VEGF with AMD3100 Endogenously Mobilizes Mesenchymal Stem Cells and Improves Fracture Healing

Richard Meeson[1,2] Anita Sanghani-Keri[1], Melanie Coathup[1,3] Gordon Blunn[1,4]

[1]Division of Surgery, University College London, Stanmore, UK; [2]Royal Veterinary College, Hertfordshire, UK; [3]University of Central Florida, USA; [4]University of Portsmouth, Portsmouth, UK

Corresponding Author

Richard Meeson

Principal Clinical Research Fellow, Division of Surgery, Institute of Orthopaedics and Musculoskeletal Science, University College London, Royal National Orthopaedic Hospital, Stanmore, HA7 4LP

Running title: Endogenous mobilization of stem cells improves fracture healing

Author contributions: RM grant funding, experimental design, experimental work and manuscript preparation. AS experimental work and manuscript review. MC grant funding experimental design, manuscript review. GB grant funding, experimental design, experimental work, manuscript preparation. All authors have read and approved the final submission.

Abstract (250)

A significant number of fractures develop non-union. Mesenchymal stem cell (MSC) therapy may be beneficial, however this requires cell acquisition, culture and delivery. Endogenous mobilization of stem cells offers a non-invasive alternative. The hypothesis was administration of VEGF and the CXCR4 antagonist AMD3100 would increase the circulating pool of available MSCs and improve fracture healing. Ex-breeder female wistar rats received VEGF followed by AMD3100, or sham PBS. Blood prepared for culture and colonies were counted. P3 cells were analysed by flow cytometry, bi-differentiation. The effect of mobilization on fracture healing was evaluated with 1.5mm femoral osteotomy stabilized with an external fixator in 12-14 week old female Wistars.

The mobilized group had significantly greater number of cfus/ml compared to controls, $P=0.029$. The isolated cells expressed 1.8% CD34, 35% CD45, 61% CD29, 78% CD90, and differentiated into osteoblasts but not into adipocytes. The fracture gap in animals treated with VEGF and AMD3100 showed increased bone volume; 5.22±1.7um^3 and trabecular thickness 0.05±0.01um compared with control animals (4.3±3.1um^3, 0.04±0.01um respectively). Radiographic scores quantifying fracture healing (RUST) showed that the animals in the mobilization group had a higher healing score compared to controls (9.6 vs 7.7). Histologically, mobilization resulted in significantly lower group variability in bone formation ($p=0.032$) and greater amounts of bone and less fibrous tissue than the control group.

Clinical significance: This pre-clinical study demonstrates a beneficial effect of endogenous MSC mobilization on fracture healing, which may have translation potential to prevent or treat clinical fractures at risk of delayed or non-union fractures.

Key words: Mobilization, mesenchymal stem cells, AMD3100, VEGF, fracture healing

47 **Introduction**

48 A significant number of bone defects and fractures do not heal. In the USA, it is estimated
49 that around 100,000 fractures per year go on to non-union[1]. The UK National Health Service
50 reports around 10% of fractures fail to heal, and treatment can be difficult requiring repeat
51 surgery with a cost of up to £80,000 per patient[2]. Successful fracture healing is reliant upon
52 the recruitment, migration and homing of cells for inflammation, blood vessel formation,
53 chondrogensis, osteogenesis[3]. Adult mammalian bone marrow has two constituent stem cell
54 populations with separate lineages, including haematopoietic stem cells which are non-plastic
55 adherent and will circulate in the peripheral blood, and stromal cells, which are plastic
56 adherent, with a fibroblastic morphology and considered non-circulating[4]. Cells isolated from
57 stroma have been shown in vitro and in vivo transplantation to be able to produce all tissues
58 require for the bone organ[4,5] and are termed mesenchymal stem cells (MSCs). There is
59 evidence for low numbers (1 in 10^{6-8} of nucleated cells) of peripheral blood circulating plastic
60 adherent, osteogenic potent cells in mice, rabbits, guinea pigs and humans[6,7]. Clinical
61 evidence from fracture patients also supports a role for circulating MSCs in fracture healing[8].

62 The chemokine stromal cell derived factor 1 (SDF1, also known as CXCL12), and its receptor
63 CXCR4, has a key role in stem cell migration from the bone marrow stroma into the
64 circulation and is believed to be important for homing of stem cells to a fracture sites[9]. Local
65 increases in SDF1 expression have been measured in distraction osteogenesis, stress fractures
66 and segmental defects[9,10]. It is suggested that when a fracture occurs there is a chemotactic
67 gradient, with high levels of SFD1 at the fracture site, and subsequently increased levels in
68 the blood steam, facilitating stem cell migration from their niches[9].

69 The SDF1-CXCR4 axis maintains HSCs and likely other stem cells in the bone marrow[9,11]
70 and probably within other body niches. An intentional forced egress of cell ‘mobilization’ has

71 been in clinical use for some time with haematopoietic stem cells for repopulation of the bone
72 marrow after treatment for certain blood related malignancies[12,13]. AMD3100, (1,1-[1,4-
73 Phenylenebis(methylene)] bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride), is a
74 bicyclam derivative that antagonises the CXCR4 receptor directly and mobilizes a population
75 of CD34+ haematopoietic stem cells into the peripheral circulation. This occurs by highly
76 selective, high affinity competitive blocking of the CXCR4 receptor which displaces stem
77 cells from the bone marrow niche by disruption of their attraction to SDF1[14]. Although
78 haematopoietic stem cells have been successfully mobilized using GCSF or AMD3100 or a
79 combination, work on MSC and other progenitors is limited and these cells do not seem to be
80 as migratory as hematopoietic stem cells, potentially due to their larger size, increased niche
81 adherence and their limited number in the bone marrow stroma[15]. Pitchford's seminal work
82 on different mobilization protocols in mice, demonstrated that AMD3100 combined with
83 VEGF rather than GCSF, preferentially mobilizes a population of MSCs rather than
84 haematopoietic stem cells[11].

85 The aim of this study was to investigate whether administration of VEGF with AMD3100,
86 could mobilize MSCs into the peripheral circulation of rats, and to determine whether
87 increasing the circulating levels of MSCs would improve fracture healing in a delayed union
88 rat femoral fracture model.

89

90

91

92

93

94 **Methods**

95

96 *Isolation of bone marrow MSCs (BMMSCs)*

97 Healthy, ex-breeder female wistar rats (n=3) (450-550g), were the donors. All procedures
98 were carried out according to the UK Home Office Animals Scientific Procedures Act of
99 1986 and were approved by the Animal Welfare Ethical Review Board at the Royal
100 Veterinary College, and aligned to the ARRIVE guidelines. The rats were euthanised and the
101 femur aseptically isolated. The femoral medullary canal was flushed with 5mls DMEM 4500
102 mg/L glucose, (Sigma-Aldrich, UK) with 20% fetal calf serum (FCS) and 1%
103 penicillin/streptomycin (termed 'media'), into a 25cm^2 polystyrene cell culture flask
104 (Corning, USA). The cells were cultured in a humidified incubator at 37°C, 95% air and 5%
105 CO_2. The media was changed after 5-7 days to remove non-adherent cells and every 3-4 days
106 thereafter. Once they had reached 70-80% confluence, they were passaged.

