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Systemic recruitment of osteoblastic cells in fracture healing

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Abstract

We hypothesise that following a bone fracture there is systemic recruitment of bone forming cells to a fracture site. A rabbit ulnar osteotomy model was adapted to trace the movement of osteogenic cells. Bone marrow mesenchymal stem cells from 41 NZW rabbits were isolated, culture-expanded and fluorescently labelled. The labelled cells were either re-implanted into the fracture gap (Group A); into a vein (Group B); or into a remote tibial bone marrow cavity 48 h after the osteotomy (Group C) or 4 weeks before the osteotomy was established (Group D), and a control group (Group E) had no labelled cells given. To quantify passive leakage of cells to an injury site, inert beads were also co-delivered in Group B. Samples of the fracture callus tissue and various organs were harvested at discrete sacrifice time-points to trace and quantify the labelled cells. At 3 weeks following osteotomy, the number of labelled cells identified in the callus of Group C, was significantly greater than following IV delivery, Group B, and there was no difference in the number of labelled cells in the callus tissues, between Groups C and A, indicating the labelled bone marrow cells were capable of migrating to the fracture sites from the remote bone marrow cavity. Significantly fewer inert beads than labelled cells were identified in Group B callus, suggesting some of the bone-forming cells were actively recruited and selectively chosen to the fracture site, rather than passively leaked into the circulation and to bone injury site. This investigation supports the hypothesis that some osteoblasts involved in fracture healing were systemically mobilised and recruited to the fracture from remote bone marrow sites.

Keywords: Fracture healing; Osteoblast recruitment; MSCs; Circulation; PHK 26

Introduction

The delayed union or non-union of a fracture is often predictable in the presence of adverse trauma biomechanics and patient characteristics. Long bone fracture non-union or delayed union rates are between 5% and 20% and cause significant patient morbidity [15,25]. Furthermore, modern orthopaedic procedures are biologically dependant on optimal osteotomy repair and such scheduled surgery provides an opportunity to manipulate the bone biology to enhance healing.

Successful fracture healing is a complex interplay of angiogenesis and osteogenesis on the stage of a broken bony scaffold. Osteogenesis is dependant on an adequate number of osteoblasts, which are recruited predominantly from the local bone marrow and periosteal cambial layer [39]. The stromal compartment of the marrow contains many multi-potential mesenchymal stem cells (MSCs) [17,18,29], which can be induced to differentiate into osteoblasts in vitro and in vivo and form bone [3,6,19]. Ongoing research into the biology of MSCs shows that they have the ability to enhance fracture

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healing in long bone fractures when locally delivered to the fracture site in several species [8,10,26]. MSCs administered intravenously have been shown to engraft into bone marrow tissues and bone preferentially [20,30]. Clinical trials have not identified any toxicity following re-implantation of ex vivo expanded stem cells [23]. Recent literature demonstrated that intravenously delivered MSCs will localise to a fracture site in murine models [12,13], and when modified with insulin-like growth factor 1 will potentate fracture healing [35].

Controversy surrounds the presence of MSCs in normal adult peripheral blood. Early studies by haematologists failed to identify MSCs in the blood [24,28]. However, recent data supports the concept that a very small number of MSCs circulate in normal adult blood [22,32,40]. The presence of stem cells in umbilical cord blood is well established, where the numbers of mesenchymal and haematopoietic stem/progenitor cells declines with gestation [9,16,34]. Unpublished data from our laboratory has shown that a larger number of osteoblast-like cells were cultured from 15 ml of peripheral blood from fracture patients in contrast to the blood of normal controls. Furthermore, there was a significant time-dependant increase in the number of the osteoblast-like cells over time post-fracture. Explanations for this phenomenon include passive cell mobilisation, via damaged vasculature at the fracture site into the blood or a systemic recruitment from remote marrow in response to the traumatic stimuli. The anabolic osteogenic effect of fracture on contralateral limbs in a fracture model is well established [15]. Therefore, based on our preliminary clinical observation, we hypothesise that in response to a distant skeletal injury, there is a systemic mobilisation and recruitment of osteoblastic precursors to the fracture site, via the peripheral circulation.

