YIELD OPTIMISATION AND MOLECULAR CHARACTERISATION OF UNCULTURED CD271+ MESENCHYMAL STEM CELLS IN THE REAMER IRRIGATOR ASPIRATOR WASTE BAG

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Abstract

Bone reconstruction requires the use of autografts from patients' iliac crest (IC); for large-volume defects bone void fillers and autologous mesenchymal stem cells (MSCs) are often added. The Reamer/Irrigator/Aspirator (RIA) device provides the means of harvesting large amounts of autograft and additionally yields a waste bag containing MSCs, which is currently discarded. The aim of this study was to enumerate and characterise native MSCs from RIA waste bag and compare them to 'gold-standard' donor-matched MSCs from IC bone marrow (BM). IC-BM from age matched trauma patients was used as control.

In RIA waste bags the median MSC yield established using a colony-forming fibroblast assay was 314333 (range 5 x 10^4-1.4 x 10^6), equivalent to approximately one litre of IC-BM aspirate. $CD271^+$ cells were present at high levels in RIA waste bags, had MSC surface phenotype ($CD90^+CD73^+CD105^+CD34^-CD61^-CD19^-CD31^-CD33^-$) and expressed genes associated with multipotentiality, osteogenesis, adipogenesis and angiogenic support. RIA-$CD271^+$ MSCs were transcriptionally similar to donor-matched IC-$CD271^+$ MSCs (76 % transcripts); with the majority of bone-related and Wnt pathway molecules being expressed at comparable levels. Lower-level expression of *MCAM*/CD146 and 5/13 hypoxia-related molecules was found in RIA-$CD271^+$ MSCs, potentially reflecting their native residence in a more hypoxic environment of the endosteum and bone cortex.

These data suggest that long bones contain very large numbers of MSCs, transcriptionally-similar to IC-BM MSCs; they can be procured by reaming using the RIA device and used, following concentration, as autologous and potentially allogeneic bone repair therapy.

Keywords: Multipotential stromal cell; bone regeneration; long bone; gene expression; Reamer/Irrigator/Aspirator.

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Introduction

Current reconstruction of large bone defects involves the use of autografts from the iliac crest (IC) of the pelvis (Dimitriou *et al.*, 2011). Although such autografts possess the correct mechanical properties, osteogenic cells and osteoinductive growth factors (Giannoudis *et al.*, 2007), their volume is small and their harvesting remains invasive and often painful to the patient. When the defect volume exceeds that of the available IC autograft, a 'graft expander' material is commonly used, in the form of a natural or synthetic scaffold (Lichte *et al.*, 2011). The advent of a reaming-irrigator-aspirator (RIA) device has opened a new opportunity for harvesting large amounts of autograft (Cox *et al.*, 2011; Masquelet *et al.*, 2012). To its advantage, RIA-harvested autograft bone has good handling properties and is amenable for use as a sole alternative to the IC graft or as a graft expander, in conjunction with autologous IC bone marrow (BM) aspirate (Calori *et al.*, 2013; Giannoudis *et al.*, 2013).

The RIA procedure for bone graft harvesting (Fig. 1) involves the collection of the saline used for irrigation in a 'waste' bag, which is normally discarded. It contains small particulate material mechanically released from the bone, with the recent data showing that it also contains live osteoprogenitor cells including mesenchymal stem cells (MSCs) (Porter *et al.*, 2009; Cox *et al.*, 2011). It has been suggested that in addition to harvesting the graft material, the waste bag could be also utilised as an additional source of MSCs; these cells could be used instead of the autologous IC BM for complex grafting (Porter *et al.*, 2009; Cox *et al.*, 2011). Our previous study showed that the liquid phase of RIA waste bags contained on average 45,000 native MSCs, which is equivalent, in some cases, to a litre of undiluted BM aspirate (Cox *et al.*, 2011; Cuthbert *et al.*, 2012).

To prove the presence of MSCs in any material, including the RIA fluid, culture-expansion and *in vitro* differentiation assays are commonly used (Porter *et al.*, 2009; Cox *et al.*, 2011). In other words, the presence of MSCs is inferred by analysing their progeny whereas their native characteristics remain unknown. We were the first group to use the $CD271^+$ phenotype to characterise native MSCs in the IC BM (Jones *et al.*, 2002; Churchman *et al.*, 2012) and in long bones (Cox *et al.*, 2012), later confirmed independently (Buhring *et al.*, 2007; Kuci *et al.*, 2011; Tormin *et al.*, 2011; Maijenburg *et al.*, 2012; Rasini *et al.*, 2013). Here we hypothesised that MSCs released using the RIA device into the waste bag have the same $CD271^+$ phenotype and a transcriptional profile very similar to

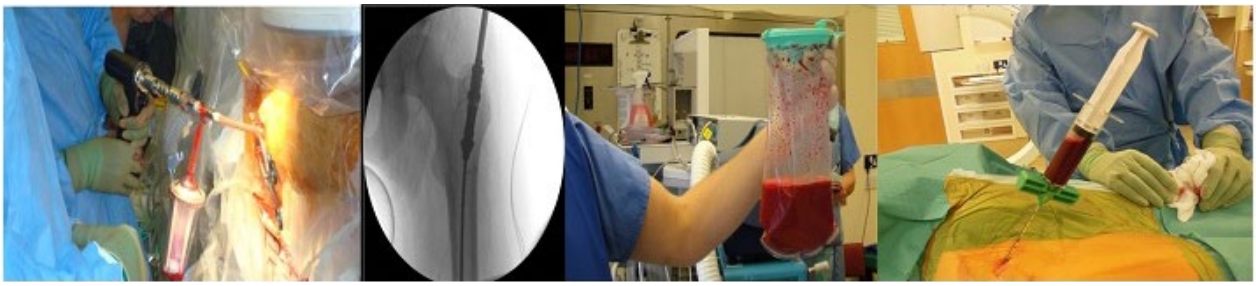

Fig. 1. **Intraoperative pictures of BM/MSC sample collection.** (Far left) The RIA device inserted in the left femoral shaft. The filter where the reaming debris is collected is clearly shown; (Left) Fluoroscopic view of the left proximal femur showing the RIA device inside the intramedullary cavity during the reaming debris (graft) harvesting process. (Right) 'Average-sized' fluid waste bag of the RIA device. (Far right) insertion of trocar into the right anterior iliac crest for BM aspiration.

gold-standard IC BM MSCs. Conversely, any observed differences could shed new light on the topographical diversity of human intra-osseous MSCs.

