (1 pellet per pore)
181 to form the BMT construct [27]. A total of 8 pellets were incorporated into the scaffold in a bi-layered
182 configuration (4 microtissues per layer). Constructs were cultured in osteogenic medium for 6 weeks.

183 **Histological and immunohistochemical analysis**

184 Following fixation in 10% neutral buffered formalin (NBF) (Cellpath, UK), samples were embedded in
185 paraffin wax and sectioned (4 µm) using a microtome (Leica, UK). Sections were stained with
186 Haematoxylin and Eosin (H&E) (Sigma-Aldrich, UK), Picosirius red (Abcam, UK)/Alcian blue (Sigma-
187 Aldrich, UK), using standard histological methods. Calcium deposits were identified with Alizarin red
188 (2% pH 4.3) for 15 mins. For semi-quantification, Alizarin red-stained samples were de-stained with
189 10% cetylpyridinium chloride (Sigma-Aldrich, UK) for 1 hour and then absorbances were read at 550
190 nm using the Varioskan Flash Multimode Microplate Reader. Mineralisation was assessed using the
191 Von Kossa Staining Kit (Atom Scientific, UK) following the manufacturer's instructions. The mean
192 mineral nodule percentage coverage was quantified using ImageJ software.

193 Extracellular matrix deposition was assessed using the EnVision™ Detection Systems Peroxidase/DAB,
194 Rabbit/Mouse (Dako, UK). Briefly, sections were placed in a PBS bath before incubation with 'Dual
195 Endogenous Enzyme Block' from the EnVision™ kit for 10 mins. Sections were washed in PBS for 5
196 mins prior to blocking by 20% Normal goat serum (Dako, UK) in PBS for 30 mins. Then, the primary
197 antibodies (supplementary table 2) were added to samples at the desired concentration in 1% BSA
198 (Sigma-Aldrich, UK) in PBS and incubated overnight at 4 °C. The next day, sections were washed in PBS

199 for 10 mins, before the addition of the secondary antibody HRP goat anti-rabbit to sections for 30
200 mins. After PBS wash for 5 mins, the Dako DAB developing solution was added to sections for 10 mins
201 before immersion in Harris Haematoxylin for 20 seconds, which was cleared in a running water bath.
202 Slides were then dehydrated, mounted and visualise under the microscope (Olympus BX50).

203 ***In vivo* implantation of BMTs**

204 All procedures for the animal studies were conducted under the approval of the University of Leeds
205 Ethics Committee and under the UK Home Office project license (PPL: 70/8549). Untreated and MI192
206 treated BMTs were fabricated as described above. The constructs (n=5) were sealed within diffusion
207 chambers (Sigma-Aldrich, UK), which was then implanted within the intraperitoneal cavity of
208 immunocompromised CD1 nude mice (30 g, 8-week old male). After 8 weeks, the chambers were
209 retrieved, and samples were fixed in 10% NBF prior to histological and immunohistochemical analysis.

210 **Data analysis**

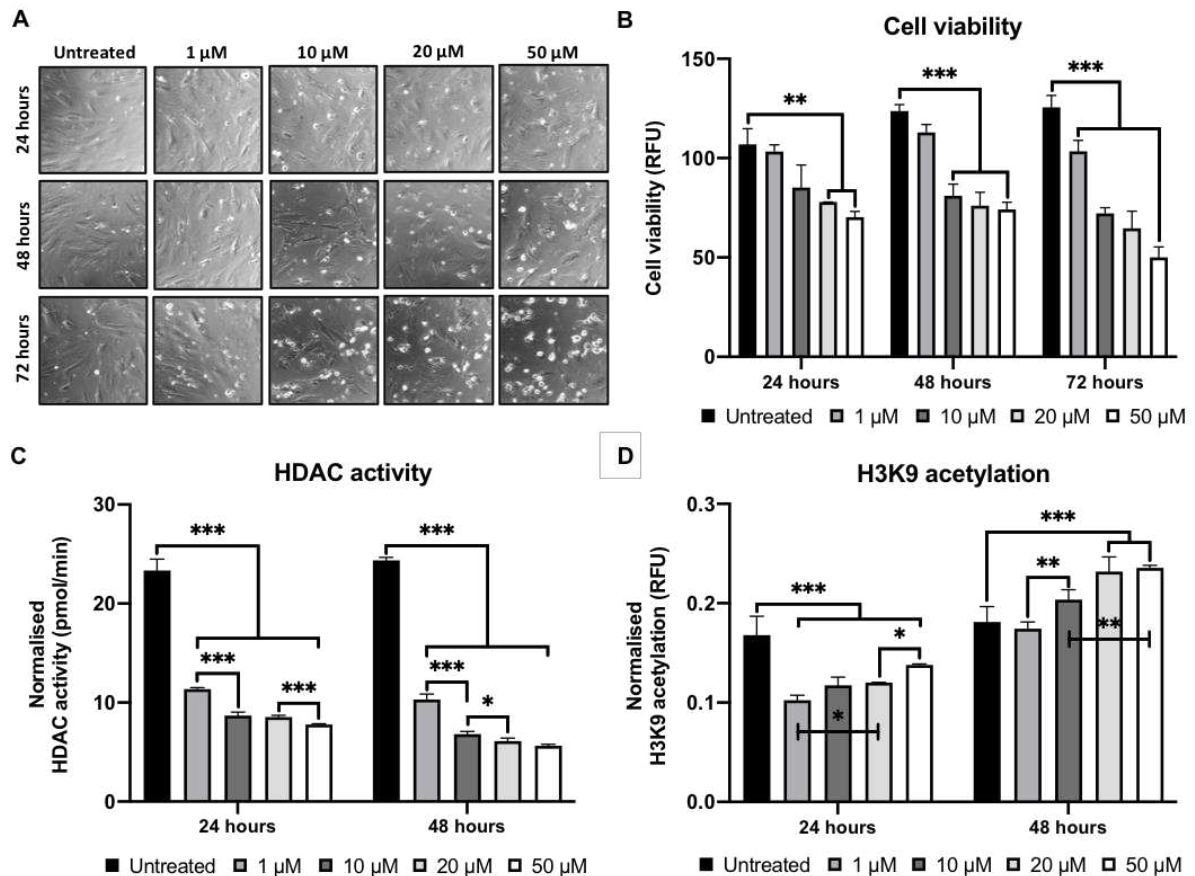
211 For all data presented, experiments were repeated at least 3 times. All statistical analysis was
212 undertaken using ANOVA multiple comparisons test with Tukey modification with IBM SPSS software
213 (IBM Analytics, version 21). P values equal to or lower than 0.05 was considered significant. *P ≤ 0.05,
214 **P ≤ 0.01 and ***P ≤ 0.001.

215 **Results**

216 **MI192 altered hBMSCs epigenetic functionality in 2D *in vitro***

217 Following 24 hours of culture, cells in the basal medium exhibited a fibroblast-like morphology (Fig
218 1A). Treatment with low concentrations of MI192 (≤ 10 μM) exhibited a similar fibroblast-like
219 morphology as the control, while treatment with higher concentrations (20 and 50 μM) resulted in a
220 dose-dependent reduction in cell density whilst also exhibiting a condensed morphology. At 48 and
221 72 hours, a more apparent dose-dependent reduction in cell density was observed with an increasing
222 quantity of floating cells in the high MI192 concentration groups. The AlamarBlue assay showed that
223 MI192 caused a time and dose-dependent reduction in hBMSCs metabolic activity when compared to
224 that of the untreated control (Fig 1B). MI192 concentrations of ≥ 20 μM, ≥ 10 μM and ≥ 1 μM
225 significantly reduced hBMSCs viability at 24, 48 and 72 hours (P ≤ 0.01, 0.001, 0.001, respectively).