Materials and methods

MSCs harvesting and culture expansion

Forty-one adult male (2–3 kg) New Zealand White rabbits had bone marrow harvested from the right tibia, at three weeks (Groups A, B and C) or 7 weeks (Group D) prior to the creation of an open 3 mm ulnar osteotomy defect. A 2 mm hole was drilled into the proximal tibia and the marrow aspirated by flushing the intramedullary cavity with heparinised saline. The mononuclear cells were isolated from the whole marrow sample by centrifugation across a Lymphoprep™ density gradient (Nycomed, Norway) and seeded at density of $2 \times 10^4/cm^2$ for culture expansion over three weeks in DMEM supplemented with 10% FCS. The culture conditions were optimised for osteoblastic differentiation of the plastic-adherent mesenchymal cells with 10^{-7} M dexamethasone, 10 mM β-glycero-phosphate and 50 μg/ml ascorbate-2-phosphate [7,38]. The mean total number of MSCs after 3 weeks of culture was 3.0×10^6, with a range of 1.2×10^6–5.5×10^6 cells. Osteoblastic phenotype was confirmed prior to re-implantation by positive immunocytological and histological staining for osteocalcin, collagen type-1, Cbfa-1, vimentin, BMP-2, BMPR-2, alkaline phosphatase and the in vitro formation of calcified nodules [4,5,7,14,33]. Our in vitro cell culture expansion data demonstrated a cell increase by a factor of 6156 over 3 weeks. Following culture expansion approximately 1.5×10^6 mesenchymal or osteogenic cells per animal were selected for labelling and re-implantation.

Cell labelling

The autologous cells were labelled with a fluorescent dye (PKH-26, PKH fluorescent cell linker kits for general cell membrane labelling, Sigma-Aldrich, Poole, UK) according to the manufacturer's instructions. PKH-26, a red fluorochrome, has excitation (551 nm) and emission (567 nm) characteristics compatible with rhodamine or phycoerythrin detection systems. This easy-to-use PKH-26 is physiologically stable for up to 4 months in vivo and showed little to no toxic side effects on cell systems; the labelled cells retain both biological and proliferative activity, and are ideal for cell tracking and cell–cell interaction studies [21,36]. Preliminary experiments were conducted in our laboratory to confirm that the dye had no effect on cell viability, morphology and marker expression. To determine the in vitro durability of the PKH-26 label, labelled cells were maintained in culture up to 21 weeks and the percentage of cells retaining label quantified at day 0, day 2 and 3, 4, 6, 12 and 21 weeks, was 100%, 98%, 80%, 80%, 60%, 40% and 20% respectively (data not shown). In all in vivo experiments, cells were rested in the standard culture conditions for 24 h between PKH-26 labelling and re-implantation.

Experimental groups

Approval from the Department of Health, Social Services and Public Safety, Northern Ireland complying with the animals (Scientific Procedures) act 1986 of UK, was obtained for this investigation. Animals were anaesthetised by intramuscular injection of Hypnorm (0.2 ml/kg, Janssen Animal Heath, High Wycombe, England) combined with Midazolam (Hypnovel, 0.5 mg/kg, Roche, Welwyn Garden City, England) for all invasive procedures.

Rabbits were randomly allocated into five experimental groups as shown in Table 1. In Groups A–D, the autologous labelled cells were either re-introduced directly into the osteotomy gap under radiological imaging (Group A, $N = 9$); into an ear vein (Group B, $N = 13$); or into the virgin left tibial bone marrow cavity (Group C, $N = 6$ and D, $N = 6$). For re-implantation into the marrow a further proximal drill hole was made and a purse-string suture placed before injecting the labelled cells. Immediately, after injection the purse string was tied and the hole plugged with 0.5 ml of autologous blood to minimise local cell ooze. In Groups A–C the labelled cells were re-implanted 48 h after the fracture had been created, to permit haemostasis. In Group D, the labelled cells were re-implanted into the left tibial bone marrow cavity 4 weeks prior to the ulnar osteotomy, to permit mesenchymal cell establishment within an in vivo niche prior to the traumatic insult. A control group (Group E, $N = 7$) did not receive any labelled cells. In addition, to quantify the rate of passive cell leakage from the blood into the osteotomy site, biologically inert, 2 μm, microspheres/beads (Polysciences, Germany) were intravenously delivered to some animals ($N = 7$) in Group B. Approximately, 1.5×10^9 green-fluorescent beads (1000 times the number of the injected PKH-labelled cells) were delivered 30 s after the PKH-labelled cells injection. If both cells and beads leaked passively we would expect to find ~1000 times more beads than cells in the tissue concerned.

Ulnar osteotomy, animal sacrifice and tissue retrieval

A 5-cm skin incision was made over the subcutaneous border of the left ulnar and a proximal ulnar osteotomy was created using an oscillating saw, leaving a 3 mm osteotomy gap. The incision was closed and the rabbits were allowed to weight-bear on the operated forearm. In all groups, animals were sacrificed at either 3 or 12 weeks after creation of the osteotomy. In addition, three animals were killed at 3 days and 1-week post-fracture, in Group B. The animals in Group B who had received green-fluorescent beads were killed at 3 days, 1 and 3 weeks post-surgery ($N = 3, 2, 2$ respectively).