Materials and Methods

Sample collection and preparation

Ethical approval was granted by the Leeds Teaching Hospital NHS Trust Ethics Committee. Seventeen patients were recruited for this study (8 female: 9 male, the median age 52, range 18-84). Patients selected were those undergoing surgical revision of a non-united long bone fracture (femur/tibia/radius), and requiring RIA-reaming of the femur to obtain bone graft material. This was carried out on the contralateral side for previous femoral fractures or the ipsilateral side for previous tibial or radial fractures, by experienced orthopaedic surgeons as previously described (Cox *et al.*, 2011). The contents of the RIA waste bag were filtered through a sterile 70 μm sieve (Endecotts, London, UK) to remove solid fatty/bony fragments, which were discarded and the fluid saved for further processing as described below. Concomitantly, donor-matched IC BM was collected via an 11G introduction needle into heparin coated syringes (Fig. 1) and immediately transferred to EDTA-containing tubes for further processing. The median volumes (range) for RIA fluid and IC BM aspirates were 700 mL (450-2000 mL) and 18 mL (15-22 mL), respectively. The volume of the RIA fluid was determined by the surgeon and related to the procedure and minimal amount of graft needed, rather than the dimensions of the bone being reamed.

In addition to the donor-matched IC BM, further control IC BM aspirates from patients undergoing surgery following trauma, and not for treating an atrophic non-union, were obtained from seven individuals (range 25-60 years, median 36 years).

MSC enumeration using colony-forming unit-fibroblast (CFU-F) assay and flow cytometry for the CD271$^+$ cell population

Triplicate CFU-F assays were performed using 100 μL sample directly plated into Nonhaematopoietic (NH) medium (Miltenyi Biotec, Bisley, UK) in 100 mm dishes (Corning, NY, USA) as previously described (Churchman *et al.*, 2012). For flow cytometry validation, the RIA fluid was centrifuged to minimise the volume, before isolating mononuclear cells (MNCs) using a Lymphoprep® density gradient (Axis-Shield, Dundee, UK); CD271$^+$ cells were subsequently enumerated as described previously (Cox *et al.*, 2012).

MSC sorting based on the CD271$^+$ phenotype

The MNCs from the RIA fluid and control IC BM aspirates were isolated using Lymphoprep and CD271$^+$ cells were first pre-enriched with Anti-Fibroblast Microbeads (Miltenyi), as described previously (Churchman *et al.*, 2012; Jones *et al.*, 2010). The MSC population: CD45$^{-/low}$CD271$^+$, together with the control haematopoietic lineage cell (HLC) population; CD45$^+$CD271$^-$, was purified by cell sorting, using CD45-FITC (Dako, Stockport, UK), CD271-PE (BD Biosciences, Oxford, UK) and 7-AAD (Sigma, Gillingham, UK), the latter used to eliminate dead/dying cells, on a MoFlo cell sorter (Dako, Ely, UK), directly into lysis buffer (Norgen Biotek, Thorold, Canada).

Quantitative real-time PCR (qPCR)

RNA (n = 12) was isolated from cell lysates using the Norgen Biotek RNA/DNA/protein kit (Geneflow, Lichfield, UK). cDNA was reverse transcribed using High Capacity cDNA reverse transcription kit for use on a Custom Taqman Array (format 48) (both Life Technologies, Paisley, UK). Based on the manufacturer's recommendation, 200 ng cDNA was used per sample where possible, alternatively all available cDNA was used. Low concentrations could result in some missing data, but this was rarely the case. Analysis was carried out using the $2^{-\Delta Ct}$ method normalising to the reference gene *HPRT*. Genes included those previously found to be specific for CD271$^+$ IC-BM MSCs (examples: *BMP2*, *SPP1*, *BGLAP*, *FABP4*, *SOX9*, *WIF1* and *FRZB*) (Churchman *et al.*, 2012) as well as new molecules, chosen to be associated with the hypoxia pathway (*HIF1A, HIF1AN, VHL, ARNT, EGLN1*) and MSC angiogenic support (*VEGFA, VEGFB, VEGFC, PGF*). Taqman assays for all 48 transcripts studied are shown in Tables 1 and 2.

Table 1 Mean fold decrease of transcripts significantly lower in RIA-CD271$^+$ cells compared to donor-matched IC-CD271$^+$ cells.

Gene	Taqman assay used	Description	IC CD271 to RIA CD271
KDR #	Hs 00911702_m1	kinase insert domain receptor (VEGFR2)	**144.21**
HIF1AN #	Hs 00215495_m1	hypoxia inducible factor 1, α subunit inhibitor	**32.18**
EGLN1 #	Hs 00254392_m1	egl nine homolog 1 (*C. elegans*)	**27.60**
LRP5 *	Hs 00182031_m1	low density lipoprotein receptor related protein 5	**20.22**
MCAM *	Hs 00174838_m1	melanoma cell adhesion molecule	**14.72**
PGF *	Hs 01119262_m1	placental growth factor	**12.29**
DDR2 *	Hs 00178815_m1	discoidin domain receptor tyrosine kinase 2	**11.80**
VEGFC *	Hs 01099206_m1	vascular endothelial growth factor C	**4.88**
BMP2 *	Hs 00154192_m1	bone morphogenetic protein 2	**4.34**
SPARC *	Hs 00277762_m1	osteonectin	**3.78**
COL1A2 *	Hs 01028971_m1	collagen, type I, α2	**2.83**

n = 12 donors. Significant differences* $p \leq 0.05$ using Wilcoxon test, and major# (> 25 fold) differences in gene expression (#no significant value since $n < 5$ pairs of data of RIA-CD271$^+$ cells prevented matched statistical analysis being performed).

Table 2. Transcripts, demonstrating no significant reduction in gene expression in RIA-CD271$^+$ compared to donor-matched IC-CD271$^+$ cells.