226



227
 228 **Figure 1 - The effects of MI192 on hBMSC viability and epigenetic functionality.** MI192 caused a time-dose
 229 dependant effect on hBMSCs A) morphology (magnification $\times 100$), B) metabolic activity, C) HDAC activity and
 230 D) H3K9 histone acetylation levels. Data are represented as mean \pm SD ($n = 3$). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq$
 231 0.001.

232 HDAC activity assay showed that MI192 treatment (1 - 50 μ M) led to a significant time- and dose-
 233 dependent reduction in HDAC activity compared to the untreated control (Fig 1C) ($P \leq 0.001$). At 24
 234 hours, there was a significant reduction in HDAC activity of the 10 μ M vs 1 μ M treated group, and 50
 235 μ M vs 20 μ M group ($P \leq 0.001$). Similarly, there were significant reductions in HDAC activity in 10 μ M
 236 vs 1 μ M group ($P \leq 0.001$), and 20 μ M vs 10 μ M group ($P \leq 0.05$) at 48 hours.

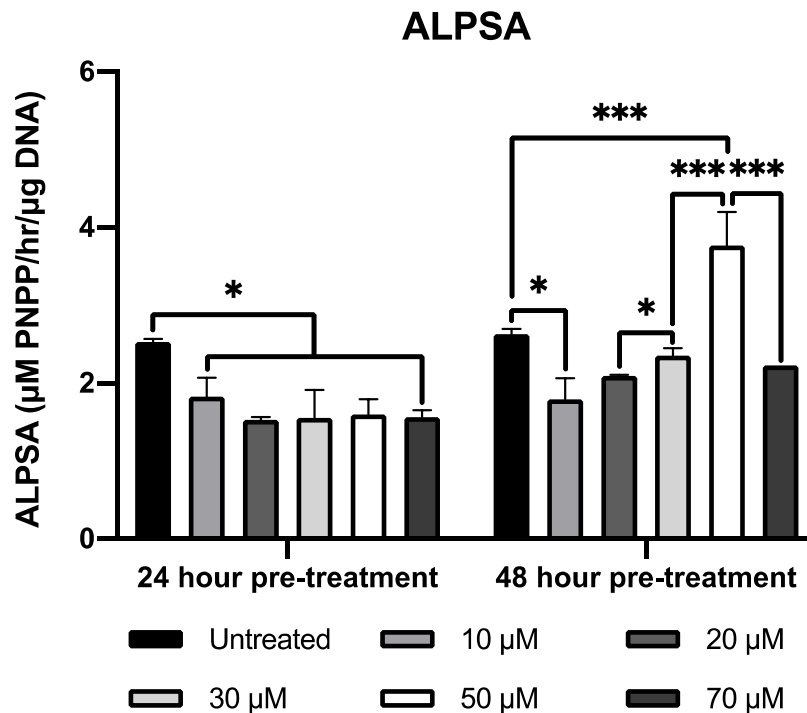
237 All test groups treated with MI192 for 24 hours caused a significant reduction in the histone
 238 acetylation levels when compared to the untreated cells ($P \leq 0.001$), with a dose-dependent increase
 239 in acetylation observed within the MI192 treated groups. The 20 μ M and 50 μ M MI192 treated cells
 240 exhibited a significant increase in acetylation levels compared to the 1 μ M and 20 μ M treated groups
 241 respectively ($P \leq 0.05$). At 48 hours, there was a dose-dependent increase in histone acetylation, with
 242 20 and 50 μ M groups exhibiting a significant increase in acetylation levels compared to the untreated
 243 cells ($P \leq 0.001$). Interestingly, there was a significant enhancement in acetylation within the 20 μ M vs
 244 10 μ M group ($P \leq 0.05$), and 10 μ M vs 1 μ M group ($P \leq 0.01$) (Fig 1D).

245

246

247 **MI192 promoted hBMSCs osteogenic differentiation and mineralisation in 2D *in vitro***

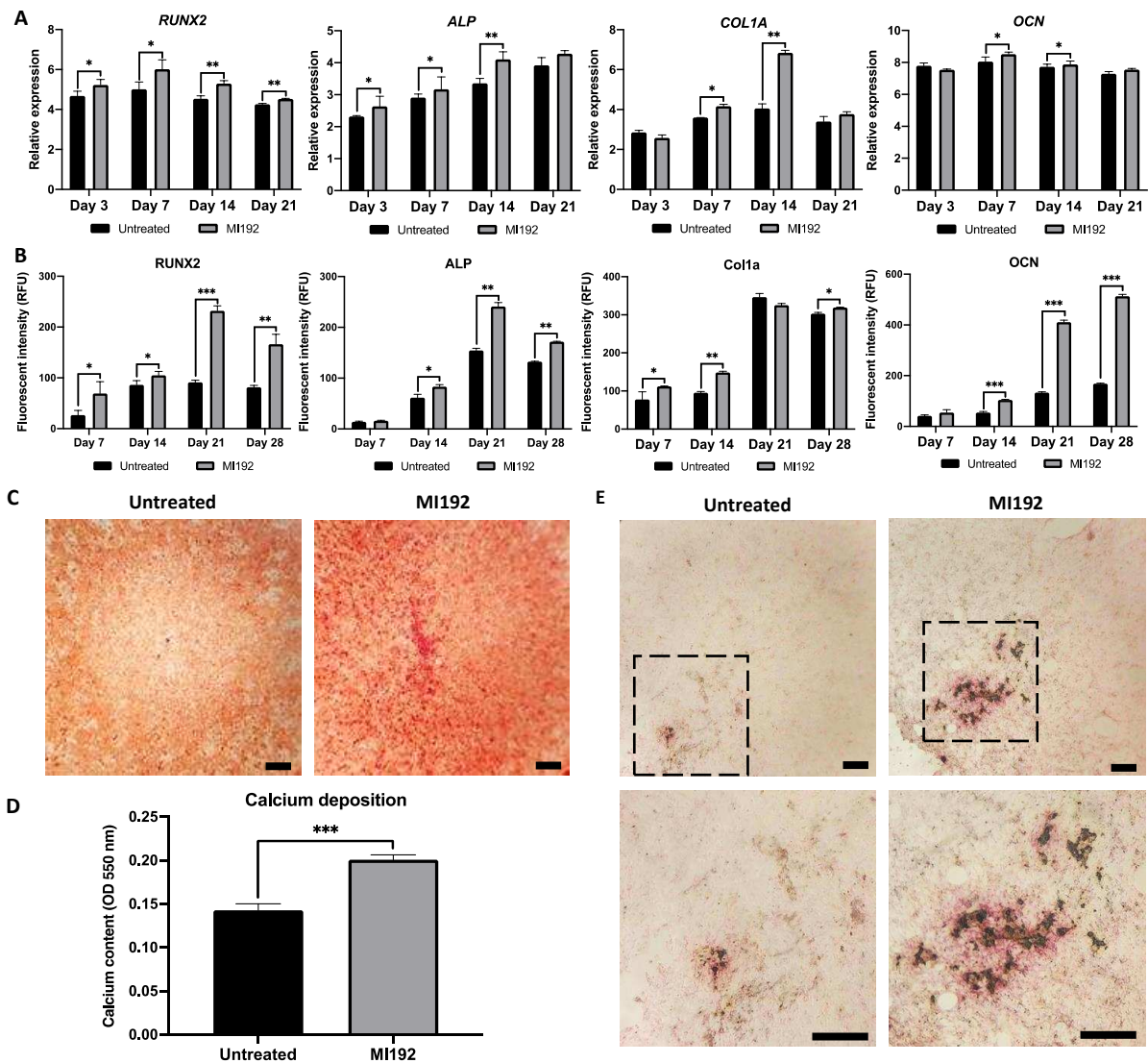
248 The influence of MI192 pre-treatment for 24 or 48 hours on hBMSCs osteogenic differentiation after
 249 14 days osteogenic culture was evaluated by assessing ALPSA (Fig 2). Cells pre-treated with MI192 for
 250 24 hours exhibited significantly reduced ALPSA when compared to untreated hBMSCs cultured in
 251 osteogenic medium (≥ 1.38 -fold) ($P \leq 0.05$). Following pre-treatment with MI192 for 48 hours, ALPSA
 252 results showed that 50 μ M MI192 significantly enhanced hBMSCs ALPSA (>1.43 -fold) when compared
 253 with other MI192 doses and the untreated control following 14 days osteoinduction ($P \leq 0.001$). Thus,
 254 50 μ M MI192 pre-treatment for 48 hours was used for subsequent experiments.