107

108 *Preparation of Growth Factors for Mobilization*

109 Rat Vascular Endothelial Growth Factor 165 (VEGF) (PeproTech, USA, 400-31) was
110 prepared by dissolving the lyophilized product in sterile water to make a 0.1mg/ml stock
111 solution. A working solution was prepared by adding 1ml of stock solution to 4mls of sterile
112 PBS + 0.1%BSA (Sigma-Aldrich, UK), to achieve a 100ug/ml injectable solution which was
113 then aliquoted and stored at -20°C until needed. AMD3100 (Sigma-Aldrich, UK A5602),
114 stock solution was prepared by dissolving 5mg lyophilized product in 0.5mls sterile water,
115 and then added to 4.5mls PBS to produce a 1mg/ml injection solution, which was then
116 aliquoted and stored at -20°C, until needed.

117 *Mobilization of Peripheral Blood MSCs (PBMSCs)*

118 For the mobilization study, the VEGF-AMD group (n=8) and PBS treated controls (n=6) were
119 healthy, ex-breeder female Wistar rats (380-600g). Rats were pre-treated with VEGF
120 (Peprotech, USA), at 100ug/kg, once daily by intra-peritoneal (i.p.) injection daily for four
121 days, at a volume of 0.5mls/100g. On day five, rats received a single i.p. 5mg/kg dose of
122 AMD3100, at a volume of 0.5mls/100g. The dosages of VEGF and AMD3100 were taken
123 from Pitchford's et al[11]based on prior pharmacokinetic work[12] and were adjusted to be
124 appropriate for each individual rat's bodyweight.

125 One hour post administration, rats were anaesthetised for terminal cardiac venipuncture.
126 Controls were treated with PBS i.p. at the same volume and time intervals (Figure 1a).
127 Isolation of stem cells was achieved by red blood cell lysis where 10mls of lysis solution (Red
128 Blood Cell lysing Buffer Hybri-Max solution Sigma-Aldrich, UK), was added per 1ml of
129 blood, and mixed in a 50ml Falcon tube (Corning, USA). After five minutes, 35mls of PBS
130 was added to neutralize the lysis solution, and then centrifuged at 400g for five minutes. The
131 supernatant was aspirated and the cells were re-suspended in media and plated into 25cm^2
132 polystyrene cell culture flasks for MSC culture. The media was changed after five to seven
133 days to remove non-adherent cells and thereafter every 3-4 days. Colonies were counted at x4
134 magnification placed under a phase-contrast light microscope, using a grid overlay. Final
135 CFU count was performed at 20±2 days. Cells were passaged when they were 70-80%
136 confluent, as previously described.

137 *Analysis of Isolated Cells*

138 P3 cells, 30,000 cells per group were fixed and assessed using a combination of positive
139 CD90 (Anti-Mouse/Rat CD90.1 (Thy-1.1) eBiosceince, UK) and CD29 (Anti-Mouse/Rat
140 CD29 (Integrin beta 1) eBioscience, UK), and negative MSC markers CD45 (Anti-Rat CD45

141 eBioscience, UK) and CD34 (Anti-CD34 abcam, UK) and were compared with appropriate
142 isotopye controls. Analysis was performed using a flow cytometer (Cytoflex, Beckman
143 Coulter, UK), with Cytexpert (Beckman Coulter, UK) software.

144 For differentiation, cells isolated from blood and from bone marrow were assessed by taking
145 30,000 P3 cells were seeded into a sterile 48 well plates, in triplicate and assessed for
146 osteogenic and adipogenic differentiation through media supplementation for 21 days in
147 comparison to standard media. Osteogenic media consisted of standard media with 100nM
148 dexamethasone (Sigma-Aldrich, UK), 50µg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich,
149 UK) and 10mM Glycerol-2-phosphate disodium salt hydrate, (Sigma-Aldrich, UK).
150 Adipogenic media consisted of standard media with 0.1mM dexamethasone (Sigma-Aldrich,
151 UK), 0.45 mM IBMX (Sigma-Aldrich, UK), 10mg/ml Insulin (1.7mmol/L) (Sigma-Aldrich,
152 UK), and 50mM Indomethacin (Sigma-Aldrich, UK). Osteogenic differentiation was assessed
153 by staining with Alizarin red stain (0.2 Molar, pH 4.32, Sigma-Aldrich, UK), to identify
154 calcium mineral deposition. Adipogenic differentiation was assessed with Oil red O staining
155 for lipid droplets.

156 *Femoral delayed union fracture model*

157 For the fracture model, 12-14 week old female Wistar rats were assigned to the control (n=7)
158 or mobilization group (n=8) (230-300g). According to the Home Office license and under
159 aseptic conditions, a lateral approach was made to the left femur. Using anatomical
160 landmarks, and a custom precision jig-guide system, four bicortical threaded 1.4mm stainless-
161 steel fixator pins were consistently placed in the craniomedial femur. Pins were exited
162 through separate stab incisions and the custom variable spacing fixator was attached. A mid-
163 diaphyseal femoral osteotomy, with no periosteal stripping was made using a diamond tipped
164 hand-saw, whilst applying sterile saline coolant/lubricant. A precision spacer ensured a fixed

165 distance between the cis cortex and connecting blocks of 9mm. The fixator was then attached
166 and the osteotomy gap distracted to 1.5mm using a second precision spacer. The biceps
167 femoris was closed over the osteotomy with a single horizontal mattress suture (1.5M PDS II,
168 Ethicon, UK), and then the skin was closed with intradermal continuous suture (1.5M
169 monocryl, Ethicon, UK). Activity was unrestricted post surgery. Twenty-four hours post-
170 surgery, rats were given a single i.p. injection of either VEGF (100ug/kg), or PBS once daily
171 for four days. On day five, they were given a single injection of AMD3100 (5mg/kg). All i.p.
172 injections including AMD3100 and sham PBS were administered at a volume of 0.5mls/100g
173 bodyweight based on the day 0 pre-surgical weight (Figure 1b). All procedures were carried
174 out at the Royal Veterinary College, North Mymms, in accordance with the Animals
175 Scientific Procedures Act 1986, and aligned to the ARRIVE guidelines. Those taking part in
176 any surgical procedure held UK Home Office licences. The dosages of VEGF and AMD3100
177 were taken from Pitchford et al.[11] and the timing of mobilisation relative to surgery was based
178 on prior work[16,17].

179

180 After five weeks, the left femur including the fixator, were retrieved from the sacrificed rats.
181 Femurs were fixed and scanned with a Bruker Skyscan 1172 micro-tomograph machine
182 (Bruker, Belgium), at 60KV, 167uA with a 0.5mm aluminum filter. A rotation step of 0.5
183 degrees, without frame averaging, and an image pixel size of 4.89um. MicroCT scans were
184 reconstructed using NRecon (Bruker, Belgium). Analysis was performed using CTAn
185 (Bruker, Belgium). The central 60% of the osteotomy gap was assessed (0.9mm = 180 slices
186 at 5um thick). Radiographic scouts were assessed for bone union, using orthogonal
187 projections isolated from the microCT image acquisition series. Radiographs were evaluated
188 by three independent assessors in a randomised and blinded manner and graded according to
189 the RUST scoring[18].

190

191 After microCT analysis, the bones were decalcified in ethylenediaminetetraacetic acid
192 (EDTA, Sigma Aldrich, UK), sequentially dehydrated in alcohol solutions, de-fatted and
193 embedded into wax with the fixator pins orthogonal to the facing surface of the block. Fixator
194 blocks and pins were removed once the wax had set and a sledge microtome (ThermoFisher
195 Scientific, UK) was used to make 5μm thick slices. The alignment of the blocks within the
196 microtome was altered as necessary to ensure a central sagittal slice through the femur,
197 assessed using the fixator pin tract holes. Slides were prepared and stained with Haematoxylin
198 and Eosin (Sigma-Aldrich, UK). Histomorphometric analyses were performed at 2.5x
199 magnification, quantified with a 1.5mm scaled width line-intercept grid, with 120
200 intersections; grid squares were 160um in both directions. Intersections were then scored as
201 bone, cartilage, fibrous tissue, vascular or void.