Gene	Taqman assay (Hs)	Description	IC CD271 to RIA CD271
18s	99999901_s1	18S ribosomal RNA	-
ACAN	00153936_m1	aggrecan	BD
ANGPT1	00181613_m1	angiopoietin 1	1.20
ANGPTL4	01101127_m1	angiopoietin-like 4	2.17
ARNT	01121918_m1	aryl hydrocarbon receptor nuclear translocator	3.14
BMP7	00233477_m1	bone morphogenetic protein 7	LD (both)
BMPER	00403062_m1	BMP binding endothelial regulator	0.46
CEBPA	00269972_s1	CCAAT/enhancer binding protein (C/EBP)	1.40
FABP4	00609791_m1	fatty acid binding protein 4, adipocyte	3.15
FLT1	01052937_m1	fms-related tyrosine kinase 1 (VEGFR1)	LD (both)
FRZB	00173503_m1	frizzled-related protein	1.91
FZD4	00201853_m1	frizzled family receptor 4	3.77
FZD5	00258278_s1	frizzled family receptor 5	1.89
GAPDH	99999905_m1	glyceraldehyde-3-phosphate dehydrogenase	=
HIF1A	00936371_m1	hypoxia inducible factor 1, α subunit	0.91
HPRT	99999909_m1	hypoxanthine phosphoribosyltransferase 1	=
IGF2	01005963_m1	insulin-like growth factor 2	2.17
MYOD1	00159528_m1	myogenic differentiation 1	LD/BD
NANOG	02387400_g1	Nanog homeobox	1.77
NGFR	00182120_m1	nerve growth factor receptor	1.40
OMD	00192325_m1	osteomodulin	2.35
PDGFRA	00998018_m1	platelet-derived growth factor receptor α	1.60
PDGFRL	00185122_m1	platelet-derived growth factor receptor-like	4.39
PECAM1	00169777_m1	platelet/endothelial cell adhesion molecule	2.35
POU5F1	00999632_g1	POU class 5 homeobox 1	1.00
PPARG	01115513_m1	peroxisome proliferator-activated receptor γ	3.46
PTPRC	00894732_m1	protein tyrosine phosphatase, receptor C	3.63
SFRP1	00610060_m1	secreted frizzled-related protein 1	1.41
SFRP4	00180066_m1	secreted frizzled-related protein 4	3.06
SOX9	00165814_m1	SRY (sex determining region Y)-box 9	2.66
SPP1	00959010_m1	secreted phosphoprotein 1	2.69
TNFRSF11B	00900360_m1	tumor necrosis factor receptor superfamily 11b	2.23
VEGFA	00900058_m1	vascular endothelial growth factor A	2.01
VEGFB	00173634_m1	vascular endothelial growth factor B	3.80
VHL	01650959_m1	von Hippel-Lindau tumor suppressor	1.09
WIF1	00183662_m1	WNT inhibitory factor 1	1.05
WNT2	00608224_m1	wingless-type MMTV integration site family 2	BD/LD

n = 12 donors, BD = below detection, LD = low detection ($n \leq 2$).

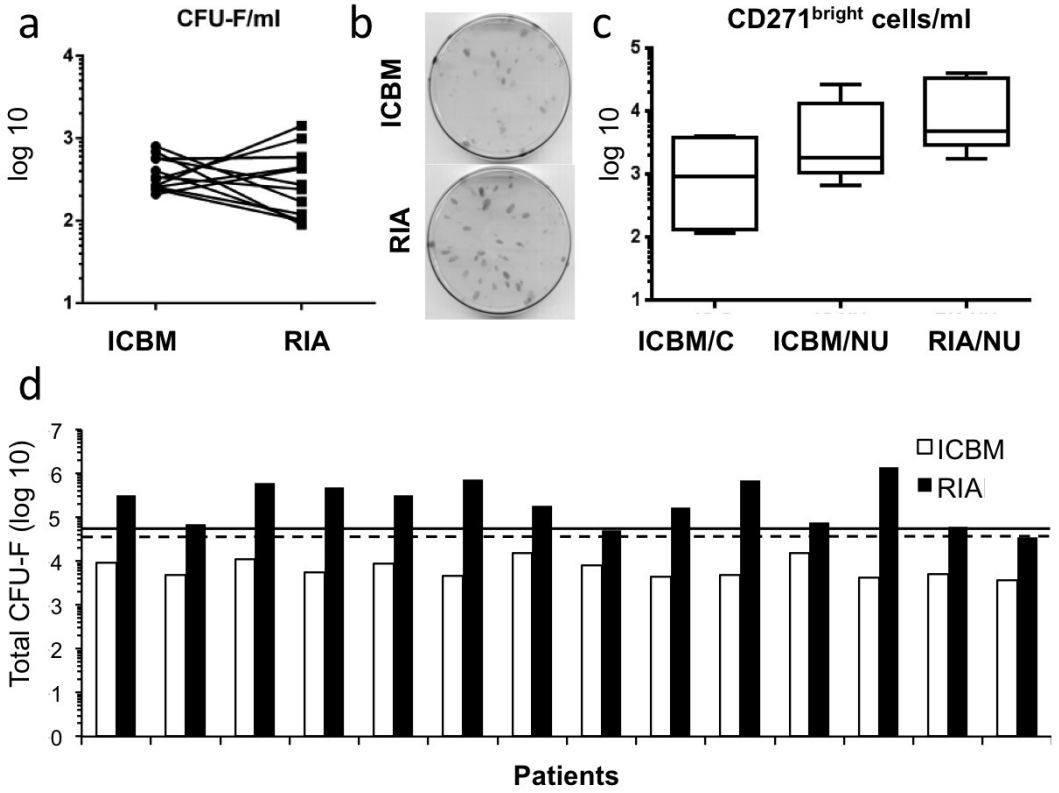

Fig. 2. **Enumeration of MSCs in IC BM aspirates and RIA waste bags from non-union patients compared to age-matched trauma controls.** (**a**) CFU-F/mL in donor-matched IC BM aspirates and RIA waste bags from non-union patients**.** (**b**) Donor-matched pair of CFU-F dishes from IC BM aspirate and RIA waste bag. (**c**) The lack of significant differences between the numbers of $CD271^+$ cells in the IC BM aspirate control (ICBM/C) and age-matched non-union patients (n = 7 and 9 patients, respectively), and between IC BM aspirate (ICBM/NU) and RIA waste bag (RIA/NU) from non-union patients (9 patients). (**d**) Donor-matched comparison of total recovered CFU-Fs; solid/dotted lines represent mean/minimum CFU-F numbers suggested by Hernigou *et al*. as required for a successful healing of non-union fractures (Hernigou *et al.*, 2005). In every patient, CFU-F numbers in RIA waste bags exceeds the minimum value.