255

256 **Figure 2 - Effect of MI192 pre-treatment on hBMSCs ALPSA.** Cells were pre-treated with/without MI192 for 24
 257 or 48 hours prior to culture in osteogenic medium for 14 days. Data are represented as mean \pm SD (n=3). * $P \leq$
 258 0.05 and *** $P \leq 0.001$.

259 After 50 μ M MI192 pre-treatment and 3 days of osteogenic culture, RT-qPCR (Fig 3A) confirmed that
 260 the mRNA levels of the early osteogenic markers (*RUNX2* and *ALP*) were significantly upregulated in
 261 the MI192 pre-treated cells ($P \leq 0.05$), while the mRNA levels of later markers (*COL1A* and *OCN*) were
 262 slightly down-regulated compared to untreated cells, however not significantly ($P > 0.05$) (Fig 3A). On
 263 day 7, the mRNA expression of all osteoblast-related genes was significantly upregulated in MI192
 264 pre-treated cells compared to that in the untreated control ($P \leq 0.05$). On day 14, a similar significant
 265 increase in gene expression was observed in the MI192 pre-treated cells ($P \leq 0.05 - 0.01$). On day 21,
 266 the mRNA expression remained upregulated in the MI192 pre-treated cells, with the expression of
 267 *RUNX2* significantly increased compared to the untreated control ($P \leq 0.01$).



268

269 **Figure 3 - The effects of MI192 on hBMSCs osteogenic differentiation and mineralisation.** A) mRNA expression
 270 levels of *RUNX2*, *ALP*, *COL1A* and *OCN* were measured in untreated/MI192 pre-treated (50 μ M for 48 hours)
 271 hBMSCs during osteoinductive culture. B) Protein expression levels of *RUNX2*, *ALP*, *Col1a* and *OCN* in
 272 untreated/MI192 pre-treated (50 μ M for 48 hours) hBMSCs analysed by ICW. C) Alizarin Red staining for calcium
 273 deposition (x100), D) Semi-quantification of Alizarin Red staining, and E) Von Kossa staining of hBMSCs
 274 untreated/MI192 pre-treated (50 μ M for 48 hours) hBMSCs after 28 days of osteoinductive culture. Black
 275 squares highlight the magnified area in the panel below. Scale bars = 100 μ m. Data are represented as mean \pm
 276 SD (n=3). *P \leq 0.05, **P \leq 0.01 and ***P \leq 0.001.

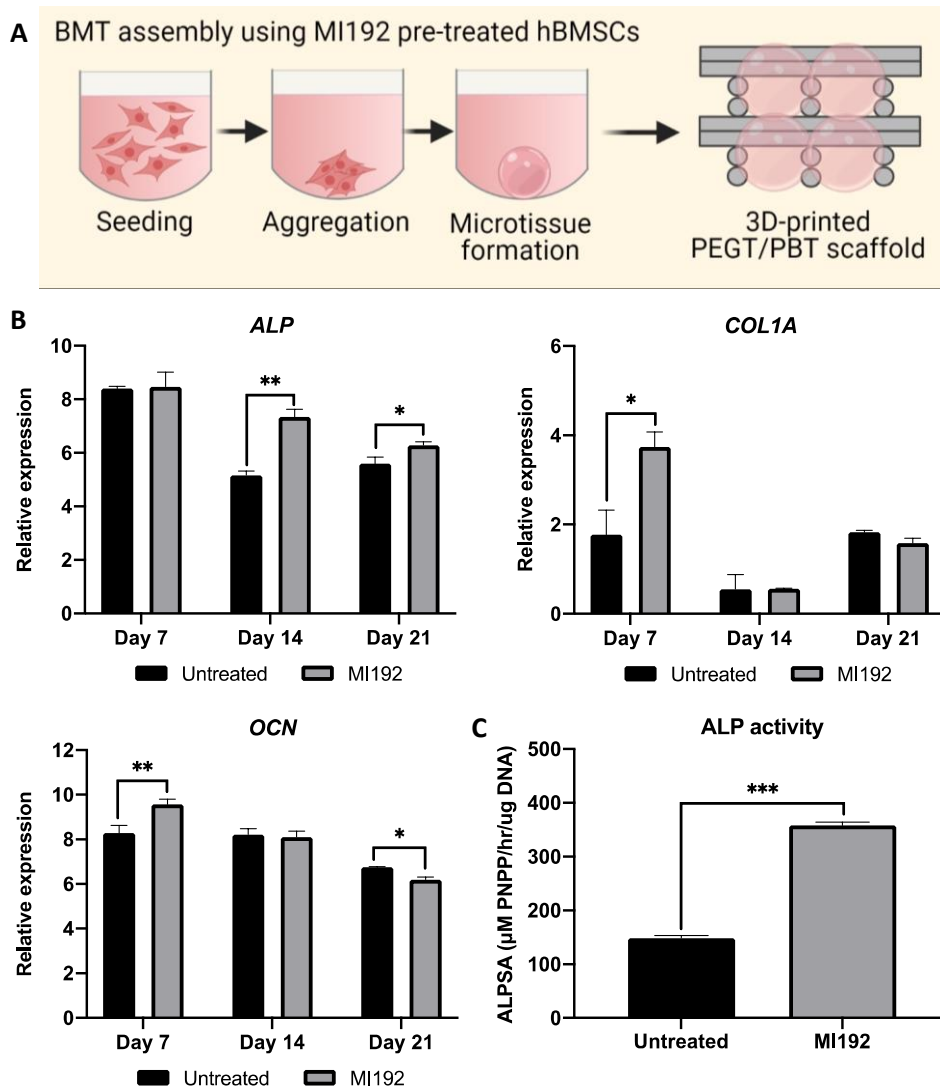
277 ICW was used to examine the effects of 50 μ M MI192 pre-treatment on the intracellular expression
 278 of osteoblast-related proteins (*Runx2*, *ALP*, *Col1a* and *OCN*) (Fig 3B). On day 7, MI192 pre-treatment
 279 significantly enhanced hBMSCs protein expression levels for *Runx2* and *Col1a* compared to that of the
 280 control group (P \leq 0.05 - 0.01). However, there was no significant difference for *ALP*, and *OCN* protein
 281 expression between the MI192 treated and untreated cells (P > 0.05) (Fig 3B). On day 14, all markers
 282 assessed were significantly increased in the MI192 pre-treated cells compared to that of the control
 283 group (P \leq 0.05 - 0.001). A similar significant upregulation was observed at day 21 (P \leq 0.01 - 0.001),
 284 with *Col1a* expression levels similar to the untreated control (P > 0.05). On day 28, the expression of

285 all osteoblast-related proteins were significantly enhanced in the MI192 treated cells compared to the
286 untreated control ($P \leq 0.05 - 0.001$).

287 Alizarin red staining showed that the MI192 treated group substantially enhanced hBMSCs
288 extracellular matrix calcium deposition compared to the untreated control (Fig 3C). The semi-
289 quantitative analysis confirmed the MI192 pre-treated cells exhibited significantly enhanced calcium
290 deposition (1.4-fold) compared to the untreated group ($P \leq 0.001$) (Fig 3D). Von Kossa staining
291 indicated that there was a substantial increase in mineral nodule formation (black staining) in the
292 MI192 pre-treated group compared to the untreated control (Fig 3E). Additionally, enhanced Van
293 Gieson's staining for collagen deposition (pink staining) was observed in the MI192 pre-treated group,
294 particularly in close proximity to the mineral nodules.

