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The biological fitness of bone progenitor cells in Reamer Irrigator Aspirator-waste

Abstract

Background

The biological waste collected during the Reamer-Irrigator-Aspirator (RIA) procedure has been described as an abundant source of bone progenitor cells **with a comparable osteogenic gene profile to donor-matched iliac crest-bone marrow (IC-BM)**. However, it is not clear if these RIA-waste (RIA-W) cells are biologically fit. We aimed to evaluate the stress levels and functions of RIA-W progenitor cells.

Methods

The Reactive Oxygen Species (ROS) levels were tested in freshly-collected bone progenitor cells (defined as $CD45^{low}$ $CD271^{high}$ cells) using flow-cytometry. Induced ROS levels in these cells were measured under hypoxia and/or oxidative stress **as well as under** experimental **simulation of RIA procedure**. Furthermore, the alkaline phosphatase (ALP) expression levels, proliferation and senescence of culture-expanded RIA-W and IC-BM Mesenchymal **Stromal** Cells (MSCs) were compared.

Results

RIA-W and donor-matched **IC-BM $CD45^{low}CD271^{high}$ cells were 97% and 98% viable, but the** ROS levels for RIA-W cells were significantly higher than IC-BM **cells** (p=0.0020). Also, hypoxia-, oxidative stress- or both-induced ROS were higher for RIA-W cells (p=0.0312, p=0.0078, and p=0.0312 respectively). The dilution with saline, suction pressure, and irrigation-effect reduced cell viability with a positive correlation of the ROS levels (p=0.0035). **The RIA-W and IC-BM colony-forming cells (average 96,100 and 11,000, respectively) showed comparable ALP levels**. Furthermore, **culture-expanded RIA-W and IC-BM MSCs showed comparable** ROS, ALP levels, death susceptibility and proliferation.

Conclusions

Although freshly-collected RIA-W bone progenitor cells seemed to be transiently stressed, these cells were as viable, but higher in numbers than IC-BM cells. The proliferation and osteogenesis of both cells were comparable.

Clinical Relevance

The RIA-W bag should not be wasted as containing bone progenitor cells with promising potential for regenerative application.

Keywords

Bone regeneration; bone progenitor cells; Mesenchymal **Stromal Cells**; Reaming-Irrigation-Aspiration; Bone marrow; Cell stress.

Introduction

Bone progenitor cells extracted from bone marrow (BM) are increasingly used for orthopaedic regenerative procedures. However, considering the donor-related variability (1-4) and rarity of these progenitor cells (5), using even BM concentrates cannot guarantee enough cells for effective bone repair. In contrast, cortical and trabecular bone cavities contain significant high numbers of progenitor cell numbers (6, 7) and this difference is related to the presence of bone lining, stromal and perivascular progenitor cells (6). Hernigou et. al. reported that around 50,000 progenitor cells **(colony-forming)** were required for non-union healing (8). Thus, huge **BM aspirate** volumes would be needed to extract adequate progenitor cell numbers helping bone regeneration (1, 2). Consequently, alternative and abundant sources of bone progenitor cells **are** highly desirable.

Recently, the Reamer-Irrigator-Aspirator (RIA) device has been introduced in the clinical setting mostly for bone harvesting procedures (9). During the RIA procedure, the femoral medullary canal **reaming** is combined with irrigation with Sodium Chloride 0.9% (saline) and suction pressure to separate the bone graft from the debris and the fatty tissue (10, 11). Alongside, a considerable volume of a biological waste bag is usually collected (10). Interestingly, RIA waste (RIA-W) has been reported to contain **an average of 398 colonies/ml comparable with 403 colonies/ml of iliac crest-bone marrow (IC-BM) aspirate (12, 13). Also, RIA-W bag contains on average 2252 $CD45^{low}CD271^{high}$ cells/ml, compared to 1313 cells/ml of IC-BM (12). These $CD45^{low}$ $CD271^{high}$ cells are positively correlated with colony-forming cell counts, unlike $CD45^{low}$ $CD271^{low}$ cells (1, 14, 15). Additionally, $CD45^{low}$ $CD271^{high}$ cells uniformly express the classic MSC markers and alkaline phosphatase (ALP) (1, 2).**

Comparing gene expression profile of uncultured RIA-W and IC-BM $CD45^{low}$ $CD271^{high}$ cells showed that Wnt pathway transcripts; FRZB (frizzled-related protein) and

SFRP1 and 4 (secreted frizzled-related proteins 1 and 4) as well as several osteogenic-related transcripts; SPP1 (Osteoprotegerin), BMP2, BMP7 (Bone morphogenic proteins), OMD (Osteomodulin), SPARC (Osteonectin), COL1A2 (Collagen 1A2), and TNFRSF11B (Osteoprotegrin) were all comparable. However, few hypoxia-related transcripts were lower in RIA-W cells (12). Another study showed that culture-expanded osteogenic RIA-W MSCs express ALP and calcium, similar to IC-BM MSCs (16). However, stress status, viability and proliferation of uncultured RIA-W cells remain unclear. This study aimed to assess the basal and induced cell stress levels of freshly collected RIA-W **$CD45^{low}CD271^{high}$ cells compared** to donor-matched IC-BM. Additionally, **colony-forming cell counts**, **osteogenesis** and proliferation of the culture-expanded **MSCs** from both sources were **tested**.