Extended phenotypic analysis of the RIA fluid by 6-colour flow cytometry

Following density gradient separation using Lymphoprep, cryogenically stored MNCs (n = 3 donors, age range 28-55) were thawed and re-suspended at 1 x 10^7 cells/mL FACS buffer (phosphate-buffered saline, PBS/0.5 % bovine serum albumin, BSA). Combinations of the following antibodies were used according to manufacturers' recommendations and incubated for 20 min: CD19-PE, CD33-FITC, CD34-PcCy5.5P, CD45-PECy7, CD61-FITC, CD73-PE (all BD Biosciences), CD31-FITC, CD90-PE, CD105-PE (all Serotec, Oxford, UK) and CD271-APC (Miltenyi). Isotype controls were from BD Biosciences. CD73, CD90 and CD105 were used as additional MSC markers (Buhring *et al.*, 2007; Cuthbert *et al.*, 2012; Jones *et al.*, 2006), whereas CD19, CD33, CD61 and CD31 were used to identify other cell lineages (B-, myeloid-, megakaryocytic and endothelial cells, respectively). The cells were washed and re-suspended in FACS buffer containing 100 ng/mL DAPI (Sigma-Aldrich, Poole, UK) before analysing on an LSRII flow cytometer. DAPI-positive dead/dying cells were excluded before gating of the $CD271^+$ cells and HLCs for the assessment of marker expression on these cell populations.

Statistical analysis

Matched statistical analysis was carried out using the Wilcoxon Signed Ranks test for paired IC BM and RIA fluid samples. Multiple grouped analysis was performed using the Kruskal-Wallis test with Bonferroni-Dunn correction. All comparative statistics and graphs were produced using Graphpad (La Jolla, CA, USA) Prism 5; where data is shown as box-whisker plots, each box represents upper to lower quartile, with the line representing the median; whiskers represent maximum and minimum values. A single dot represents one value.

Correlations were determined using Spearman's *rho* and normality testing (Kolmogerov-Smirnov test) of sample information (i.e., volumes and CFU-F counts) were performed using IBM SPSS Statistics 19; only sample volumes were found to be normally distributed.

Results

MSC yield of RIA fluid compared to IC aspirate

We initially endeavoured to validate our previous calculations of the high yield of MSCs in the RIA waste

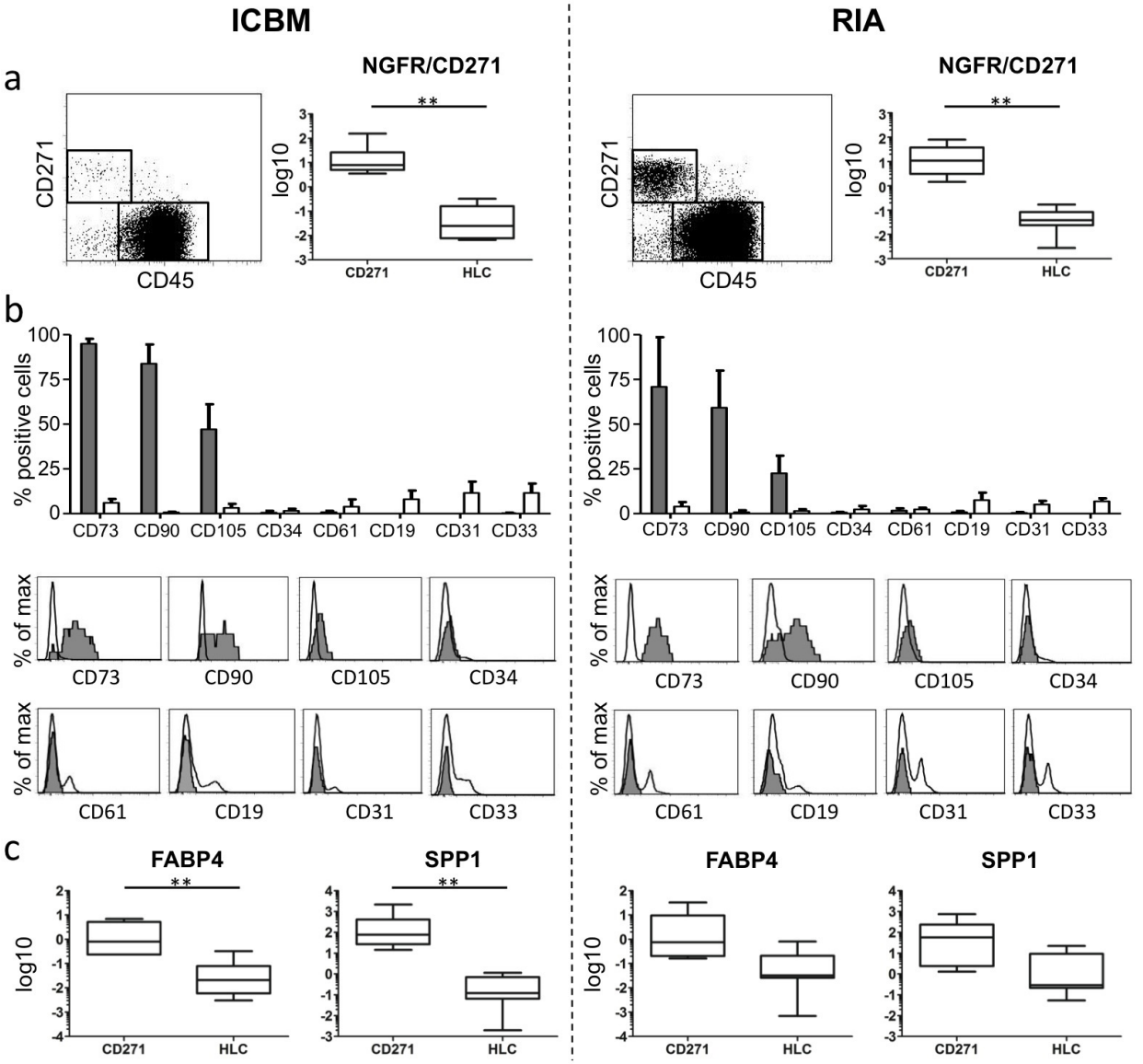

Fig. 3. **Confirmation of the CD271$^+$ phenotype of native MSCs from RIA waste bags (right) compared to donor-matched IC-BM aspirates (left).** (**a**) Gating strategy for CD271$^+$ cells (CD45$^{-/low}$CD271$^+$) and control HLCs (CD45$^+$CD271$^-$) (left panels) and purity control of the sorted fractions based on *NGFR*/CD271 gene expression (right panels). (**b**) CD271$^+$ MSCs (filled bars/histograms) and HLCs (open bars/histograms) from IC-BM and RIA-BM. CD271$^+$ cells show a 'classic' MSC phenotype (n = 3 donors, error bars indicate SDs). Representative marker histograms are shown in lower panels. (**c**) The expression of *FABP4* (fat-lineage) and *SPP1* (osteopontin; bone-lineage) transcripts (normalised to *HPRT*) in CD271$^+$ MSCs and HLCs of donor-matched IC-BM aspirates and RIA waste bags, confirming over-expression in CD271$^+$ MSCs compared to HLCs. Variability of both surface marker and gene expression is commonly greater in the RIA CD271$^+$ population. **$p < 0.01$