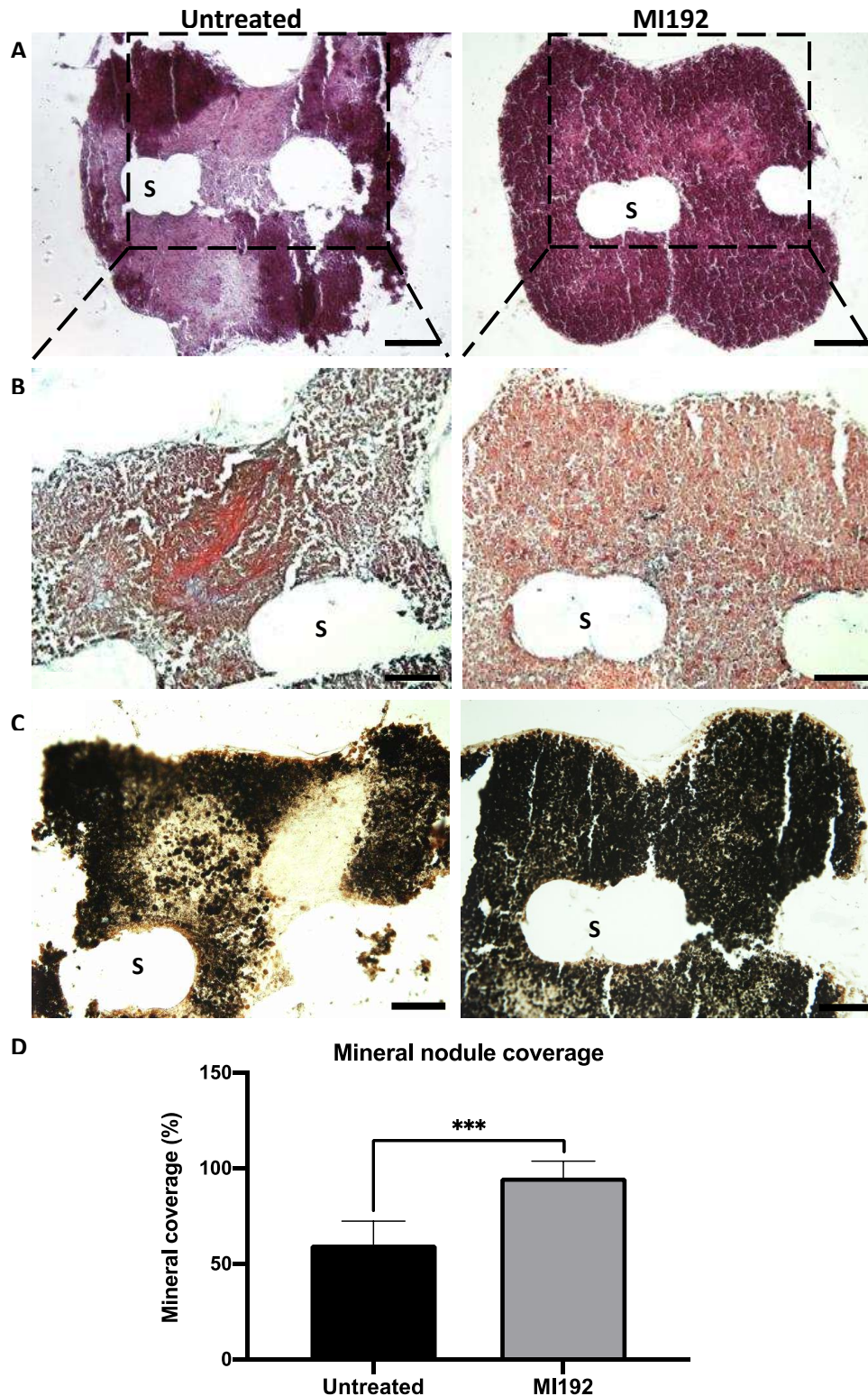
295 **MI192 enhanced hBMSCs osteogenic differentiation and mineralisation in 3D BMT *in vitro***

296 Following pre-treatment with/without 50 μ M MI192 for 48 hours, hBMSCs were cultured as
297 microtissues in osteogenic conditions for up to 21 days, and the mRNA levels of osteoblast-related
298 genes were assessed (Fig 4A). On day 7, the expression of *Col1a* and *OCN* were significantly
299 upregulated in the MI192 pre-treated cells when compared to that in the untreated group ($P \leq 0.05 -$
300 0.01). On day 14, the expression of *ALP* was significantly enhanced in the MI192 pre-treated group (P
301 ≤ 0.01). The mRNA expression levels of *ALP* were significantly increased in the MI192 pre-treated cells
302 when compared to that in the untreated group at day 21 ($P \leq 0.05$), while *OCN* was significantly down-
303 regulated in the MI192 pre-treated group ($P \leq 0.05$). Following 2 weeks in osteogenic conditions,
304 MI192 pre-treatment significantly enhanced hBMSCs ALPSA when compared to that of the untreated
305 BMT construct (> 2.41 -fold) ($P \leq 0.001$) (Fig 4B).



306
 307 **Figure 4 - The effects of MI192 on hBMSCs osteogenic differentiation within the 3D BMT construct *in vitro*.** A)
 308 Schematic diagram represents the procedure of microtissues formation and the creation of the BMT construct
 309 (Created with BioRender.com). B) mRNA expression levels of *ALP*, *COL1A* and *OCN* were measured in
 310 untreated/MI192 pre-treated hBMSCs microtissues during osteoinductive culture. C) ALPSA of hBMSCs
 311 microtissues pre-treated with/without MI192, followed by 14 days in osteogenic conditions. Data are
 312 represented as mean \pm SD (n=3). *P \leq 0.05, **P \leq 0.01 and ***P \leq 0.001.