202

203 *Statistical Analysis*

204 Due to the relatively small group sizes ($n<9$), non-parametric tests were performed to
205 compare groups including Mann-Whitney U (MWU). Assessment of data spread was made
206 with a Levene's test for equality of variance. Significance was set a $p<0.05$ and tests were
207 analysed with SPSS version 24 (IBM, Chicago, USA). Data is expressed as mean± SD unless
208 otherwise specified.

209

210 **Results**

211

212 *Comparison of BMMSCs to PBMSCs*

213 BMMSCs formed fibroblastic colonies (CFUs) with spindle shaped cells. P3 cells were able
214 to differentiation down osteogenic and adipogenic lines, with positive staining with Alizarin
215 red and Oil red O respectively. BMMSCs had mean expression levels of the following
216 markers: CD45 5.7±5.0%, CD34 0.2±0.1% CD29 98.4±4.1% and CD90 98.9±1.0%.

217 No CFUs were isolated from the non-mobilized control blood. The mobilized group formed
218 CFU-Fs in 6 out of the 8 individual cultures. Initially, round mononuclear cells were present
219 with some immature elongating cells. Over time, the cells began to take on a similar
220 morphology to those obtained by bone marrow isolation; spindle or fibroblastic-shaped cells,
221 with a centrifugal arrangement of cells to form a colony. Notably however, they took longer
222 to form clear CFUs, typically around 14-18 days, when compared with bone marrow isolated
223 cells (5-7 days). The mean CFU-F/ml for the mobilized group was significantly higher at
224 2.9±1.8 CFU-F/ml (p=0.029) than the controls. These cells were passaged up to P3 (Figure 2a
225 shows bone marrow derived and 2b shows blood mobilised MSCs).

226 Flow cytometry of P3 cells showed cells were CD34 negative, however CD45 which is
227 typically negative in MSCs, was positive on some cells with both CD45 positive and CD45
228 negative cells present, varying from 9 to 60% positive within any population. MSC markers
229 CD90 and CD29 were relatively highly expressed at 78%, and 64% respectively (See table 1
230 for details).

231 In differentiation assays, mobilized P3 PBMSCs formed a monolayer within seven days.
232 During supplementation with osteogenic media, the cell morphology became less spindaloid
233 and more cuboidal and multiple small granules became apparent. Staining for Alizarin red
234 after 21 days demonstrated red stained calcium deposits in all samples, consistent with
235 osteoblast activity (n=5) (see Figure 3), however despite adipogenic supplementation there

236 was no evidence of adipogenic differentiation, with no positive Oil Red O staining evident in
237 4/5 and one showing a very small amount. This was in contrast to the cells isolated directly
238 from the bone marrow, which demonstrated mineralisation and lipid production when
239 differentiated down the respective lineages.

240

241 *Influence of mobilization on fracture healing*

242 RUST scoring gave a mean of 7.71±2.7 for the control group and 9.63±1.3 for the VEGF-
243 AMD group (Figure 4 shows a low scoring (a) and high scoring (b) example). On microCT
244 analysis, mobilized group (n=8), showed a higher mean bone volume than controls
245 (5.22±1.7um^3, vs 4.3±3.1um^3), as well as increased trabecular thickness (0.048±0.007um,
246 vs 0.042±0.003 um). The overall data spread was significantly reduced in the VEGF-
247 AMD3100 group when looking at tissue volume (TV um^3, p=0.036) and trabecular number
248 (p=0.048).

249 Histomorphometric analysis revealed proportionally greater levels of bone in the osteotomy
250 of the mobilized group; 55.1±7.8% bone, 40.9±9.8% cartilage, 0.3±0.8% fibrous and
251 3.9±3.9% vascular tissue (Figure 5). The combined bone and cartilage percentage was
252 96±3.7. This compared with control osteotomy tissue composition of 39.1±23.9% bone,
253 43.1±24.6% cartilage, 15.3±37.4% fibrous and 2.4±2.0% vascular tissue; and the combined
254 bone and cartilage within the gap was 82.3±36.4%. Levene's test for equality of variances
255 showed a significant decrease in the variability of healing in the mobilized group for bone
256 (p=0.032) and fibrous tissue (p=0.026).

257

258

259 **Discussion**

260 This study has demonstrated a significant increase in peripheral blood circulating PBMSCs
261 post VEGF and AMD3100 administration. Notably, they were morphologically similar to
262 BMMSCs, but were osteoblastic lineage limited, and had lower levels of MSC markers
263 (CD90, and CD29) and higher levels of CD45, likely indicating a heterogeneous population
264 of adherent fibroblastic expandable cells. Critically, the mobilization of this population with
265 VEGF and AMD3100 was associated with an improvement in fracture healing. Mobilized
266 fractures had increased bone volume and thicker struts of woven bone on micro CT analysis,
267 near doubling of union rates, with a significant reduction in data variation for the treated
268 group on bone volume from microCT data, and a smaller standard deviation with the RUST
269 radiographic score, with improved means. This suggests mobilisation may preferentially have
270 influence on the poorest healing individuals, which may be clinically beneficial. Histology
271 corroborated the findings on microCT with an increase in bone volume, vascularisation and
272 reduced fibrous tissue within the osteotomy.

273 Under the in vitro protocol, no PBMSCs were isolated from the non-mobilized blood samples.
274 Others have had more success[19], however, they used younger animals. The isolation of cells
275 was performed in ex-breeders, which are adult through to geriatric, and age related changes in
276 stem cell differentiation, proliferation and metabolism[20] are reported. Older rats have fewer
277 CFUs, fewer MSCs in the bone marrow[21], possibly explaining the reduced yields in non-
278 mobilized controls and mobilized groups compared with Pitchford et al[11], who used young
279 mice. Humans also show reduced CFUs with ageing[6] and a recent study also showed an age
280 relationship with CXCR4 dependent migration[22] and hence the effect of antagonism of the
281 CXCR4-SDF1 axis may also reduce with age. Most of the studies looking at peripheral blood
282 circulating stem cells and mobilization in animal models have used young immature
283 animals[11,17,19,23], and therefore there may be merit in evaluating the role of age in mobilization

284 potential. Although the rats were not ovariectomised, assessing mobilization in ex-breeders is
285 more relevant, as the likely target population for translation will be fractures in older patients
286 and in those with osteoporosis where fracture healing is more likely to be impaired than in
287 young individuals.

288 It is assumed that the mobilized cells are of bone marrow origin, however with systemic
289 administration of the mobilising agents, they may be from other niches, particularly when
290 considering the conservation of SDF1-CXCR4 axis goes beyond the bone marrow
291 niche[10,24,25]. It is certainly suggested that the bone marrow makes a contribution to the
292 mobilized peripherally circulating cells, as demonstrated by isolating the femoral artery and
293 vein[11] to evaluate mobilization from an isolated body compartment. The authors suggest that
294 the cells are therefore bone marrow in origin, however an isolated femoral component
295 actually only informs of all possible niches within the hind limb, and not exclusively the bone
296 marrow compartment. It remains possible that these cells were mobilized from sources other
297 than the bone marrow, such as the periosteum, muscle or perivascular niches[26]. Interestingly
298 the mobilized cells did not have adipogenic potential, but had effective osteoblastic
299 differentiation, which may be important for bone tissue engineering. The traditional view is
300 that a single MSC would be capable of differentiation into osteoblasts, chondrocytes or
301 adipocytes. Even classical bone marrow stem cell work has shown different clonal
302 populations frequently do not have full tri-differentiation, whilst interestingly, the osteogenic
303 lineage is always present[27]. Other studies have suggested a hierarchy of sequential lineage
304 loss with the potential for osteogenic differentiation remaining[28].