bag, compared to IC BM aspirate, using a larger and different cohort of patients (14 *versus* previous 6 (Cox *et al.*, 2011)). The frequency of MSCs, measured as CFU-F per mL was similar in the two sources (p = 0.660, Fig. 2 a,b). We were mindful that patients suffering from non-union could be characterised by a reduced IC BM MSC frequency (Seebach *et al.*, 2007). However, when the CFU-F assay for MSC enumeration was performed using IC BM aspirate from control trauma patients and compared to an age-matched subgroup of IC BM from the non-union cohort, no significant differences were found (485 *versus* 700 CFU-F/mL, n = 7 and 8 patients, respectively). Flow cytometry enumeration of CD271$^+$ cells to quantify MSCs (Cuthbert *et al.*, 2012) revealed a slight increase in the RIA-derived MSCs compared to donor-matched (non-union) IC BM MSCs and age-matched trauma IC BM MSCs (not significant, Fig. 2c).

Given that the volume of the RIA fluid (including saline used for irrigation) was 39-fold larger than IC BM aspirate, the total CFU-F yield in the RIA fluid was 59-fold higher (p = 0.0001). For every donor tested, the total number of RIA-CFU-Fs was greater (Fig. 2d) than the minimum therapeutic MSC dose required for non-union fracture repair according to Hernigou (Hernigou *et al.*, 2005). Based on the median number of CFU-F/mL in IC BM aspirate (328/mL from the whole non-union group),

the total MSC yield of an 'average' waste bag (median value 314,333 cells) would be equivalent to approximately 958 mL of IC aspirate. Since there was no decline in IC BM MSC frequency in patients suffering non-union, we concluded that our calculations on the 'volume equivalents' of IC BM would be valid in relation to any similarly drawn 'average' BM aspirate (Cuthbert *et al.*, 2012).

Confirmation of the MSC identity of CD271$^+$ cells from RIA fluid

Having detected CD271$^+$ cells in the RIA fluid, we next used flow cytometry and basic transcriptional profiling to investigate their identity as MSCs; CD271$^+$ cells from donor-matched IC BM aspirate were used as controls (Fig. 3 a,b). In our previous studies, we used the CD45$^+$CD271$^-$ cell population (haematopoietic lineage cells, HLCs) as non-MSC 'negative' controls (Churchman *et al.*, 2012; Cox *et al.*, 2012), the same strategy was used in the present study (Fig. 3a, left panels).

As seen on Fig. 3b, CD271$^+$ cells derived from both IC BM aspirate and RIA fluid were positive for classical MSC markers CD73 and CD90 (Dominici *et al.*, 2006; Veyrat-Masson *et al.*, 2007). The expression of CD105, another well-known MSC marker (Barry *et al.*, 1999; Pittenger *et al.*, 1999) was slightly lower compared to CD73 and CD90, as described previously (Martinez *et al.*, 2007; Cox *et al.*, 2012), but remained highly specific for CD271$^+$ cells. Consistent with their non-haematopoietic nature, CD271$^+$ cells from both sources did not express markers for haematopoietic progenitors (CD34), megakaryocytes/platelets (CD61), B-cells (CD19), endothelial cells (CD31) and myeloid cells (CD33). Representative histograms are shown on Fig. 3b, bottom panels. Following culture expansion, trilineage differentiation was performed on MSCs from this source in Cox *et al*. (Cox *et al.*, 2011).

IC BM- and RIA fluid- derived CD271$^+$ cells were next sorted for gene expression analysis. The purity of the sorted fractions was validated by qPCR for *NGFR*, the gene encoding the CD271 protein (Fig. 3a, right panels). For both IC- and RIA fluid-derived samples, the expression of *NGFR* in CD271$^+$ cells was over 300-fold higher compared to the corresponding HLCs (367- and 386-fold, respectively), confirming their purity.

The gene expression analysis revealed that genes typically associated with MSC lineage potentiality: *FABP4* (fat-lineage specific transcript) and *SPP1* (osteopontin, bone-lineage specific transcript) were preferentially expressed in RIA-CD271$^+$ cells compared to RIA-HLCs; a pattern similar to IC BM CD271$^+$ cells and HLCs (Fig. 3c).

Together, these results revealed that RIA-CD271$^+$ cells had phenotypic and molecular characteristics consistent with MSCs. This was further confirmed by a significant correlation ($rho = 0.538$, $p = 0.047$) observed between total CFU-F and the number of CD271$^+$ cells sorted from the RIA fluid.

Molecular comparison between iliac crest and RIA fluid: bone- and Wnt pathway-related molecules

The Wnt pathway plays a key role in bone development and differentiation of MSCs towards bone, cartilage and fat