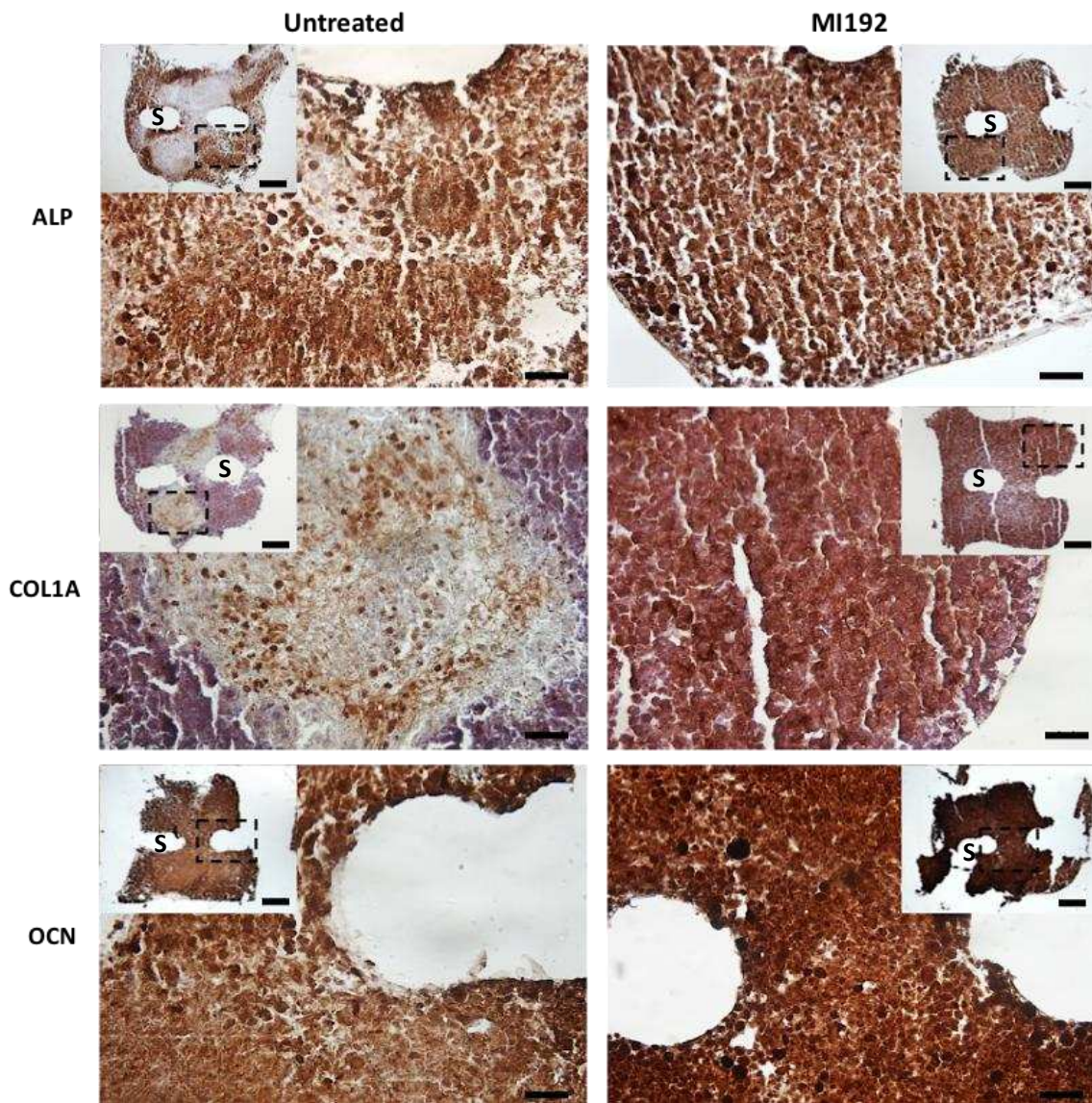
313 Histological analysis confirmed that the incorporated microtissues fused within the 3D printed scaffold
 314 after 6 weeks *in vitro* culture in osteogenic conditions (Fig 5A). The MI192 treated constructs exhibited
 315 enhanced uniformity in tissue formation when compared to the distribution observed within the
 316 untreated BMT. Moreover, the MI192 pre-treated group exhibited strong picosirius red staining for
 317 collagen deposition throughout the construct. However, collagen staining in the untreated BMT was
 318 observed in an aberrant manner (Fig 5B). Additionally, GAGs deposition highlighted by alcian blue
 319 staining was substantially increased in the untreated group compared to the MI192 construct (Fig 5B).
 320 Von Kossa staining showed that both groups exhibited positive staining for functional mineral nodules
 321 (black staining). However, the mineralisation within the MI192 pre-treated group was distributed
 322 more homogenously throughout the construct when compared to the untreated control (Fig 5C).
 323 Semi-quantitative analysis indicated a significant increase in mineral nodule coverage in the MI192
 324 pre-treated BMT constructs (1.67-fold) compared to the untreated control (Fig 5D) (P \leq 0.001).



325

326 **Figure 5 - MI192 promote hBMSCs *de novo* tissue formation in 3D BMT constructs after 6 weeks *in vitro***
 327 **culture.** A) H&E staining showing tissue formation within the BMT, B) Picosirius red/alcian blue staining
 328 identifying collagen/GAG depositions within the construct, C) Mineral nodule formation highlighted by Von
 329 Kossa staining, D) Percentage mineral nodule coverage on BMT samples. Scale bars = 200 (A) and 100 μ m (B, C),
 330 respectively. Data are represented as mean \pm SD (n=3). ***P \leq 0.001. 'S' denotes the spaces of '3D printed
 331 PEGT/PBT scaffolds', which were removed during histological processing.

332 Immunohistochemical analysis showed that both untreated and MI192 pre-treated BMT constructs
 333 exhibited positive staining for the osteoblast-related markers (ALP, Col1a and OCN), with enhanced
 334 protein deposition situated at the outer regions of individual microtissues and the periphery of the
 335 construct (Fig 6). The MI192 pre-treated BMTs displayed substantially increased deposition of ALP,
 336 Col1A and OCN when compared to the untreated construct. Moreover, protein expression was
 337 distributed more uniformly throughout the MI192 construct when compared to the untreated BMT.

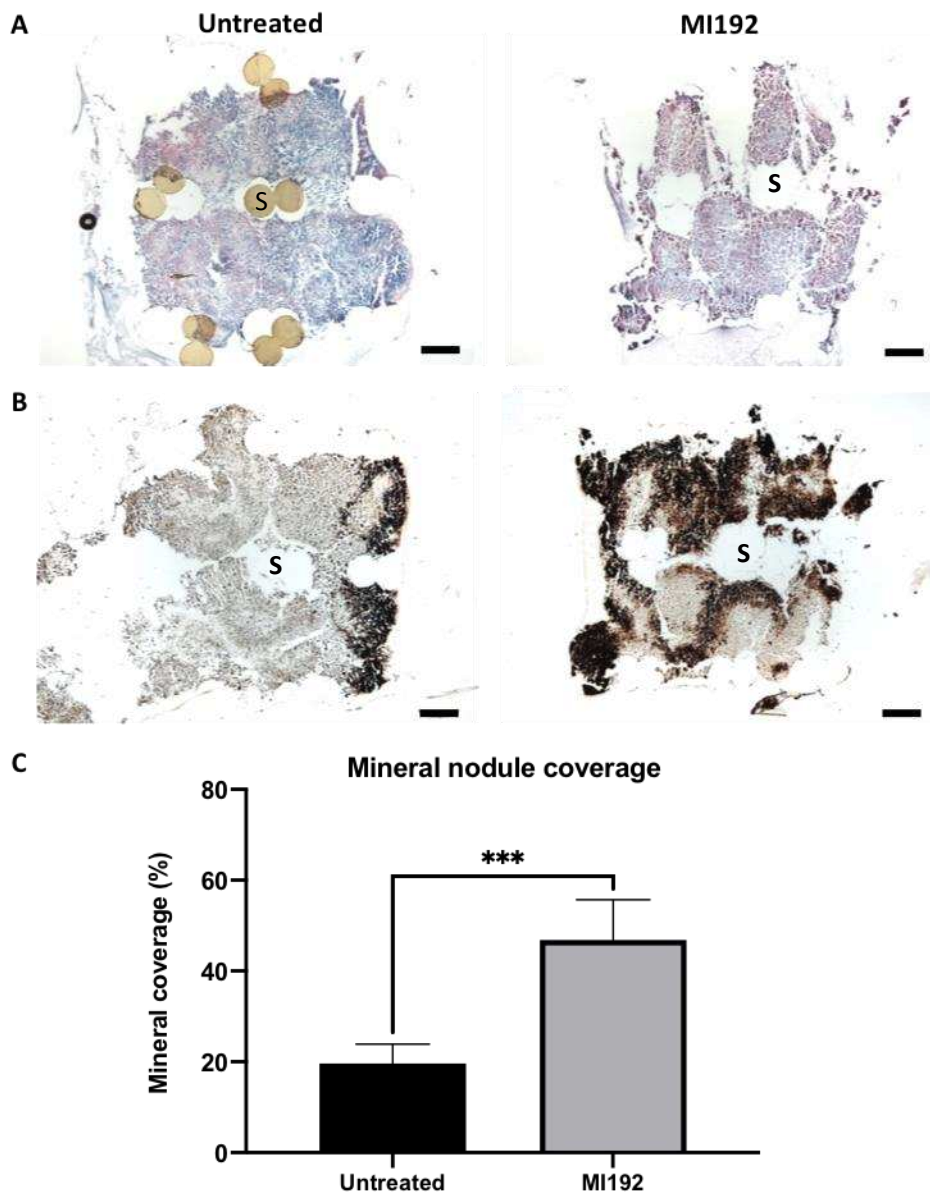


338
 339 **Figure 6 - Immunostaining of osteoblast-related proteins within MI192 pre-treated/untreated BMTs after 6**
 340 **weeks of osteogenic culture *in vitro*.** Positive immunohistochemical staining (brown), with a Harris
 341 haematoxylin counterstain (purple). Black dotted box highlight the magnified area. Scale bars = 200 and 50 μ m
 342 (low and high magnification, respectively). 'S' denotes the spaces of '3D printed PEGT/PBT scaffolds', which were
 343 removed during histological processing.