At the time of sacrifice, representative samples from the osteotomy gap callus tissue, lung, liver, spleen, kidney, blood and marrow (from the original harvested tibia) were selected from all animals. All solid tissue samples were rapidly frozen in liquid nitrogen and stored in

Table 1
Details of experimental groups

Experimental group	Treatments	Number of animals sacrificed at different time following osteotomy			
	(Short descriptor)	3 days	1 week	3 weeks	12 weeks
A (gap)	Labelled bone marrow cells were directly injected into fracture gap 48 h after ulnar osteotomy	–	–	6	3
B (cells)	Labelled bone marrow cells were delivered intravenously 48 h after ulnar osteotomy	3	3	4	3
B (beads)	Microspheres (beads) were delivered IV immediately after the cells delivery at 48 h after ulnar osteotomy	3	2	2	–
C (after)	Labelled bone marrow cells were implanted into remote tibia marrow site, 48 h after ulnar osteotomy	–	–	3	3
D (prior)	Labelled bone marrow cells were implanted into remote tibia marrow site, 4 weeks prior to ulnar osteotomy	–	–	3	3
E (none)	Animals had osteotomy but no labelled cell re-implanted	–	–	4	3

the dark at −70 °C until sectioned. Fluid samples were centrifuged over a Lymphoprep™ density gradient and the mononuclear cells were spun onto glass slides and stored at −70 °C until further examination.

Quantification of PKH-26-labelled cells

Seven micrometers of frozen sections were cut from all solid organ tissues and examined under a confocal fluorescent microscope. The numbers of red PKH-26-labelled fluorescent cells were recorded for each tissue from five randomly selected high powered (×400) fields, in each animal and a mean number was derived. Cytospin slides from the blood and marrow samples were examined in a similar fashion and the labelled cells expressed as a percentage of the total cells in five randomly selected fields. For inter-observer reliability, two independent observers examined six random sections and the differences in the numbers of PKH-labelled cells or inert beads identified by each observer did not significantly differ, ($p = 0.94$).

Statistical analysis

The mean numbers of PKH-labelled cells in each tissue for each animal were transferred to statistical spreadsheets and analysed using a commercially available statistical program SPSS (Version 11, Chicago, Illinois, USA). The non-parametric Mann-Whitney-U test was applied and any difference was considered significant at $p < 0.05$.

Results

PKH-labelled cells in the osteotomy gap callus at 3 and 12 weeks

At 3 weeks following osteotomy, the labelled cells were found on the trabecular surfaces in all sections of callus in Groups A–D (Fig. 1A and B) and mean numbers of labelled cells found in the callus tissues were 28.4 in Group A; 11.8 in Group B; 23.5 in Group C and 3.0 in Group D (Fig. 2A). No PKH-labelled cells were identified in the callus tissues of control Group E. The number of labelled cells in the gap tissues of Group A and Group C was significantly greater than those in Group B ($p = 0.05$ and $p = 0.03$ respectively). There was no statistical difference ($p = 0.3$) between the number of labelled cells in the callus of Groups A (the cells were directly implanted back into osteotomy site) and Group C (the cells were re-implanted into the remote bone marrow). The number of cells identified in the fracture gap tissue from experimental group D was statistically less than those seen in Group C at 3 weeks, $p = 0.05$.

At 12 weeks there was evidence that small numbers of PHK labelled cells in all groups had characteristics of osteocytes, confirmed by surrounding osteoid, see confocal microscopy sequential images (Fig. 1D–I). At 12 weeks following osteotomy, the mean numbers of labelled cells in the callus tissues in Groups A–E were 1.1, 3.5, 0.8, 1.5 and 0 respectively (Fig. 2B). There was no statistical difference in the numbers of labelled cells between Groups A and C ($p = 0.38$). There were significantly less labelled cells in the osteotomy gap tissues in Groups A and C than that in Group B ($p = 0.05$ and $p = 0.05$ respectively). At 12 weeks, there is no difference in the numbers of labelled cells identified in the fracture gap tissue between experimental groups C and D, $p = 0.26$ (The cells in experimental group D were labelled 4 weeks earlier than those in experimental group C.).

The changes in the numbers of labelled cells in the callus tissues over time are summarised in Fig. 2C. Only in Group D (the labelled cells were permitted to engraft in the marrow 4 weeks prior to the osteotomy) that there was no significant decrease in the number of labelled cells in the osteotomy gap tissue, between week 3 and 12 post-surgery ($p = 0.28$). In Groups A, B and C the number of labelled cells in the osteotomy gap decreased significantly over time between 3 and 12 weeks ($p = 0.02, 0.03$ and 0.05 respectively).