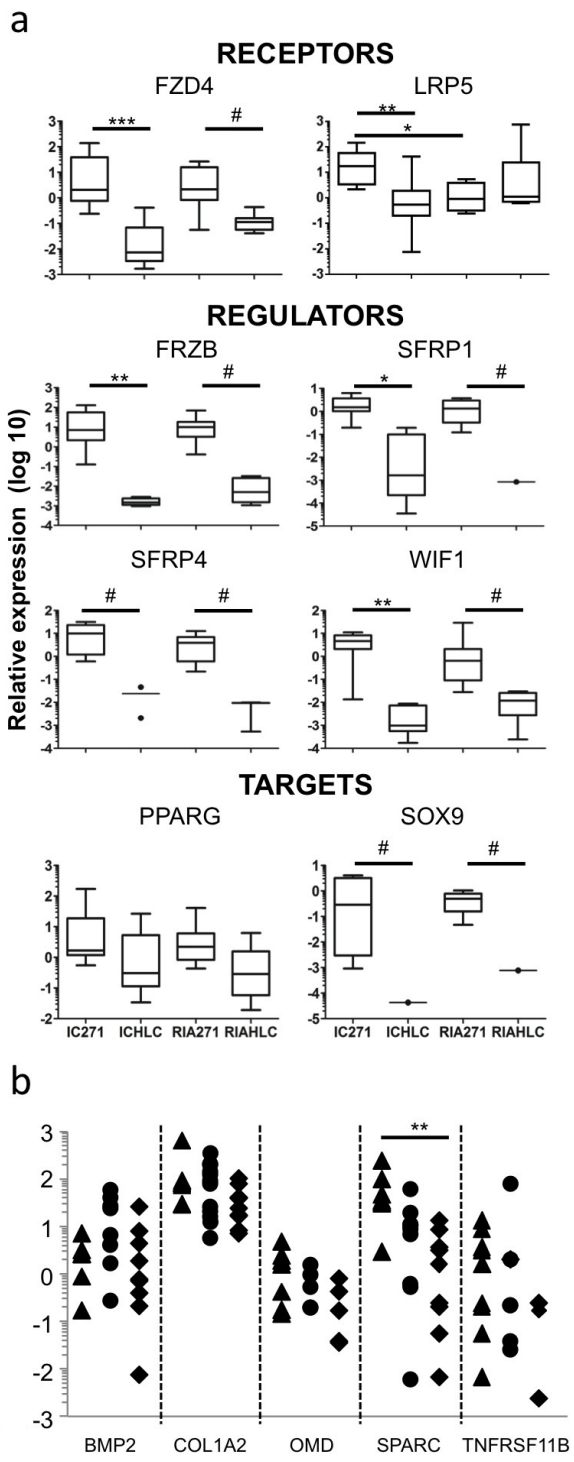

Fig.4. The gene expression of bone- and Wnt pathway-related molecules. (**a**) Relative expression of Wnt pathway related molecules in CD271$^+$ MSCs and HLCs of both IC-BM and RIA-BM. Kruskal-Wallis statistics: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, # $n < 5$ data points, therefore not statistically valid, but >25-fold difference. (**b**) Relative expression (normalised to *HPRT*) of five bone-related molecules in CD271$^+$ MSCs of patients suffering non-union (circles: IC BM, diamonds: RIA waste bag) compared to IC BM CD271$^+$ MSCs of control trauma patients (triangles). Most transcripts show no reduction in CD271$^+$ MSCs from RIA waste bags.

lineages (Etheridge *et al.*, 2004; Takada *et al.*, 2009). In our previous study we demonstrated extensive Wnt pathway-signalling activity in CD271$^+$ MSCs from IC BM aspirate (Churchman *et al.*, 2012). Amongst the seven detectable canonical Wnt pathway transcripts chosen for this study, six transcripts (two surface receptors and four regulatory molecules) were highly specific for CD271$^+$ MSCs (Fig. 4a); amongst those only *LRP5* expression was reduced in the RIA-CD271$^+$ group compared to donor-matched IC-CD271$^+$ (Fig. 4a, Table 1). Interestingly and in agreement with our previous study (Churchman *et al.*, 2012), Wnt pathway regulators, particularly *FRZB,* displayed the highest specificity for CD271$^+$ MSCs (Fig. 4a) suggesting that these molecules can potentially serve as novel markers of MSCs in cell tracking studies using animal models (Kurth *et al.*, 2011).

We were aware that patients suffering non-union could be characterised by reduced expression of bone-related molecules (Seebach *et al.*, 2007). We therefore compared the expression of such molecules in IC and RIA CD271$^+$ MSCs from a subgroup of the non-union patients to the CD271$^+$ MSCs of age-matched control trauma patients (Fig. 4b). A multiple comparison test (Kruskal-Wallace, with multiple group correction) revealed no significant differences in the expression of *BMP2*, *COL1A2*, *OMD* (osteomodulin) and *TNFRSF11B* (osteoprotegrin) in the three groups studied. The only exception was the trend for a lower expression of *SPARC* (osteonectin) in RIA-MSCs from non-union patients compared to trauma IC controls, which reached statistical significance for RIA CD271$^+$ MSCs (Fig. 4b).

Altogether, these data confirmed our previous findings pertaining to highly-specific Wnt signalling activity in CD271$^+$ MSCs and extended these observations to MSCs resident in long bones (those harvested in RIA bags). Secondly, we showed that the expression of bone-related molecules in RIA CD271$^+$ MSCs was similar to donor-matched IC BM CD271$^+$ MSCs. Finally, together with our numerical data on the abundance of MSCs in RIA waste bags (Fig. 3c), the above gene transcript data indicated that patients suffering non-union had a broadly similar bone transcript expression profile (IC- and RIA-derived MSCs), to that of a control group of trauma patients.

Molecular comparison between iliac crest and RIA fluid: molecules related to local MSC topography

Despite their intra-osseous origin, topographical differences in MSCs recovered from different types of bones do exist (Akintoye *et al.*, 2006). More recently, MSCs have been topographically linked to both pericytes (Caplan, 2008; Crisan *et al.*, 2008; Meirelles *et al.*, 2008; Caplan