344 **MI192 enhanced hBMSCs bone-like tissue formation in 3D BMT *in vivo***

345 Following the assembly of untreated/MI192 pre-treated BMTs, the constructs were sealed within
 346 diffusion chambers, and these chambers containing BMT constructs were implanted intraperitoneally

347 within CD1 nude mice for 8 weeks. Histological analysis revealed the MI192 pre-treated BMTs
 348 exhibited increased intensity and uniform deposition of collagen when compared to the control,
 349 identified by picrosirius red analysis (Fig 7A). Moreover, the untreated constructs displayed enhanced
 350 accumulation of GAGs, where the distribution of collagens and GAGs were observed aberrantly within
 351 the BMT. Following Von Kossa staining, mineral nodules were observed throughout both BMT groups,
 352 primarily towards the outer regions of individual microtissues (Fig 7B). Mineral nodule formation was
 353 more isolated in the untreated BMT, located towards one face of the constructs. However, the MI192
 354 BMT exhibited substantially increased mineral depositions, distributed more homogenously through
 355 the construct. The semi-quantitative analysis confirmed a significantly greater mineral nodule
 356 coverage within the MI192 pre-treated BMT (2.39-fold) compared to the untreated control (Fig 7C) (P
 357 ≤ 0.001).

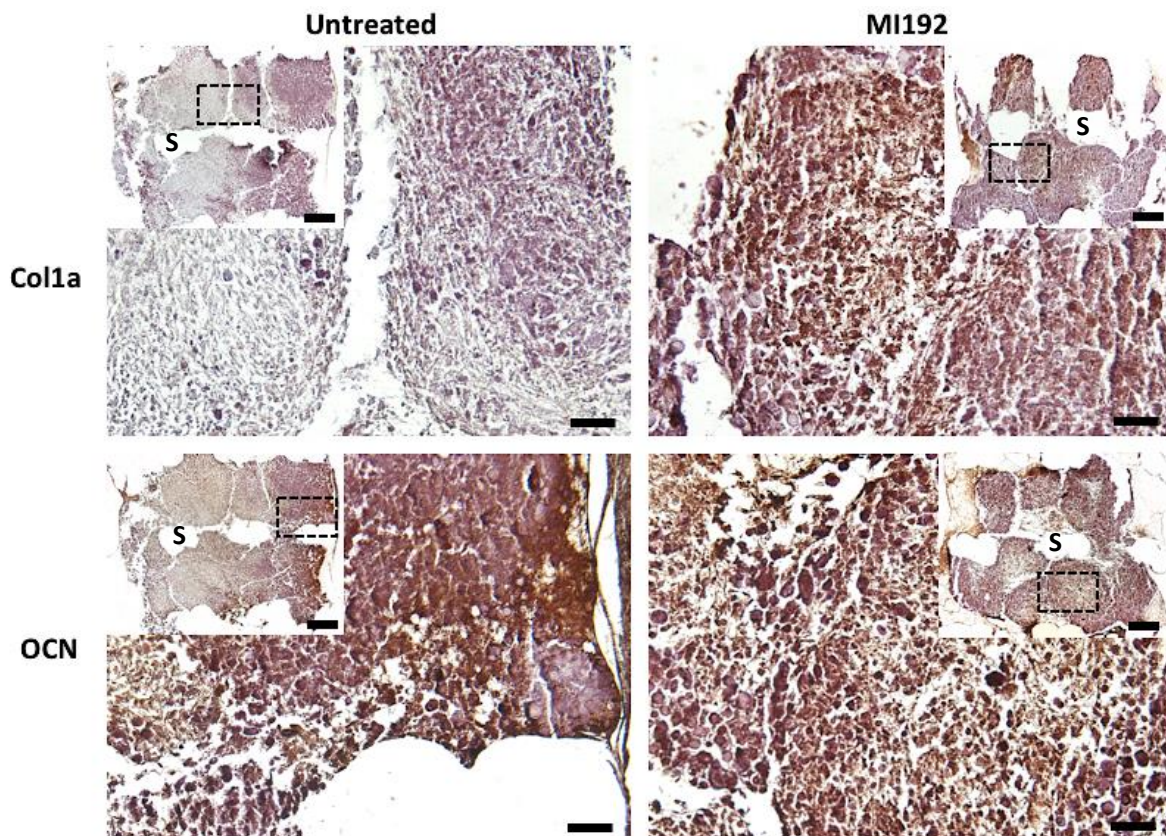


358

359 **Figure 7 - Histological analysis of MI192 pre-treated hBMSC BMT constructs after *in vivo* implantation.** A)
 360 Picrosirius red/Alcian blue staining for collagen/GAG depositions. (B) Von Kossa staining for mineral nodule
 361 formation. C) Percentage mineral nodule coverage on BMT samples. Scale bars = 200 μ m. Data are represented

362 as mean \pm SD (n=3). ***P \leq 0.001. 'S' denotes the '3D printed PEGT/PBT scaffolds' (brown colour), the white
363 spaces were due to the scaffolds removed during histological processing.

364 Immunohistochemical analysis showed that both untreated and MI192 treated BMT constructs
365 exhibited positive staining for the Col1a and OCN (Fig 8). There was substantially increased Col1a
366 expression within the MI192 BMT when compared to the untreated control. Both untreated and
367 MI192 BMT constructs exhibited strong OCN expression. However, protein deposition was more
368 uniformly expressed within the MI192 treated construct when compared to the untreated control.



369
370 **Figure 8 - Immunostaining of osteoblast-related proteins within MI192 pre-treated/untreated BMTs after *in***
371 ***vivo* implantation.** Positive immunohistochemical staining (brown), with a Harris haematoxylin counterstain
372 (purple). Black dotted box highlight the magnified area. Scale bars = 200 and 50 μ m (low and high magnification,
373 respectively). 'S' denotes the spaces of '3D printed PEGT/PBT scaffolds', which were removed during histological
374 processing.

375 Discussion

376 Reprogramming the epigenome has been shown to substantially affect key cellular functions without
377 altering the DNA sequence, therefore, possibly providing a safer alternative to controlling MSCs fate.
378 In particular, inhibiting HDAC enzymes has shown to promote MSCs osteogenic capacity [31, 32].
379 Studies have demonstrated that HDAC3 is intimately linked to osteogenic differentiation [14, 15].
380 Hence, this study aimed to determine the effects of the HDAC2 & 3 selective inhibitor - MI192 on
381 hBMSCs therapeutic efficacy for bone augmentation.

382 As MSCs are highly receptive to epigenetic changes [33, 34], the use of HDACis may provide a valuable
383 tool for controlling MSCs functionality for bone tissue engineering. Numerous studies have

384 demonstrated the ability of these epigenetic regulators to initiate cell death, essential to HDACi
385 success in cancer therapeutics [35, 36]. Due to the potential side-effects of this compound, the
386 influence of MI192 on hBMSCs viability was assessed. MI192 caused a time-dose dependant reduction
387 in hBMSCs viability confirmed via morphological and metabolic assessment, consistent with previous
388 studies utilising this HDACi on leukaemia and cholangiocarcinoma cell line [16, 36]. Moreover, the
389 findings of this study showed that MI192 significantly reduced HDAC activity in a time-dose dependent
390 manner, consistent with previous studies with HeLa and PC3 [16, 37]. Importantly, the HDAC inhibition
391 observed had a downstream effect on histone acetylation [31]. Therefore, MI192 treatment of ≥ 20
392 μM for 48 hours likely resulted in a transcriptionally permissive chromatin structure, enhancing the
393 cell's differentiation capacity. In this study, a pre-treatment strategy was adopted due to the effects
394 of prolonged MI192 exposure on causing a time-dose dependent reduction in hBMSCs viability, with
395 this approach commonly implemented in the literature with the use of panHDACis such as TSA [38].
396 To initially determine the effects of MI192 on hBMSCs osteogenic differentiation, ALPSA, an early
397 marker of osteogenesis, was quantified. Our findings showed that 50 μM MI192 for 48 hours prior to
398 osteogenic culture significantly enhanced hBMSCs ALPSA compared to the untreated control.
399 Interestingly, hBMSCs pre-treated with MI192 for 24 hours exhibited reduced ALPSA when compared
400 to the untreated cells. These findings correlated with the histone acetylation results, indicating a
401 causative link between the state of chromatin acetylation and the differentiation capacity of MI192
402 treated hBMSCs.