305 They showed that only 1/3 clones were capable of tri-differentiation, and the majority were
306 osteo or chondro orientated. Due to the paucity of cells and the long duration of culture required
307 with PBMSCs, there were insufficient cells for pellet culture and chondrogenic differentiation,
308 and therefore it remains unclear if the PBMSCs could also be differentiated into chondrocytes.

309 The PBMSCs mobilized had similar cell surface marker expression as the BMMSCs, although
310 CD29 and CD90 were not as highly expressed. Notably there appeared to be the presence of
311 two CD45 populations; CD45- and CD45+ groups, both without CD34 expression. CD45 is a
312 key leukocyte marker and MSCs are universally considered to be CD45-[29,30], however, CD45
313 has been demonstrated on cultured bone marrow MSCs from patients with haematological
314 malignancies. These cells could differentiate and were morphologically similar to CD45- MSCs,
315 and hence under certain circumstances the CD45 rule may be broken[31]. It is impossible to tell
316 if these cells were simply contaminants or did represent a CD45+ MSC population. Other
317 studies in rabbits [32] and a comprehensive comparison of rat bone marrow to blood MSCs
318 showed no difference in CD marker expression, morphology and tri-lineage potential, although
319 growth and differentiation potential are reduced in peripheral blood isolated MSCs[33].

320 Although VEGF with AMD3100 statistically increased the yields of PBMSCs, they were still
321 relatively low. Conceptually, a single CFU is produced from a single stem/progenitor[5]. In this
322 model system, the average yield of three stem/progenitor cells per ml of blood combined with
323 a maximum 6-10mls available made evaluation challenging. To that end, in vitro
324 identification with expansion was necessary, and allowed a wider platform of evaluation,
325 although cell expansion was also slow. This methodology was similar to Pitchford's work in
326 mice[11], however they showed in young mice greater stem cell mobilization of 15 MSCs/ml
327 blood. This finding, may be an artefact of *in vitro* culture and the processing performed to
328 remove red blood cells prior to culture, or relate to species or age differences in the donors.
329 The problems of isolating stem cells from the peripheral circulation is not new however, and
330 other groups have used fibrin microbeads that bind matrix-dependent cells to concentrate the
331 proportion of MSCs within MNCs to improve subsequent plating density and yields[34]. A
332 further consideration is the single time point of sampling, which essentially provides only a
333 'snap-shot' of the circulating pool.

334 Evidently, the mobilization of a peripheral blood MSC like osteoblastic progenitor was
335 beneficial in a compromised fracture healing environment. Previous work using a similar
336 fixator system showed complete union at 5 weeks with a 0.5mm osteotomy and an atrophic
337 non-union with a 3mm osteotomy[35]. A 1.5mm osteotomy was chosen as a half-way measure
338 with the expectation of compromised healing as a good test base for the effect of mobilization
339 on fracture healing. This study was the first study to evaluate the potential effects of stem-
340 progenitor mobilization on fracture healing in rats. Critically, it evaluated the potential to
341 recue a compromised healing environment, rather than simply augmenting an uncomplicated
342 healing situation.

343 Mobilization using VEGF with AMD3100, improved fracture healing, with an increased
344 RUST score. The trabecular thickness and the space between trabecular (trabecular
345 separation) evaluation output provides commentary on the nature of the woven bone formed
346 within the osteotomy in this instance. The mobilized group had an increased the trabecular
347 separation, implying of the bone formed was more porous, but increase trabecular thickness
348 suggested the individual struts were thicker. Perhaps this represents a more advanced or more
349 rapidly developed stage of endochondral ossification. In any case, VEGF with AMD3100
350 increased the bone formation over the controls. Others have seen improvements in fracture
351 healing using AMD3100 alone[17], or combined with IGF1[16]. These models were in young
352 mice and showed improved healing, in a non-compromised situation. There is therefore cross-
353 species merit in this strategy, and the ideal mobilization protocol remains unclear, particular
354 in a compromised healing environment which may be a more appropriate test system
355 considering the likely translation. Notably, on microCT and histomorphometric analysis, the
356 variation in healing was significantly reduced with VEGF-AMD3100 treatment, perhaps
357 indicating it improved the poorer healing individuals more than the better healing ones, and
358 thus decreased the variability of healing seen in the controls.

359 Clearly the nature of the disturbance to the SDF1-CXCR4 axis is important. It is likely that a
360 short duration blockade will mobilize more stem and progenitor cells and hence increase the
361 total pool available to the fracture site. However, the homing to the fracture site also relies
362 upon the very interaction being antagonised. The short half-life of AMD3100[14], likely
363 provides a 'pulse' in the early inflammatory phase of fracture healing has more mobilising
364 effect at the bone marrow or other niches, rather than significantly impairing the recruitment
365 of cells to the fracture site over days to weeks. Longer term blockade throughout the period of
366 fracture healing will significantly reduce callus cartilage, callus size and bone formation, with
367 reduced expression of genes associated with endochondral ossification[10]. Continued
368 AMD3100 administration also reduces new bone formation in distraction osteogenesis
369 models[36], and therefore the timing of the administration of VEGF and AMD3100 relative to
370 the stage of fracture healing is likely to be crucial and warrants further investigation.

371

372 A difference between the CFU mobilization analysis and the subsequent evaluation in fracture
373 healing in this study is presence and influence of the osteotomy. It is well know that fractures
374 release increased levels of growth factors[37]. Clinically, VEGF has been shown to be increased
375 in human patients with long bone fractures within the first couple of weeks, lasting up to six
376 months post trauma[38]. Conceivably the administration of VEGF which is thought to pre-prime
377 the bone marrow to preferentially release MSCs when ADM3100 is given[11], could have had
378 direct humoral influence on the fracture healing itself, irrespective of stem cell mobilization.
379 Histomorphometric assessment of vascularisation in the mobilized group showed higher
380 levels of blood vessels, and VEGF is a known potent angiogenesis promoter with a clear role
381 in endochondral and intramembranous bone formation[39,40]. Exogenous VEGF administration
382 will inevitably have both direct and indirect effects on bone formation. VEGF can act
383 indirectly through its receptors on endothelial cells, influencing the development of a new

384 vascular network, allowing bone orientated stem and progenitors to migrate into the fracture
385 callus and differentiate into osteoblasts[41].

386

387 An important aspect of any evaluation of fracture healing is associated with the functional
388 results. There have been a number of articles that assessed the strength of repair and the
389 degree of bone formation. The majority of these show good correlation, however, this was not
390 performed in this proof of concept study due to the increased numbers of rats that would have
391 been required to achieve sufficient statistical power. Now that we have established efficacy,
392 future dose regime evaluations would include this aspect.