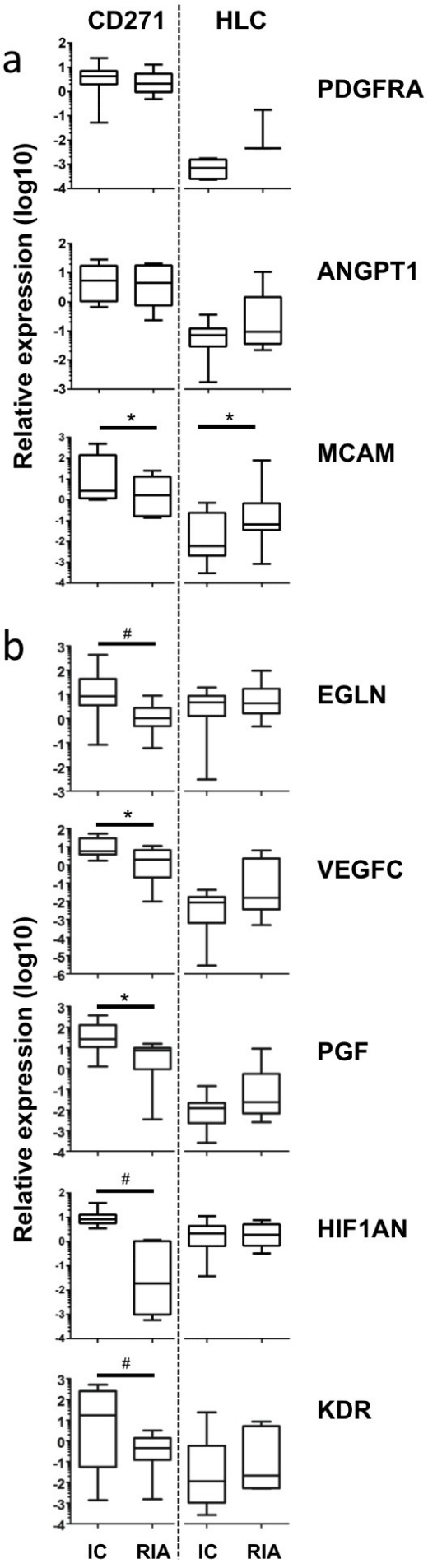

Fig. 5. **The expression of molecules related to local MSC topography and hypoxia signalling. (a)** Pericyte-related molecules. (**b**) Angiogenesis- and hypoxia- related molecules. Gene expression normalised to *HPRT*. Wilcoxon matched paired statistics are shown on the graphs. *$p < 0.05$, **$p < 0.01$, # $n < 5$ pairs of data, therefore not statistically valid, but > 25-fold difference.

and Correa, 2011) and bone-lining cells (Tormin *et al.*, 2011; Rasini *et al.*, 2013). In agreement with the 'MSC as a pericyte' theory (Caplan, 2008; Caplan and Correa, 2011), pericyte-specific *PDGFRA*, *ANGPT1* and *MCAM* (Churchman *et al.*, 2012) were expressed in both IC BM and RIA CD271$^+$ MSCs (Fig. 5a). *MCAM* expression was however significantly reduced in RIA CD271$^+$ MSCs (~15-fold, Table 1). Interestingly, we observed a trend for the reduced *MCAM* expression in relation to the volume of the RIA fluid harvested (*rho* = 0.55, not significant).

We additionally tested the expression of angiogenesis- and hypoxia- related molecules, based on a hypothesis that long bone MSCs could have originated from a lower oxygen environment compared to IC BM MSCs. Amongst 13 angiogenesis- and hypoxia-related molecules (*HIF1A*, *HIF1AN*, *ARNT* (HIF1β), *EGLN1*, *ANGPT1*, *ANGPTL4*, *PGF*, *VEGFA*, *VEGFB*, *VEGFC*, *FLT1* (VEGFR1), *KDR* (VEGFR2) and *VHL*, five were down-regulated in RIA-CD271$^+$ MSCs (Fig. 5b, Table 1). These molecules included two HIF pathway regulators *EGLN* and *HIF1AN* and two target genes *PGF* and *VEGFC* (Fig. 5b). Two other target genes however, *VEGFA* and *VEGFB*, displayed no down-regulation in RIA-CD271$^+$ MSCs (Table 2) suggesting that the observed down-regulation in RIA CD271$^+$ MSCs was unlikely to be vital.

Molecules with very low levels of expression

Of the gene transcripts tested in the 48-gene array, Wnt ligand *WNT2, BMP7* and the myogenic transcription factor *MYOD1* were detected in two or fewer samples for all fractions and the late chondrogenic marker aggrecan (*ACAN)* was undetected in all samples (Table 2). The lack of transcript expression for this mature chondrogenesis-related protein in native CD271$^+$ MSCs is consistent with our previous study (Churchman *et al.*, 2012) and indicates the absence of active chondrogenesis in both cancellous and cortical bone. Lower levels of endogenous *BMP7* expression, as opposed to *BMP2*, are additionally noteworthy given the widespread use of both agents in the treatment of fractures and their differential expression during fracture repair (Bishop and Einhorn, 2007; Yu *et al.*, 2010, Kloen *et al.*, 2012).

Altogether, the transcript expression data showed that 34/45 (75.6 %) tested molecules were similar in their expression between IC and RIA CD271$^+$ MSCs, which support the use of RIA waste bags, instead of IC BM aspirate concentrate (Kasten *et al.*, 2008), as a source of MSCs in complex bone grafting procedures.

Discussion

Earlier findings pertaining to RIA-harvested MSCs have relied on MSC culture-expansion and testing of their tripotentiality *in vitro* (Porter *et al.*, 2009; Cox *et al.*, 2011). This study, for the first time, explored the molecular signature of highly-pure MSCs immediately following their extraction, which represents the MSC's native state *in vivo* and is unaffected by any culture manipulations, which usually lead to transcript down-regulation (Churchman *et al.*, 2012; Qian *et al.*, 2012). Common molecular signatures of IC and RIA MSCs suggest that RIA waste bags may be a viable alternative to IC BM aspirate in complex bone grafting procedures.

Initially, we tested the practicality of this approach in terms of actual numbers of MSCs harvested. In full agreement with our previous study (Cox *et al.*, 2011), the calculated quantity of MSCs obtained by the average reaming of a single long bone (femur) was found to be equivalent to 958 mL of IC aspirate; notably, such a large volume aspiration would be neither ethical nor practical due to a gradual dilution of marrow with blood (Cuthbert *et al.*, 2012). Such a significant number of MSCs obtained from a single donation suggests that RIA waste bags could represent a unique resource of MSCs not only for autologous, but also for allogeneic applications. To explore this possibility and to address potential concerns pertaining to the 'inferiority' of MSCs from patients suffering non-union, we additionally investigated donor-matched IC BM MSCs and MSCs from a control cohort of trauma patients and no major differences in the MSC numbers or their bone-related gene expression was found. Overall, the 'quality' of RIA MSCs was similar to IC MSCs (75.6 % similarity based on our gene panel); this confirms the possibility of developing new technologies and devices for the concentration and purification of RIA MSCs to be used as therapy (Porter *et al.*, 2009). Parallel removal of immune cells and selection of immuno-privileged MSCs (Tyndall *et al.*, 2007) could permit their allogeneic transplantation; a proof-of-principle animal study would be required to demonstrate this point.