403 HDACis have been shown to regulate gene expression due to their ability to modify the epigenome
404 [10, 33, 35]. In this study, the expression of Runx2, a key osteogenic transcription factor [39], was
405 significantly upregulated in the MI192 pre-treated cells at both the gene and protein level throughout
406 osteogenic culture when compared to the untreated cells. These findings indicate that MI192 induced
407 chromatin hyperacetylation stimulated the expression of this osteogenic transcription factor.
408 Moreover, Jeon *et al.* (2006) demonstrated that Runx2 acetylation enhances its transcriptional activity
409 and stability via protection from Smurf-1 mediated degradation [40]. Therefore, MI192 treatment
410 probably increased Runx2 expression and transcriptional activity, likely attributed to modifications to
411 the chromatin structure, protection from Smurf-1 mediated degradation and relieving HDAC3
412 repression. However, this would require further investigation. Our data also showed that ALP mRNA
413 and protein expression levels were significantly upregulated in the MI192 pre-treated cells compared
414 to the untreated control, consistent with the enhancements observed in the functional ALPSA analysis.

415 It is well known that both Col1a and OCN are late osteoblast-related markers. MI192 pre-treatment
416 significantly upregulated the mRNA and protein expression levels of both markers compared to that
417 of the untreated group. Paino *et al.* (2014) knocked out HDAC2 in dental pulp stromal cells and found
418 that the lack of HDAC2 resulted in the downregulation of OCN gene expression [33]. Our data showed
419 that MI192 had a positive effect on OCN expression, likely due to the MI192 specificity to HDAC3. This
420 suggests the importance of HDAC3 in regulating hBMSCs osteogenic maturation. Studies have
421 demonstrated that HDACis have the capacity to enhance the expression of osteoblast-related markers
422 [32, 33], although, with the use of panHDACis, it is probable their mechanism of action relies heavily
423 on histone modifications rather than the additional modifications to Runx2 transcriptional activity due
424 to MI192 selective inhibition of HDAC3. Importantly, it was confirmed that MI192 promoted hBMSCs
425 extracellular matrix mineralisation, correlating with enhanced mineralisation induced by panHDACis
426 in the literature [12, 33]. Importantly, this was consistent with the previously reported effects of

427 MI192 on enhancing hDPSCs mineralisation capacity [19], indicating the plasticity of this HDACi in
428 stimulating MSCs from different origins.

429 Based on these findings, the effects of MI192 on hBMSCs bone-like tissue formation was further
430 evaluated within the BMT model due to the advantages microtissue culture provides for osteogenesis.
431 Several studies have reported the enhanced osteogenic induction of cells cultured as microtissues
432 when compared to conventional 2D culture. For example, Yamaguchi *et al.* (2013) reported enhanced
433 *in vitro* mineralisation and *in vivo* bone formation capacity of rat MSCs when cultured as spheroids
434 compared to 2D cultured cells [22]. Similarly, Baraniak *et al.* (2012) described a 5-fold increase in
435 murine BMSCs mineralisation when compared to cells culture in 2D culture [23]. Moreover, as cells
436 exhibit altered receptiveness to bioactive signals when cultured in 3D, it is important to investigate
437 the influence of epigenetic reprogramming induced by MI192 on hBMSCs osteogenesis within the
438 BMT. Our findings showed that MI192 significantly increased hBMSCs osteoblast-related gene
439 expression during microtissue culture, consistent with the 2D culture observations. Moreover, ALPSA
440 within the MI192 pre-treated BMT was significantly enhanced (2.41-fold) when compared to that of
441 the untreated construct. This enhancement in ALPSA was 1.43-fold higher compared to that observed
442 in the 2D studies, indicating the BMT microenvironment potentiates MI192 efficacy in stimulating
443 hBMSCs osteogenesis when compared to 2D monolayer culture.

444 Histological analysis showed that the incorporated microtissues fused *in situ* with neighbouring
445 microtissues, consistent with previous studies [27]. The MI192 pre-treated BMTs exhibited
446 substantially increased expression of osteoblast-related proteins distributed more homogeneously
447 throughout the construct when compared to the aberrant protein expression observed in the
448 untreated BMTs. This differential pattern of protein expression may indicate the role of MI192 pre-
449 treatment in priming all cells for osteogenic differentiation in this model when compared to the
450 untreated hBMSCs. The most intense protein deposition within the constructs was located at the
451 periphery and at the microtissue/scaffold interface, likely attributed to increased proximity to
452 osteogenic medium and the influence of scaffold stiffness on MSCs osteogenesis [41-43]. It is
453 important to assess the mineralisation within the newly formed extracellular matrix, a key attribute
454 for engineering functional bone-like tissue. Both untreated and MI192 treated hBMSCs exhibited
455 extensive mineral deposition within the constructs, likely attributed to the favourable microtissue
456 environment in facilitating mineralisation, such as the increased cell-cell/cell-matrix interactions
457 inducing key cellular differentiation pathways [44-46]. In addition to promoting cellular
458 differentiation, several studies have reported the influence of spheroid culture on the production of
459 soluble paracrine factors such as extracellular vesicles (EVs) [47, 48], which have been reported to
460 influence extracellular matrix mineralisation [49]. Moreover, it has been reported that EVs derived
461 from epigenetically-modified osteoblasts significantly promoted the mineralisation of hBMSCs [50],
462 indicating the potential role of MI192 treated hBMSCs secretome on stimulating extracellular matrix
463 mineralisation, although this would require further investigation. In this present study, we observed
464 a more homogenous mineral deposition throughout the MI192 BMT construct when compared to the
465 atypical deposition observed in the untreated group, consistent with the extracellular matrix protein
466 expression observed in this study. This provides further evidence regarding the capability of MI192
467 pre-treatment in “priming” all hBMSCs with increased osteogenic capacity resulting in
468 enhanced/homogenous bone-like tissue formation within the BMT. The influence of panHDACis on
469 MSCs osteogenesis in pellet culture has been previously investigated. For example, El-Serafi *et al.*

470 (2011) reported that TSA pre-treatment did not increase the mineralisation capacity of hBMSCs when
471 cultured as cell pellets [51], which did not replicate the findings of this study. This further emphasises
472 the importance of using selective HDACis which exhibits increased potency by targeting osteogenic-
473 related HDAC isoforms.

474 There have been limited investigations in the literature examining the use of HDACis in promoting
475 MSCs bone formation *in vivo*, with the majority of these studies utilising panHDACis. For example,
476 Jung *et al.* (2010) reported increased osteoblast differentiation when α -calcium scaffolds loaded with
477 the HDACis Trichostatin A (TSA) and NaB were implanted within a critical-sized rat bone defect model
478 [52]. Similarly, Lee *et al.* (2011) demonstrated pre-soaking collagen scaffolds with the HDACi Largazole
479 enhanced the regeneration of calvarial bone defect [53]. However, studies have reported the
480 detrimental effect of HDACis on bone formation *in vivo*. McGee-Lawrence and Westendorf (2011)
481 reported SAHA had a negative effect on the trabecular skeleton in C57BL/6 mice, with a reduction in
482 osteoblast numbers [54]. Consequently, there is growing precedence to investigate the efficacy of
483 selective HDACi compounds to stimulate bone formation *in vivo*.