393

394 In order to progress this study further it may be necessary to optimise the administration of
395 both VEGF and AMD3100. In this instance we chose to administer the protocol immediately
396 after fracture, during the early inflammatory phase of fracture healing, when stem cells will be
397 recruited, and is consistent with other studies[16,17]. This is sensible based on the translational
398 potential of this treatment as it would have to be given after a fracture occurred, but could be
399 instigated early on as a prophylactic in 'at risk' fracture patients such as fragility fractures.
400 Nonetheless, the optimal timing of the administration of VEGF and AMD3100 relative to the
401 stage of fracture healing could impact on its influence on fracture healing and warrants further
402 investigation. Different dosing or timings, such as repeated injections of AMD3100, may
403 increase the circulating pool of MSCs, however, for these cells to be effective they have to
404 home to the site of fracture through the SDF1 CXCR4 interaction. Fortunately the half-life of
405 AMD3100 is short allowing the cells to re-establish their homing capacity, and only studies
406 that gave AMD3100 throughout the entire fracture healing process showed impaired
407 healing[10].

408

409 Future mechanistic work could include spiking the circulation with labeled stem cells at the
410 same time that AMD3100 is administered, and then investigating the efficiency of these cells
411 to migrate to the fracture suite over time. However, there is an issue of determining cell
412 migration characteristics from cultured cells as cell surface protein expression critical for
413 migration and homing are influenced by in vitro cell culture. Whether or not cultured labelled
414 cells would reflect the in vivo kinetics of uncultured cell migration is unclear. Only bone
415 marrow ablation with recapitulation with labelled marrow, or parabiotic studies could
416 adequately answer this question and they are highly problematic to perform due to significant
417 animal welfare implications.

418

419 There are other methods that may be used to enhance mobilisation of stem cells. For example
420 Meng et al. transfected MSCs to express Aquaporin, a regulator of endothelial cell migration,
421 and showed the cells had enhanced migration in a transwell assay[42]. SOX 11 transfected stem
422 cells transcriptionally activate Runx2 and CXCR4 expression, and when administered in a rat
423 femoral fracture model they showed a larger number of MSCs migrated to the fracture site
424 and improved bone fracture healing[43]. Whilst these studies show that migration of cells can be
425 manipulated and can improve bone healing in fracture models, the advantage of our approach
426 where the CXCR4/SDF1 axis is disrupted means that cells are not manipulated in vitro, and
427 the approach is entirely endogenous.

428

429 Further work to determine the influence of age on mobilization, the role of other cells
430 populations mobilized and optimization of both the in vitro assessment and the dose/timing of

431 therapy, and combinations of other growth factors with AMD3100 should be pursued. In
432 conclusion, VEGF combined with AMD3100 can mobilize a population of MSC-like
433 osteoblastic lineage determined cells into the peripheral circulation and this leads to increases
434 in bone formation in a delay union osteotomy model. Notably, this approach shows promise
435 in a challenging fracture-healing environment, which differentiates this work from prior
436 studies augmenting normal healing, and for the first time, a MSC mobilising protocol of
437 VEGF-AMD3100 has been evaluated. Clinically there could be significant benefit to fragility
438 and other at risk fracture to provide a 'biological boost' to the fracture healing process in at
439 risk patients, through this non-invasive endogenous strategy.

440

441 **Acknowledgments**

442 This study was supported by Medical Research Council, grant, UK: MR/N002318/1. We
443 would also like that thank the technical support with the fixator manufacture from Keith
444 Raynor.

445

446 **Tables**

Rat	CD45+	CD34+	CD29+	CD90+	CD34-	CD34+	CD45+	CD45+
					CD45-	CD45-	CD34-	CD34+
1	60.9	3.0	76.2	77.8	37.0	2.6	59.3	1.0
2	35.4	1.8	60.7	83.9	61.3	2.2	36.1	0.4
3	8.6	1.8	55.2	70.7	87.0	2.3	10.6	0.2
Mean ±SD	35.0±26.2	2.2±0.7	64.0±10.9	77.5±6.6	61.8±25.0	2.4±0.2	35.3±24.2	0.5±0.4

447

448 Table 1: Flow cytometry analysis of cell surface marker expression of VEGF AMD3100
449 mobilized PBMSCs. The three right hand columns show the co-expression of CD markers. In
450 cells from all the rats there is low expression of CD34 but in 2 of the animals investigated
451 there is a relatively high co- expression of CD45, which is usually not found on MSCs. There
452 is high expression of CD29 and CD90, which are known MSC markers.

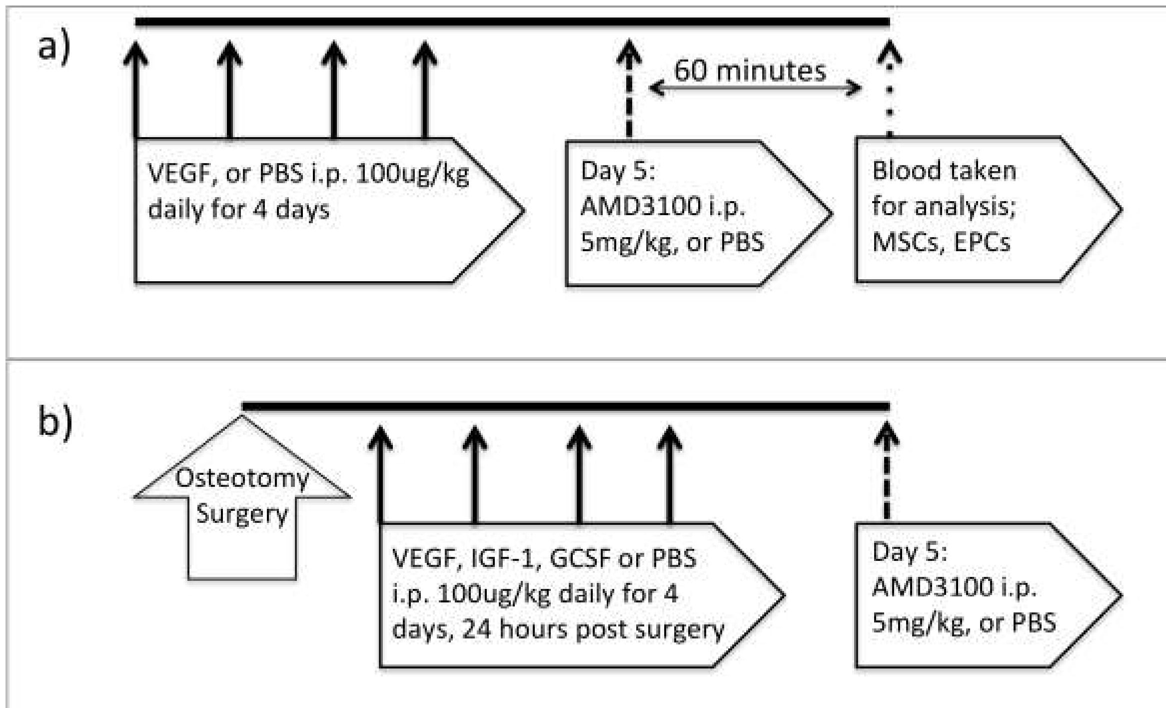
453

MicroCT parameter	1.5mm Control	VEGF-AMD
TV (um^3)	9.23±6.14	10.03±3.22
BV (um^3)	4.31±3.08	5.22±1.71
TV/BV (%)	53.79±20.82	52.52±5.85
TS (um^2)	62.83±45.55	63.56±19.88
BS (um^2)	326.15±220.05	355.52±130.15
Tb.Th (um)	0.04±0.01	0.05±0.01
Tb.Sp (um)	0.07±0.03	0.08±0.02
Tb.N (1/um)	14.09±9.32	10.99±1.08
Total Porosity (%)	46.21±20.82	47.48±5.85

454 Table 2: MicroCT quantitative morphometry indices of bone formation within the 60% of the
455 osteotomy gap where TV (um^3)= tissue volume, BV (um^3) = bone volume, TV/BV (%) =
456 percentage bone volume, TS (um^2) = tissue surface, BS (um^2) = bone surface, Tb.Th (um)
457 = trabecular thickness, Tb.Sp (um) = trabecular separation, Tb.N (1/um) = trabecular
458 number. MicroCT analysis of the mobilized group showed a higher mean bone volume than
459 controls as well as increased trabecular thickness. The overall data spread was analyzed
460 using a Levene's test for equality of variance and found to be significantly reduced in VEGF-
461 AMD3100 group for tissue volume (TV um^3, p=0.036) and trabecular number (p=0.048),
462 suggesting less variation in degree of healing between individuals.