Following intravenous delivery, in Group B the animals were sacrificed at 4 time points. There was an initial insignificant fluctuation in the number of labelled cells in the osteotomy gap tissue between 3 days and 1 week ($p = 0.18$); a significant increase from 1 week to 3 weeks ($p = 0.03$); and a decrease from 3 weeks to 12

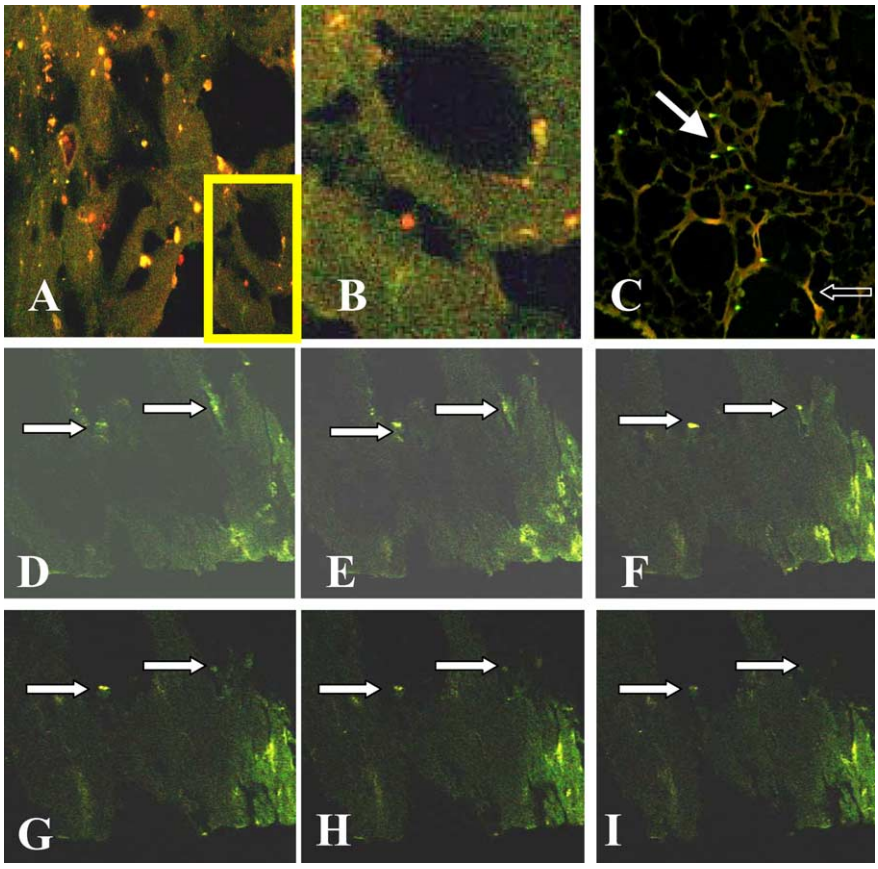

Fig. 1. (A) Representative histology of a specimen of osteotomy gap callus from Group C. The red-yellow PKH-labelled cells visualised at the surfaces of newly formed callus. (B) A higher magnification of boxed area in A, showing the PKH-labelled cells on the bone surface in a position corresponding to osteoblasts. (C) At 12 weeks post-osteotomy, green beads/microspheres were identified in the osteotomy gap tissues (solid arrow heads) together with the red PKH-labelled cells (empty arrow heads). (D–I) Sequential confocal microscopy images through a specimen of callus retrieved from Group C at 12 weeks after the osteotomy. Two yellow-red PKH-labelled cells (arrow heads) on the bone surface (D), are surrounded by bone matrix as the confocal images moved to deeper layers (E–I), confirming that the two PKH-labelled cells are indeed osteocytes. A, ×200; B, ×400; C, ×100; D–I, ×200 magnifications.

weeks ($p = 0.04$). Overall, the number of labelled cells in the osteotomy gap tissues in Group B decreased with time ($p = 0.05$) between 3 days and 12 weeks.

PKH-labelled cells identified in other tissues

At 3 and 12 weeks post-osteotomy, labelled cells were also identified in the marrow, blood, liver, spleen and lung in all animals in Groups A–D, whereas PKH-labelled cells were rarely visualised in the renal cortex (Fig. 3). No labelled cells were identified in any of the tissues from control Group E. In the groups where the PKH-labelled cells were delivered either intravenously or within the left tibial bone marrow cavity (Groups B–D), a large number of cells homed to the right tibial marrow sampled at 3 weeks (Fig. 3B) and 12 weeks post-osteotomy (Fig. 3D). At 3 weeks, the number of labelled cells in the marrow of Group D (labelled cells had been re-implanted into the right tibial bone marrow cavity 4 weeks before the left ulnar osteotomy) was less than that in Group C (labelled cell re-implanted into the right tibial bone marrow cavity 48 h after the left ulnar osteotomy). In each of the groups where PKH-labelled cells were re-implanted (Groups A–D), a large number of labelled cells were visualised in the spleen at 3 or 12 weeks after the osteotomy (Fig. 3A and C). In Groups A–C, a number of PKH-labelled cells were identified within the peripheral blood at 3 weeks after re-implantation and in Group D 7 weeks after marrow delivery (Fig. 3B). Whilst comparisons between different tissue types assumes identical engraftment mechanisms, the numbers of labelled cells visualised in the osteotomy gap tissues, spleen and marrow in Groups A–D, were generally greater than those in the other tissues at 3 weeks post-implantation (Fig. 3A–D).