484 In the present study, MI192 pre-treated BMTs were implanted within the intraperitoneal cavity of
485 mice to determine their ability to promote bone formation within a more physiological environment.
486 The use of diffusion chambers provides a 'closed' environment within the host animal to allow free
487 exchange of nutrients/waste for the implanted HDACi-induced hBMSCs for bone formation within the
488 BMT constructs without the direct interaction with host cells. The advantage of this model is to ensure
489 any tissue formed within the diffusion chamber must originate from the implanted cells [55], which
490 overcome the limitation of subcutaneous or renal capsule implantation models. Several studies have
491 reported the use of diffusion chamber model for the assessment of bone formation *in vivo* [56-58]. It
492 was observed that MI192 pre-treatment controlled the lineage-specific differentiation of hBMSCs,
493 where the untreated constructs exhibited aberrant deposition of osteogenic and chondrogenic
494 proteins, which is in support of our *in vitro* findings. Moreover, the MI192 treated groups showed
495 substantially enhanced osteoblast-related protein expression and mineralisation within the construct,
496 correlating with the *in vitro* analysis and several studies in the literature with panHDACis [8, 52, 53].
497 Interestingly, a greater fold increase in mineralisation between the MI192 treated and untreated
498 BMT constructs were observed *in vivo* compared to *in vitro* (2.39 vs 1.67-fold). This possibly
499 indicates the dependency of untreated hBMSCs within the BMT on the osteoinductive conditions
500 *in vitro* to promote mineralisation, while MI192 enhanced the osteogenic capacity of hBMSCs,
501 increasing its efficacy for bone formation *in vitro* and *in vivo*. Taken together, these *in vivo* findings
502 provide increased pre-clinical evidence into the potential utility of epigenetic reprogramming of
503 hBMSCs for bone formation. It would be of interest to investigate the influence of MI192 on promoting
504 bone regeneration within a critical-sized load-bearing defect, as this would provide greater evidence
505 into the effectiveness of this approach in a clinical setting.

506 **Conclusion**

507 In conclusion, these findings demonstrate that the HDAC2 & 3 selective inhibitor - MI192 is capable of
508 reprogramming the epigenetic functionality of hBMSCs, enhancing its osteogenic capacity *in vitro* and
509 *in vivo*, which indicates the potential of using epigenetic regulation as an engineering approach to
510 improve MSCs therapeutic efficacy for bone augmentation strategies.

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518 **CRedit author statement**

519 KM: Conceptualisation, methodology, formal analysis, investigation, data curation, writing – original
520 draft. NM: resources. KL: resources, writing – review & editing. LJ: conceptualisation, supervision,
521 writing – review & editing. TW: resources, writing – review & editing. XY: conceptualisation,
522 supervision, project administration, methodology, data curation, writing – review & editing and final
523 proof.

524 **Ethical consideration**

525 All animal studies were conducted under procedures approved by the University of Leeds Ethics
526 Committee and under the UK Home Office project license (PPL: 70/8549).

527 **Declaration of interest:** None.

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529

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Supplementary figures

Supplementary Table 1 - Primers used for TaqMan gene expression assays

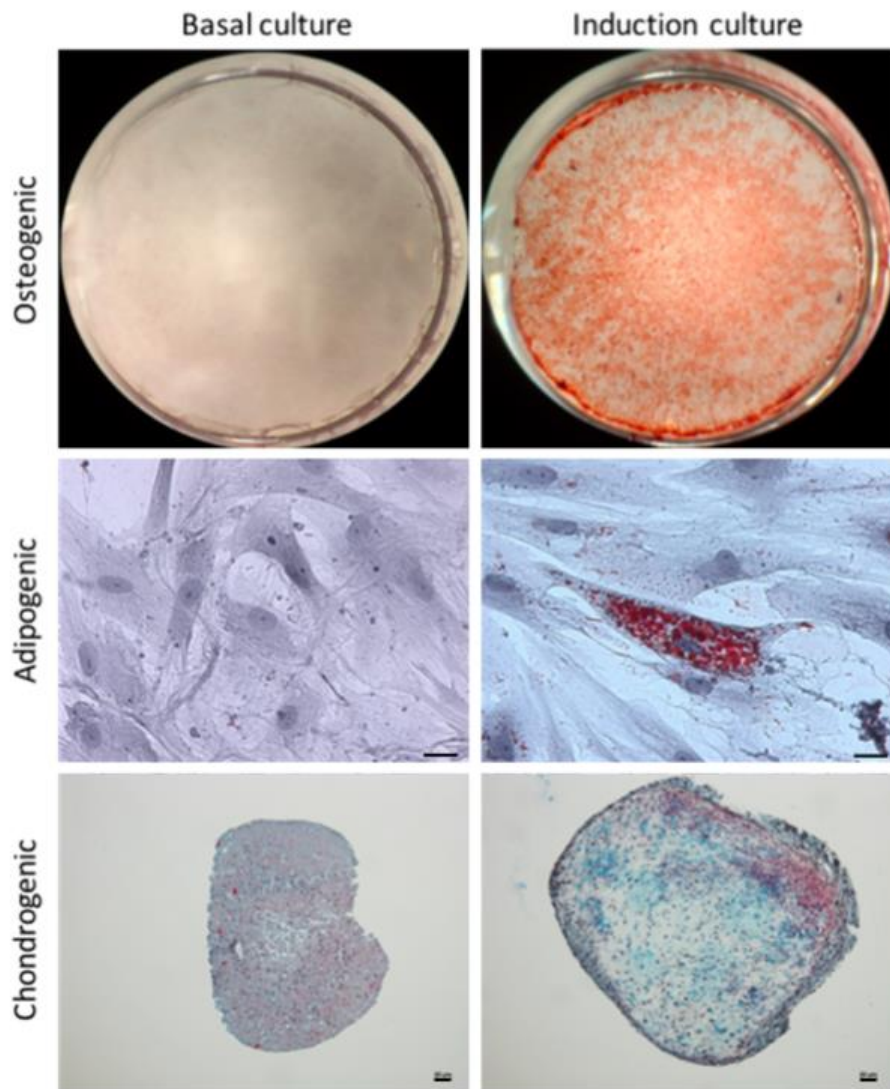
Gene symbol	Description	TaqMan identification
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
<i>RUNX2</i>	Runt-related transcription factor 2	Hs00231692_m1
<i>ALPL</i>	Alkaline phosphatase	Hs01029144_m1
<i>COL1A1</i>	Collagen, type I	Hs00164004_m1
<i>OCN/BGLAP</i>	Osteocalcin/PMF-bone gamma- carboxyglutamate (gla) protein	Hs00609452_g1

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Supplementary Table 2 - Primary antibodies used for ICW and immunohistochemistry

Antibody	Catalogue number	Dilution
<i>In vitro studies</i>		
Runx2	ab76956	1/600
Alkaline Phosphatase	ab126820	1/500
Collagen type I	ab6308	1/100
Osteocalcin	ab13420	1/800
<i>In vivo studies</i>		
Collagen type I	ab138492	1/100
Osteocalcin	ab198228	1/200

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752 **Supplementary Figure 1 - Multi-lineage potential of hBMSCs.** Osteogenic differentiation: Positive
 753 calcium accumulation was observed after 28 days cultured in osteogenic medium confirmed by
 754 Alizarin Red staining; Adipogenic differentiation: lipid droplet formation was observed in hBMSCs
 755 after 14 days culture in adipogenic induction medium after Oil red O staining (Scale bar = 50 μ m);
 756 Chondrogenic differentiation: hBMSCs were capable of forming cartilage like pellets after 21 days
 757 cultured as pellets in chondrogenic induction medium, stained blue for GAGs (Alcian blue staining)
 758 and red for collagen (Picosirius red staining). Scale bar = 20 μ m.

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