463
464

465 **Figure Legends**



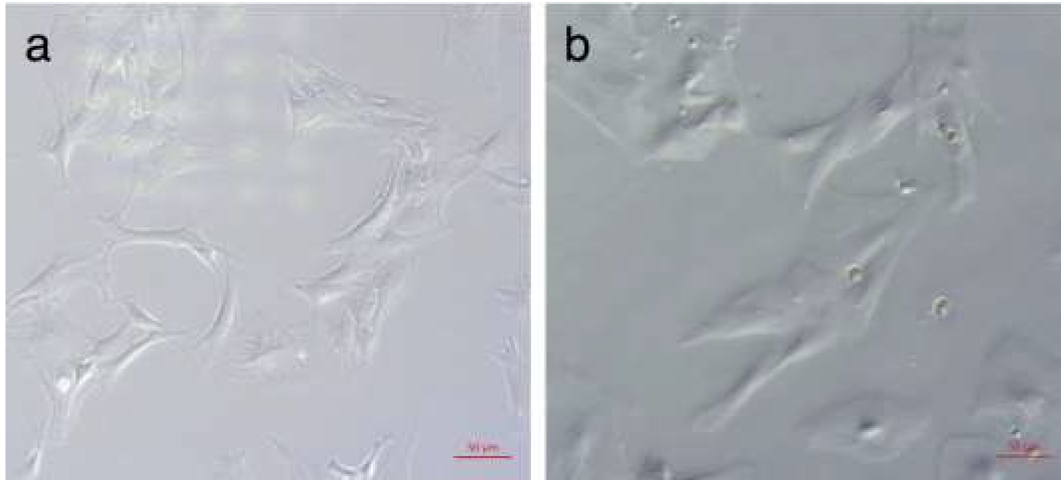
466

467 Figure 1: Experimental design and dosing schedule. Peripheral blood MSC mobilisation study

468 (a). Endogenous enhancement of fracture healing study (b).

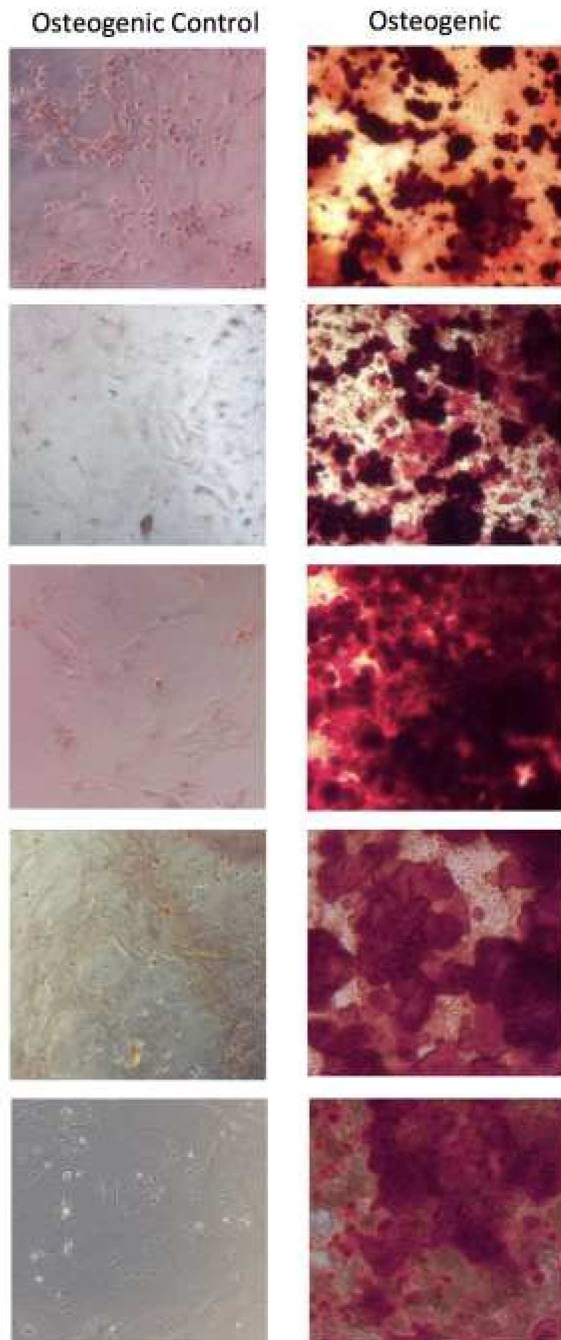
469

470



471 Figure 2: Light microscopy image (x10 magnification) of third passage bone marrow derived
472 MSCs at day seven (a), compared third passage day seven peripheral blood MSCs mobilized
473 with VEGF and AMD3100 (b). Bone marrow MSCs were obtained from the femoral shaft of
474 rats of a similar age and isolated by plastic adherence. Peripheral blood MSCs were obtained
475 by cardiac puncture 60 minutes after a single dose of AMD3100, preceded by a 4 day course
476 of VEGF, once daily, every 24hours. The cells were isolated by plastic adherence after lysing
477 the red blood cells.

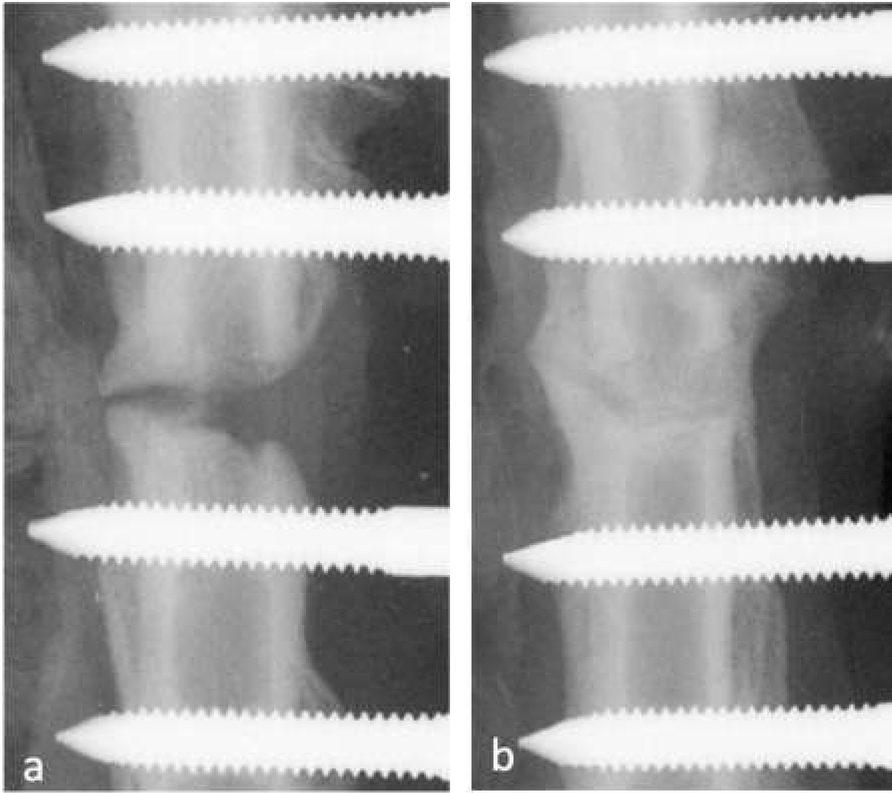
478



479

480 Figure 3: Light microscopy images (x10 magnification) of third passage cells from peripheral
481 blood MSCs in rats treated with VEGF and AMD3100. The cells were cultured with
482 osteogenic supplements for 21 days and stained with Alizarin red demonstrating mineral
483 formation. Each row represents a culture from a different rat.