Numbers of inert beads and PKH-labelled cells in all the tissues

In Group B some animals had inert beads injected immediately after the intravenous labelled cells and these beads were quantified in all harvested tissues.

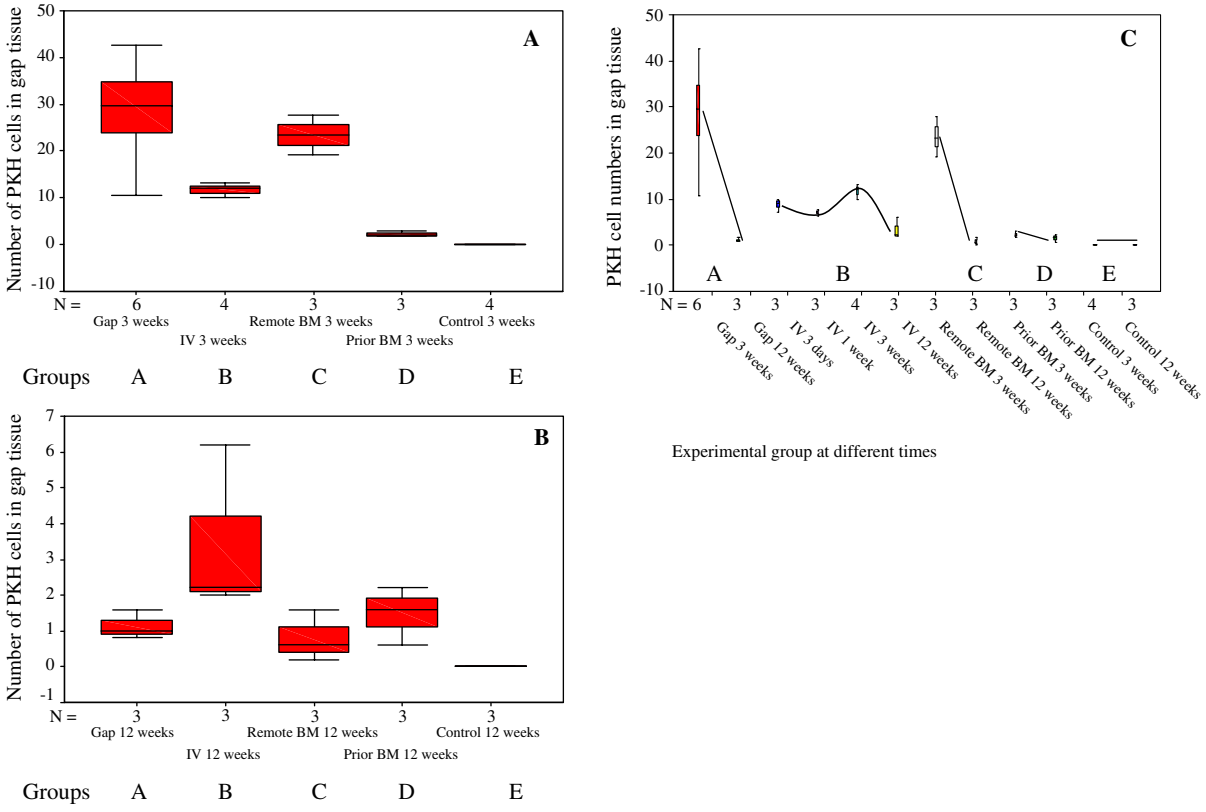

Fig. 2. (A) Scatter plot showing the number of PKH-labelled cells in each experimental group in the callus tissues harvested at 3 weeks after the osteotomy. (B) The number of PKH-labelled cells identified in the callus tissues when the animals were sacrificed at 12 weeks after creation of the osteotomy. (C) Trend of PKH-labelled cells decline over time within osteotomy gap tissues in all groups.