From a basic science point of view, we observed that transcripts from the Wnt and hypoxia signalling pathways, known to regulate bone formation and angiogenesis (Etheridge *et al.*, 2004), were overall similar between IC and RIA MSCs suggesting that the steady-state MSC activity in both locations (IC and long bone) was comparable. Gene expression profiles of CD271$^+$ cells from both locations were consistent with native MSCs (Mendez-Ferrer *et al.*, 2010; Tormin *et al.*, 2011; Churchman *et al.*, 2012; Qian *et al.*, 2012) and only subtle differences in expression of the selected genes were found. As shown recently, the expression of Wnt pathway transcripts in particular is considerably down-regulated or even lost during standard culture expansion (Churchman *et al.*, 2012; Qian *et al.*, 2012); this highlights the importance of purifying and analysing native MSC populations, and not cultured MSCs, when comparisons of 'MSC fingerprints' in different tissues are sought.

When BM is removed from human long bones manually (by scooping), it contains only 2- to 5-fold more MSCs, compared to IC BM (Cox *et al.*, 2012). This suggests that significantly greater numbers of MSCs recovered by reaming were most likely derived from the endosteum, which is disrupted by reaming but left intact during manual collection. This notion is supported by a recent demonstration that bone-lining cells are positive for CD271 but largely negative for CD146 (Tormin *et al.*, 2011; Rasini *et al.*, 2013). This study, in which significantly lower levels of *MCAM*/CD146 were found in RIA-CD271$^+$ MSCs compared to those of the IC, supports the idea that at least some of RIA-harvested MSCs have originated from the

inner bone surface and cortex (Tormin *et al.*, 2011). This is further supported by an observed reduction in *MCAM*/ CD146 expression with the increased volume of RIA fluid harvested, the latter being primarily related to the depth of cortex reamed, and by the reduction in the expression of several hypoxia pathway-related transcripts as cortex is known to be more hypoxic.

This study is limited by the use of IC BM samples from trauma patients, not healthy volunteers, as controls; this was limited by ethical considerations. Additionally, we cannot exclude the possibility that the observed differential expression for some molecules between IC and RIA MSCs was a result of the more invasive harvesting process; however, this is rather unlikely as the same significant decreases were not observed in the control HLCs. The use of purified MSCs and negative control HLCs from the same patient was a major advantage of this study. In a recent molecular study of bony chips obtained by RIA (Sagi *et al.*, 2012), whole tissues extracted from IC and long bones were used. The interpretation of gene expression data was therefore limited by the heterogeneous nature of tissues studied making conclusions relating to MSCs less robust (Sagi *et al.*, 2012).

In summary, this study has identified a normally discarded RIA by-product that can provide an abundant, non-inferior, alternative source of native MSCs compared to IC. Such is the wealth of these cells that they may not only become an osteogenic source for future 'single-surgery' autologous use, but also – with further study – may present a reservoir of cells for allogeneic bone repair applications. The present findings therefore bode well for the *in vivo* testing of bone-forming capability of MSCs concentrated from RIA waste bags in animal models.

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Discussion with Reviewers

T. Yamaza: This manuscript demonstrated the isolation and identification of mesenchymal stem cells (MSCs) from a medical waste by Reamer/Irrigator/Aspirator procedure in bone graft. The authors showed the phenotype of their colony forming capability and broad and multiplexed profiles of cell surface markers and signal molecules. However, the current form lacks a critical part to identify their isolated cells as MSCs. The fundamental criteria as MSCs contain two factors: (1) the expression of stem cell markers and (2) the capacity of multi-differentiation, at least, in three lined cells. In the current form, the first factor was demonstrated, but the second one was completely lacking. Therefore, the authors should add the multi-differentiation capacity, at least, in three lined cells likely osteoblasts, chondroblasts, adipocytes, etc., to evaluate the MSC properties. Please comment.

Authors: This study concerns native $CD271^{+}CD45^{-/low}$ MSCs; here we have endeavoured to provide a study of the same cell from both 'gold standard' iliac crest bone marrow (ICBM), and donor-matched RIA derived long bone marrow (RIA-BM) as an alternative source of MSCs. We have comprehensively identified these cells according to the ISCT surface marker phenotype (Dominici *et al*., 2006, text reference) and found them to be identical to those from ICBM. We acknowledge that tri-lineage differentiation was not performed here on account of the cell numbers required to perform the assays. In order to validate the RIA-BM as a source of similar MSCs we would have been required to perform the differentiations and molecular studies from each ICBM and donor-matched RIA-BM. Since the yield of MSCs from IC-BM is so low (below 5000) (Churchman *et al*., 2012, text reference), then this is not possible without culture expansion as the chondrogenic assay alone requires one million cells (for pellet cultures). In our previous study, these same cell sources (donor matched ICBM and RIA-BM) were used in Cox *et al.* (Cox *et al*., 2011, text reference) and following culture expansion to passage 2, trilineage differentiation was achieved, the surface phenotype was also assessed and was in agreement with the recognised surface phenotype of cultured MSCs (Dominici *et al*., 2006, text reference).

M. Stoddard: The clinical outcome of the treated patients after 6 of 12 months would also strengthen the study. Please comment.

Authors: As the major focus of this study was to characterise the molecular signature of the RIA waste bag MSCs, we did not feel that it was applicable to refer to the clinical results of the patients. Whilst all patients were seen at regular intervals in the outpatient clinic for clinical and radiological assessment, we believe that the sample is too small (17 patients) to report in detail on their outcomes. A bigger cohort of patients using validated health related quality of life assessment and anatomically based functional assessment tools would represent a study on its own.

Nonetheless, a table has been prepared below including the site of the non-union and the time to radiological healing (Table 3).

Table 3. Clinical characteristics of the patients.

Patient	Sex	Non-union site	Union	Time to union (months)*
1	M	Tibia	Yes	6.5
2	M	Tibia	Yes	5
3	M	Femur	Yes	8
4	M	Femur	Yes	9
5	M	Tibia	Yes	4
6	M	Radius	Yes	3
7	M	Tibia	Yes	4
8	M	Femur	Yes	6
9	F	Tibia	Yes	6
10	F	Tibia	Yes	4.5
11	F	Femur	Yes	5
12	F	Femur	Yes	6
13	F	Femur	Yes	6
14	F	Radius	Yes	3
15	F	Tibia	Yes	5
16	F	Tibia	Yes	6
17	F	Tibia	Yes	5.5

* Mean time to radiological union 5.45 months (range 3-9).