484

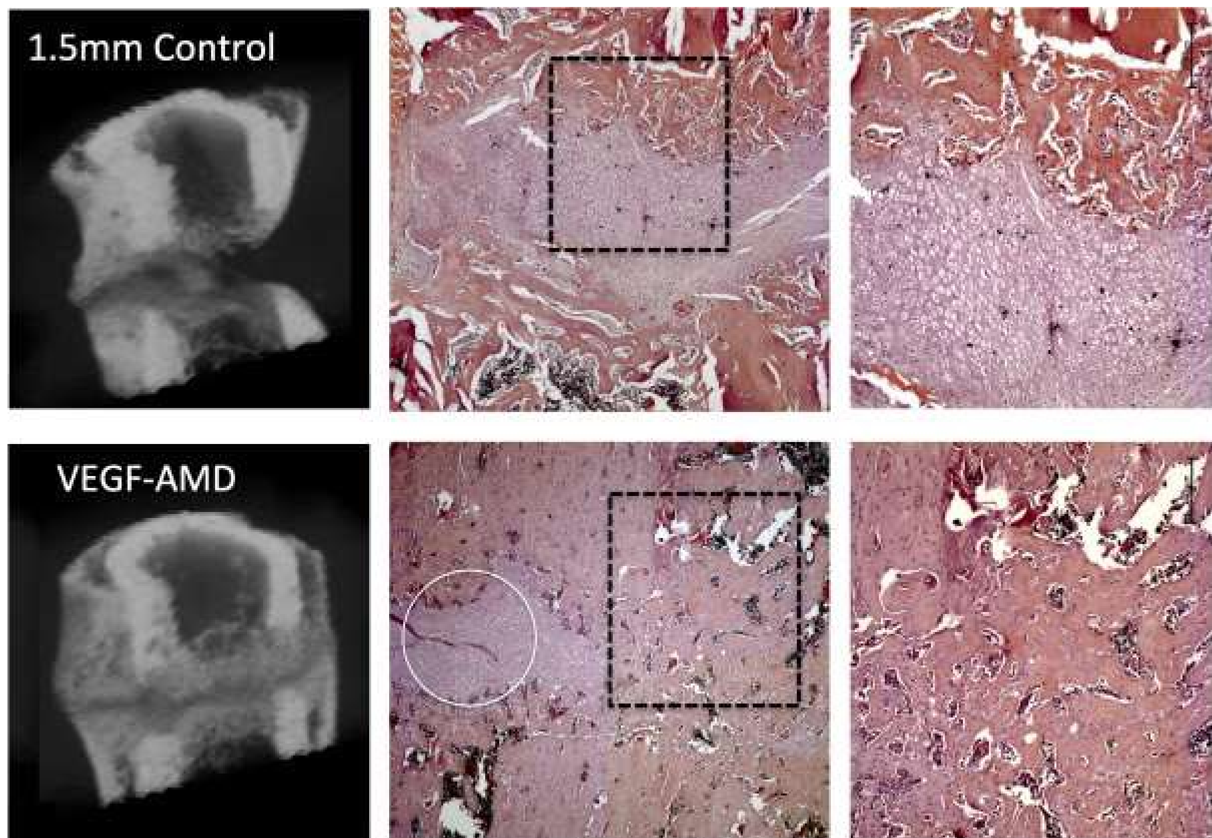


485 Figure 4: MicroCT scout radiographs, showing an example of the 1.5mm gap control group

486 (a) and the VEGF AMD3100 treated group (b) taken after 5 weeks This shows a non-union at

487 5 weeks in the control animal with union and bone formation in the gap in the treated animal.

488



489 Figure 5: MicroCT 3D reconstructions of mid femoral regions, with a mid-sagittal reveal
490 showing the associated mid sagittal histology section, stained with hematoxylin and eosin,
491 centered on the osteotomy at x2.5 magnification, and then enhanced region at x5
492 magnification. An example from a non-treated control animal is shown in the upper images
493 and a VEGF/AMD3100 treated animal in the lower images. The microCT in control groups
494 show limited union with a large gap, which has not been filled-in with bone. In the control
495 animal there is also evidence of cortical bone resorption although a periosteal callus has
496 formed. The microCT from the VEGF-AMD3100 group shows almost complete bone union
497 in the periosteal callus, and incomplete union in the endosteal callus, with the osteotomy
498 filled with mostly mineralised tissue. Histology shows that the tissue in the gap of the control
499 animal is composed of cartilage whilst cartilage is present in the gap in the treated animal
500 there are regions where bone bridges between the fracture ends have formed. The white line
501 encircles a small region of remaining cartilage within the osteotomy in the treated animal.

502 REFERENCES

503 1. Nandra R, Grover L, Porter K. 2015. Fracture non-union epidemiology and
504 treatment.Trauma 18(1):3–11.

505 2. Mills LA, Simpson AHRW. 2013. The relative incidence of fracture non-union in
506 the Scottish population (5.17 million): a 5-year epidemiological study.BMJ Open
507 3(2):e002276–e002276.

508 3. Marsell R, Einhorn TA. 2011. The biology of fracture healing.Injury 42(6):551–
509 555.

510 4. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. 1968. Heterotopic
511 of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic
512 tissues.Transplantation 6(2):230–247.

513 5. Bianco P, Cao X, Frenette PS, et al. 2013. The meaning, the sense and the
514 significance: translating the science of mesenchymal stem cells into medicine
515 [Internet].Nat Med 19(1):35–42Available from:
516 http://dx.doi.org/10.1038/nm.3028.

517 6. Kuznetsov SA, Mankani MH, Gronthos S, et al. 2001. Circulating Skeletal Stem
518 Cells.The Journal of Cell Biology 153(5):1133–1140.

519 7. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, et al. 2000. Mesenchymal
520 precursor cells in the blood of normal individuals.Arthritis Res. 2(6):477–488.

521 8. Alm JJ, Koivu HMA, Heino TJ, et al. 2010. Circulating plastic adherent
522 mesenchymal stem cells in aged hip fracture patients.Journal of Orthopaedic

523 Research 28(12):1634–1642.

524 9. Yellowley C. 2013. CXCL12/CXCR4 signaling and other recruitment and
525 homing pathways in fracture repair.Bonekey Rep 2:300.

526 10. Toupadakis CA, Wong A, Genetos DC, et al. 2012. Long-term administration of
527 AMD3100, an antagonist of SDF-1/CXCR4 signaling, alters fracture
528 repair.Journal of Orthopaedic Research 30(11):1853–1859.

529 11. Pitchford SC, Furze RC, Jones CP, et al. 2009. Differential mobilization of
530 subsets of progenitor cells from the bone marrow.Cell Stem Cell 4(1):62–72.