The numbers of PKH-labelled cells in the osteotomy gap tissues were greater than the numbers of florescent beads seen at 3 days, 1 week and 3 weeks ($p = 0.05, 0.08$ and 0.06 respectively, Fig. 4). Statistical analysis also demonstrated there was no significant difference between the numbers of beads visualised in the osteotomy gap callus at all time points examined: $p = 0.24$, between 3 and 1 week; $p = 0.68$ between 1 and 3 weeks; $p = 0.14$, between 3 days and 3 weeks. In contrast to the inert beads, there was a significant increase in the numbers of PKH-labelled cells in the osteotomy gap tissues between weeks 1 and 3 ($p = 0.03$) and between 3 days and 3 weeks ($p = 0.04$). There was no significant difference between the number of labelled cells seen between 3 days and 1 week ($p = 0.18$). There was an overall significant decrease in PKH-labelled cell number in the osteotomy gap callus at 12 weeks post-osteotomy ($p < 0.05$, between 3 days and 12 weeks, Fig. 4).

The numbers of beads identified in all tissue types examined are summarised in Table 2. The numbers of beads identified in the osteotomy gap tissues were significantly smaller than the number visualised in other solid tissues, particularly early in the spleen. There was no significant change in the number of beads visualised in each tissue type over time. However, there was a trend for the number of beads to decrease in the lung, liver and spleen over time, at a rate that almost reaches significance ($p = 0.08$ for each). Within the first 3 weeks, there was a significant decrease in the numbers of PKH-labelled cells seen in the lung, liver, spleen and blood of the host animal. There was a significant decrease in the number of PKH-labelled cells in the blood ($p = 0.05$) or in the bone marrow ($p = 0.05$) within the first week, see Table 2 for the p values.

Discussion

This study was undertaken to test the hypothesis that a proportion of the osteoblasts integral in fracture healing are systemically recruited from the bone marrow (or circulation) to the fracture site. The presence of PKH-26 labelled cells in the callus of Groups C and D animals supports this hypothesis. However, on further analysis we found something interesting. Our data at 3 weeks showed no significant difference in the number of PKH cells found in the fracture callus between Groups A and C. This can be rationalised in 2 ways, firstly, it is accepted that not all cells that appear at the trauma site initially are retained there for 3 and 12 weeks. Secondly, the engraftment to marrow may have permitted rapid proliferation of these cells so greater numbers were

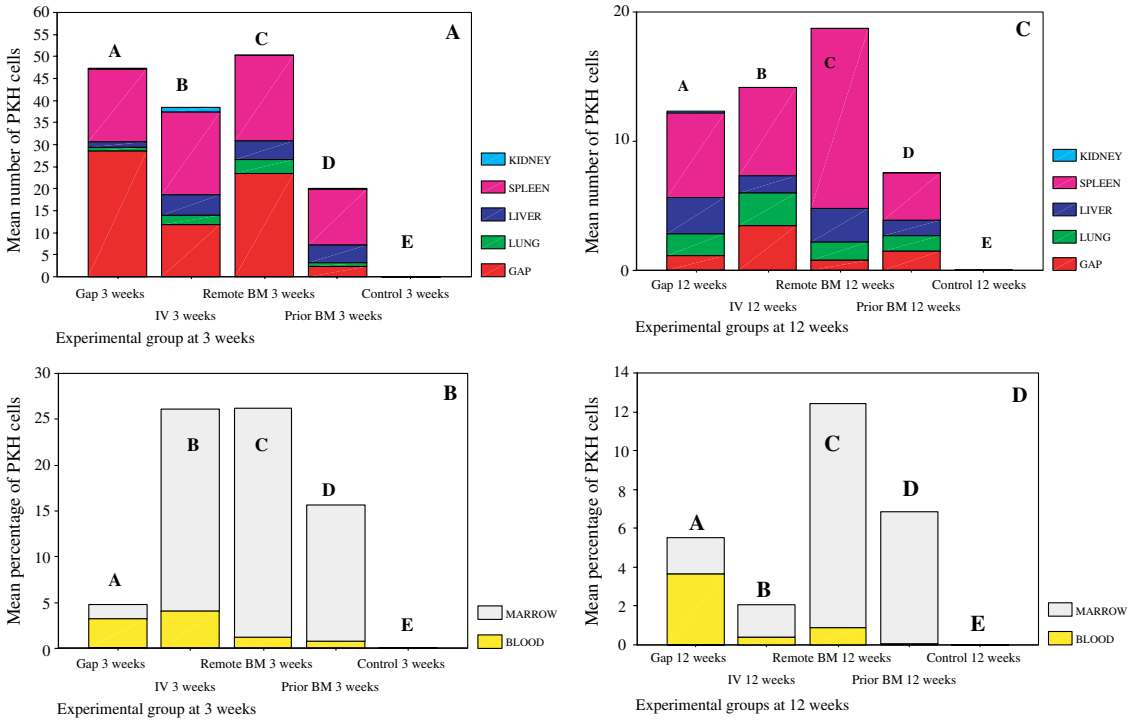

Fig. 3. (A) A bar graph comparing the mean number of PKH-labelled cells in samples of various solid tissues, harvested 3 weeks after osteotomy in each group. (B) A bar graph comparing the mean percentage of PKH-labelled cells in representative samples of blood and marrow at 3 weeks after osteotomy in each group. (C) The number of PKH-labelled cells identified in the various samples of solid organ tissues 12 weeks after osteotomy. (D) The number of PKH-labelled cells identified in the samples of blood and marrow at 12 weeks after osteotomy. Name of each group is labelled on the top of the bars.