531 12. Broxmeyer HE, Orschell CM, Clapp DW, et al. 2005. Rapid mobilization of
532 murine and human hematopoietic stem and progenitor cells with AMD3100, a
533 CXCR4 antagonist.J. Exp. Med. 201(8):1307–1318.

534 13. Martin C, Bridger GJ, Rankin SM. 2006. Structural analogues of AMD3100
535 mobilise haematopoietic progenitor cells from bone marrow in vivo according to
536 their ability to inhibit CXCL12 binding to CXCR4 in vitro.Br. J. Haematol.
537 134(3):326–329.

538 14. De Clercq E. 2009. The AMD3100 story: The path to the discovery of a stem cell
539 mobilizer (Mozobil).Biochemical Pharmacology 77(11):1655–1664.

540 15. Lévesque J-P, Winkler IG, Larsen SR, Rasko JEJ. 2007. Mobilization of bone
541 marrow-derived progenitors.Handb Exp Pharmacol (180):3–36.

542 16. Kumar S, Ponnazhagan S. 2012. Mobilization of bone marrow mesenchymal
543 stem cells in vivo augments bone healing in a mouse model of segmental bone
544 defect.Bone 50(4):1012–1018.

545 17. Toupadakis CA, Granick JL, Sagy M, et al. 2013. Mobilization of endogenous
546 stem cell populations enhances fracture healing in a murine femoral fracture
547 model.Cytotherapy 15(9):1136–1147.

548 18. Kooistra BW, Dijkman BG, Busse JW, et al. 2010. The radiographic union scale
549 in tibial fractures: reliability and validity.J Orthop Trauma 24 Suppl 1:S81–6.

550 19. Kamal A, Iskandriati D, Dilogo I, et al. 2014. Comparison of cultured
551 mesenchymal stem cells derived from bone marrow or peripheral blood of rats.J
552 Exp Integr Med 4(1):17.

553 20. Fafián-Labora J, Fernández-Pernas P, Fuentes I, et al. 2015. Influence of age on
554 rat bone-marrow mesenchymal stem cellspotential.Nature Publishing Group :1–
555 20.

556 21. Sethe S, Scutt A, Stolzing A. 2006. Aging of mesenchymal stem cells.Ageing
557 Res. Rev. 5(1):91–116.

558 22. Sanghani-Kerai A, Coathup M, Samazideh S, et al. 2017. Osteoporosis and
559 ageing affects the migration of stem cells and this is ameliorated by transfection
560 with CXCR4.Bone and Joint Research 6(6):358–365.

561 23. Fu W-L, Li J, Chen G, et al. 2015. Mesenchymal Stem Cells Derived from
562 Peripheral Blood Retain Their Pluripotency, but Undergo Senescence During
563 Long-Term Culture.Tissue Eng Part C Methods 21(10):1088–1097.

564 24. Lee DY, Cho T-J, Lee HR, et al. 2010. Distraction osteogenesis induces
565 endothelial progenitor cell mobilization without inflammatory response in
566 man.Bone 46(3):673–679.

567 25. Kidd LJ, Stephens AS, Kuliwaba JS, et al. 2010. Temporal pattern of gene
568 expression and histology of stress fracture healing.Bone 46(2):369–378.

569 26. Knight MN, Hankenson KD. 2013. Mesenchymal Stem Cells in Bone
570 Regeneration.Advances in Wound Care 2(6):306–316.

571 27. Pittenger MF, Mackay AM, Beck SC, et al. 1999. Multilineage potential of adult
572 human mesenchymal stem cells.Science 284(5411):143–147.

573 28. Muraglia A, Cancedda R, Quarto R. 2000. Clonal mesenchymal progenitors from
574 human bone marrow differentiate in vitro according to a hierarchical
575 model.Journal of Cell Science 113 (Pt 7):1161–1166.

576 29. Boxall SA, Boxall SA, Jones E, Jones E. 2012. Markers for Characterization of
577 Bone Marrow Multipotential Stromal Cells.Stem Cells International 2012(5):1–
578 12.

579 30. Dominici M, Le Blanc K, Mueller I, et al. 2006. Minimal criteria for defining
580 multipotent mesenchymal stromal cells. The International Society for Cellular
581 Therapy position statement.Journal of Cytotherapy 8(4):315–317.

582 31. Yeh S-P, Chang J-G, Lo W-J, et al. 2006. Induction of CD45 expression on bone
583 marrow-derived mesenchymal stem cells.Leukemia 20(5):894–896.

584 32. Fu WL, Zhou CY, Yu JK. 2014. A New Source of Mesenchymal Stem Cells for
585 Articular Cartilage Repair: MSCs Derived From Mobilized Peripheral Blood
586 Share Similar Biological Characteristics In Vitro and Chondrogenesis In Vivo as
587 MSCs From Bone Marrow in a Rabbit Model.The American Journal of Sports
588 Medicine 42(3):592–601.

589 33. Fu W-L, Zhang J-Y, Fu X, et al. 2012. Comparative study of the biological
590 characteristics of mesenchymal stem cells from bone marrow and peripheral
591 blood of rats.Tissue Eng Part A 18(17-18):1793–1803.

592 34. Kassis I, Zangi L, Rivkin R, et al. 2006. Isolation of mesenchymal stem cells
593 from G-CSF-mobilized human peripheral blood using fibrin microbeads.Bone
594 Marrow Transplant. 37(10):967–976.

595 35. Harrison LJ, Cunningham JL, Strömberg L, Goodship AE. 2003. Controlled
596 induction of a pseudarthrosis: a study using a rodent model.J Orthop Trauma
597 17(1):11–21.

598 36. Xu J, Chen Y, Liu Y, et al. 2017. Effect of SDF-1/Cxcr4 Signaling Antagonist
599 AMD3100 on Bone Mineralization in Distraction Osteogenesis.Calcif Tissue Int
600 100(6):641–652.

601 37. Devescovi V, Leonardi E, Ciapetti G, Cenni E. 2008. Growth factors in bone
602 repair.Chir Organi Mov 92(3):161–168.

603 38. Sarahrudi K, Thomas A, Braunsteiner T, et al. 2009. VEGF serum concentrations
604 in patients with long bone fractures: A comparison between impaired and normal
605 fracture healing.J Orthop Res 27(10):1293–1297.

606 39. Street J, Bao M, deGuzman L, et al. 2002. Vascular endothelial growth factor
607 stimulates bone repair by promoting angiogenesis and bone turnover.Proc Natl
608 Acad Sci USA 99(15):9656–9661.

609 40. Gerber HP, Vu TH, Ryan AM, et al. 1999. VEGF couples hypertrophic cartilage
610 remodeling, ossification and angiogenesis during endochondral bone

611 formation.Nat Med 5(6):623–628.

612 41. Stegen S, van Gastel N, Carmeliet G. 2015. Bringing new life to damaged bone:
613 the importance of angiogenesis in bone repair and regeneration.Bone 70:19–27.

614 42. Meng F, Rui Y, Xu L, et al. 2014. Aqp1 enhances migration of bone marrow
615 mesenchymal stem cells through regulation of FAK and β-catenin.Stem Cells
616 Dev 23(1):66–75.

617 43. Xu L, Huang S, Hou Y, et al. 2015. Sox11-modified mesenchymal stem cells
618 (MSCs) accelerate bone fracture healing: Sox11 regulates differentiation and
619 migration of MSCs.FASEB J. 29(4):1143–1152.

620