available for recruitment. This second argument is supported by the observation that a larger number of PKH-labelled cells were identified in Group C callus when compared to Group B. This supports an advantage of remote marrow re-implantation of labelled MSCs, over their intravenous delivery, which can be explained by in vivo proliferation and subsequent active recruitment.

However, at 3 weeks the number of PKH cells found in the osteotomy gaps of Group D animals was statistically less than that in Group C. The expected advantage of permitting PKH-labelled cell establishment within marrow niches, before onset of skeletal trauma, was not supported by this data. However, this result may be a reflection of the failing PKH longevity as those cells in Group D were labelled and re-implanted in vivo for 4 weeks longer than those in Group C, by the time of sacrifice. In Groups A, B and C there was a significant drop off in the number of PKH-labelled cells identified in the osteotomy callus between 3 and 12 weeks. If we accept that the decline is invariably due, at least in part, to those parameters discussed below, the observation of no differences between the 3 and 12 weeks number of PKH-labelled cells in Group D gap tissues is important. This can be explained if we accept that any cells recruited are integral and therefore not lost, so supporting an advantage to the prior engraftment to physiological niches on subsequent donor cell activity.

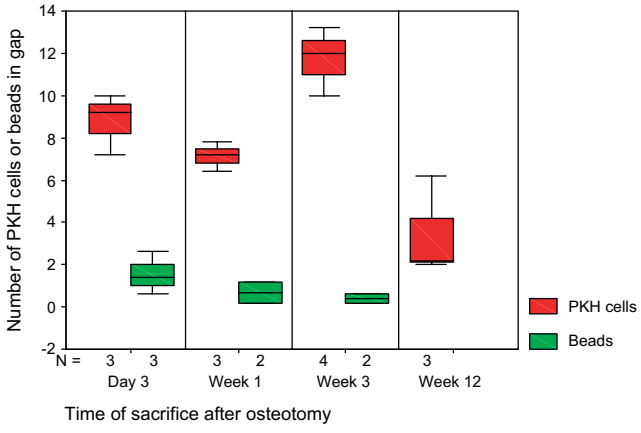

Fig. 4. The number of PKH-labelled cells and the florescent inert beads identified in the osteotomy gap tissue at various time points of sacrifice when the florescent inert beads were delivered immediately after the PKH-labelled cells injection 48 h after osteotomy.

It is possible that in Groups C and D the PKH cells are only present in the callus due to random leakage into these tissues as would be expected to occur into any vascularised and traumatised tissue. One way to show selective recruitment is to show preferential recruitment to a particular tissue. Unfortunately, it is a certainty that the mechanisms by which the PKH-labelled cells were captured in each tissue will differ for each tissue type so that

Table 2
Comparison the number of labelled cells/beads in all tissues over time in Group B

Tissue sampled	Cell/bead delivered	Time of comparison				Significant difference
		3 days–1 week	1 week–3 weeks	3 weeks–12 weeks	3 days–3 weeks	
Gap	Cells	0.18	0.03	0.03	0.05	↑
	Beads	0.25	0.68	–	0.14	–
Lung	Cells	0.51	0.03	1.00	0.05	↓
	Beads	0.08	0.12	–	1.00	–
Liver	Cells	0.51	0.03	0.29	0.16	↓
	Beads	0.08	0.68	–	0.08	–
Spleen	Cells	0.13	0.29	0.21	0.03	↓
	Beads	0.25	0.44	–	0.08	–
Kidney	Cells	0.38	0.59	0.08	0.29	–
	Beads	0.54	0.32	–	0.20	–
Blood	Cells	0.05	0.29	0.08	0.03	↓
	Beads	0.76	0.44	–	0.25	–
Marrow	Cells	0.05	0.74	0.03	0.03	↑
	Beads	0.37	1.00	–	0.37	–

direct comparisons between tissues of our data cannot be reliably drawn. There is literature published that records preferential homing of stem cells to skeletal tissues and that there is significant capture within the lympho-haemopoietic tissues and lungs [1,2,11–13,27,31,32,35].

Our 3-week data supports the preferential homing of the PKH-labelled cells to the marrow and sites of skeletal injury, and capture in the spleen as shown in Fig. 3 and Table 2. At 12 weeks there were more PKH labelled cells in the marrow of Group C than in Groups A and B. In Group B, it was only within the marrow and gap tissues that a significant increase of PKH-labelled cells occurred over time, following their intravenous delivery. This was in agreement with previous findings in rodent bone repair models [13,20,37]. At 12 weeks the continued presence of PKH-labelled cells found in the spleen, liver and lung may represent a secondary mobilisation of cells within the animal, suggesting that the systemic recruitment of bone marrow MSCs into peripheral circulation is a continuous process throughout the entire period of fracture healing. An alternative explanation would be that the captured PKH-labelled cells in these tissues have integrated into these tissues and became parts of the tissue cells. It is unlikely the labelled cells were captured in these tissues and left in situ for up to 12 weeks without degrading or differentiating. Another possible but unlikely explanation is that some of the PKH-labelled cells have been degraded by macrophages, and that PKH-labelled membranes phagocytosed and then incorporated within the macrophage membranes cannot be distinguished from the intact PKH-membrane labelled cell.

The presence of PKH cells in the blood at each time point supports the concept that these cells migrate within the circulation in animals from the marrow niches in Groups C and D. It has been proposed that passive leakage of cells can occur at traumatised capillaries. Our experimental model in Groups A–C permitted 48 h after fracture, before the cells were re-implanted. This would have permitted the haematoma stage to be developed and haemostasis to be established. Furthermore, when re-implanting the cells into the marrow cavity any leakage into soft tissues via the drill hole was minimised by the suture and blood clot.

The issue of passive cell leakage was further investigated and quantified in the present study by the addition of inert beads. The number of beads delivered was approximately 1000 times the number of labelled cell delivered at each time. Also some of the beads were ~10 times smaller than many mature osteoblastic labelled cells and so potentially more diffusible. A significant increase in the labelled cell number at the osteotomy site was found between 3 days and 3 weeks, whereas the number of beads found at the osteotomy site did not differ over time. There were significantly more PKH-labelled cells than beads found in the osteotomy gap tissues at 3 days, 1 week and 3 weeks. It is probable, that the beads found in the osteotomy gap and other tissues were within the vasculature, however, the PKH tracking method was not robust enough to permit simultaneously immuno-histochemical identification of blood vessels in the present study.

Taken together, this data strongly support that any passive leakage at the traumatised capillaries was minimal and supports active recruitment and homing of the MSCs to the site of bone injury. However, some limitations must be highlighted when interpreting these results: the rate of PKH decay and the effect of cellular proliferation. The PKH durability in vitro demonstrated significant label loss by cells between day 1 and week 3

(20%) and between day 1 and week 12 (50%), so any decrease in the number of PKH-labelled cells identified over time in vivo may represent a false finding due to failed PKH longevity. Furthermore this may represent an increase in cell proliferation leading to the division of labelled membrane. In addition, the percentage of cells in more mature stages of differentiation with decreased proliferation, increase with time in vivo. At 12 weeks, relatively small numbers of PKH-labelled osteocytes were identified per field compared to the 3-week time point and in part this reflects the effect of bone maturity on cell density. In addition, the lacunae visualised without a labelled cell may represent one of 3 variables: the osteocyte within is non-labelled, or the osteocyte missing as a result of the sectioning process or the lacunae is vacant. As some of these missing cells may have been PKH labelled, we may have underestimated the numbers and any comparisons between time-points are made with caution.

In summary, the data from this study confirmed that PKH labelling is a useful tracking tool for following the recruitment of osteoblastic precursors in vivo. The significantly larger number of labelled cells than the inert beads in the injured tissues supports the active recruitment of MSCs to the fracture site. Furthermore, the data presented supports the preferential, active homing of PKH labelled, autologous, mesenchymal osteoblastic cells to the marrow and fracture sites, following delivery either intravenously or into the remote marrow cavities. This study is a "proof of concept", demonstrating that bone-forming cells can be systemically recruited from remote bone marrow sites via the circulation and become involved in fracture healing. The mechanisms of how these bone marrow cells are recruited to the site of injury needs further investigation and indeed how differentiated the cells are during this process. The ability to minimise patient morbidity associated with bone graft harvesting for secondary surgery, to the less invasive process of venesection (with laboratory culture expansion) is an obvious advantageous prospect. Furthermore, the subsequent delivery of osteogenic cells either intravenously of intramarrow in conjunction with elective osteotomies has major potential for therapeutic enhancement. Understanding this phenomenon could provide new treatment alternatives for non-unions, planned iatrogenic insults and theoretically other osteopenic bone pathology.

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