Patients and Methods

Clinical samples

Patients undergoing RIA femoral procedures (autologous bone graft harvesting) for the treatment of **long bone fracture** non-union were invited to participate in this study. Polytrauma patients with chest injuries undergoing RIA procedures for acute stabilisation of femoral shaft fractures were excluded. All included patients (total **35 patients, table 1**) provided informed written consent according to the ethical approval with NREC number: 06/Q1206/127, National Research Ethics Committee Yorkshire & Humber–Leeds East. For comparative analysis, donor-matched RIA-W bag (**average 650ml**, 400-900 ml) and 15 ml of BM **was harvested by the same (the senior) surgeon from the anterior iliac crest, collected into 20 ml syringe with multiple suctions and changing angles. Samples were transferred immediately into EDTA-containing tubes** (4, 11).

Processing of RIA-W bags and BM aspirates

The donor-matched RIA-W and IC-BM samples were filtered to exclude clumps/debris then were treated with red blood cell lysis buffer (NH_4Cl, KCL and EDTA). **Total live cells were counted using Trypan blue.** Separated cells were processed without culture **or** expanded in StemMACS MSC Expansion medium **with Foetal-calf serum and L-glutamine (130-104-182,**

Miltenyi-Biotec) and analysed for colony-forming cells or at passage 3. The antibodies against CD73, CD90, CD105 (positive MSC markers) and CD45, CD14, CD19 and HLA-DR (negative MSC markers) (Miltenyi-Biotec and **Beckton-Dickson**) were used to **prove that culture-expanded cells were MSCs** (17). The BD LSRII 4-laser flow-cytometer and the DIVA software (**Beckton-Dickson**) were used for data acquisition and analysis, respectively.

Measuring cell stress levels and death susceptibility

The reactive oxygen species (ROS) levels were measured using **Green CellROX® kit (C10492**, ThermoFisher Scientific) that **includes a dye exhibiting** bright fluorescence upon oxidation by ROS. Freshly-collected $CD45^{low}$ $CD271^{high}$ cells (1, 2) or culture-expanded MSCs (17, 18) were analysed similarly for ROS levels. **The** SYTOX dye **(ThermoFisher Scientific)** was used to exclude the dead cells.

For induced stress, cells were kept in 1% oxygen (H35 HEPA Hypoxystation, Don Whitley Scientific) for one hour for freshly-collected cells or five days for culture-expanded MSCs. Anti-hypoxia induced factor (HIF, **Beckton-Dickson**) antibody was used to confirm hypoxic cell response. Alternatively, cells were exposed to an oxidative stress-inducing chemical, tetra-butyl hydroperoxide (TBHP, 800µM, ThermoFisher), or hypoxia and TBHP together. Cells treated with a higher dose of TBHP (15mM) were analysed using SYTOX for dead cell susceptibility.

Experimental mimicking of the RIA procedure

To simulate the RIA procedure, IC-BM samples were **processed subsequently with** different treatments. Each IC-BM sample was mixed 1:10 with phosphate buffered saline (Sigma-Aldrich) **only or** this IC-BM/saline mixture was exposed to impulse mixing using Vortex (V-1 plus, Biosan) for continuous 3 minutes mimicking the reaming/ irrigation effect. The suction pressure effect was tested via multiple aspirations of 10 ml of **the IC-**BM/saline mixture into 50 ml syringe. All samples were analysed for CellROX and **stained with** a live cell marker, **Calcein** violet (ThermoFisher).

The osteogenic assays

Freshly-collected RIA-W and IC-BM cells were **equally** seeded **($1x10^6$ cells/dish)** for one week in the expansion medium to allow cell plastic-adhesion then in the osteogenic medium

(**130-091-678,** Miltenyi-Biotec) for two more weeks. **Numbers of colonies formed by colony-forming cells (progenitor cells) were counted manually**. The ALP expression in colonies **was visualised using** fast-blue dye kit (Sigma-Aldrich). Colony area and integrated density were analysed using ImageJ software (NIH).

For the quantitative measurement of ALP in culture-expanded MSCs maintained in the osteogenic medium for two weeks, the surface **ALP levels were tested** using **flow-cytometry and specific** antibody (Miltenyi-Biotec).

The proliferation of culture-expanded MSCs

The proliferation was assessed using the **XTT assay kit (11465015001, Merck). The XTT buffer containing Tetrazolium salts was added to MSCs cultures in 1% or 21% oxygen for six days. The dye cleaves to coloured formazan by the succinate-tetrazolium reductase in metabolically intact cells, indicating cell proliferation.**

To assess the **MSC** senescence status **in cultures, a kit (75707, ThermoFisher) was used to measure lysosomal Beta-Galactosidase (β-gal) that catalyses the hydrolysis of terminal β-linked galactose residues. In senescent cells, high β-gal activity is detectable at pH-6, and a** reagent **containing O-nitrophenyl-beta-D-galactopyranoside will be lysed, giving coloured O-nitrophenyl that was measured optically.**

Statistics

The statistical analysis and figure preparation were performed using GraphPad Prism 7. The comparative tests for paired RIA-W and IC-BM groups were performed as indicated for each figure.

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Results

Freshly-collected RIA-W progenitor cells **appeared alive but** more stressed than IC-BM cells

The stress status of **RIA-W** cells was evaluated **with** flow**-cytometry;** using **forward and side** cell scatters (FSC and SSC) to identify cells **and excluding debris based on size and granularity respectively** (Figure 1, A) then SYTOX dye to exclude dead cells before gating on $CD45^{low}CD271^{high}$ cells (Figure 1, A). **The total median numbers of viable cells were comparable ($20x10^7$ and $31x10^7$, respectively, Figure 1, B). Additionally, an average of 98% and 97% viable $CD45^{low}CD271^{high}$ cells were detected in IC-BM and RIA-W samples, respectively.** The cell stress **was** measured via quantifying the **mean fluorescence intensity (**MFI**)** of CellROX dye **indicating the expression of ROS levels** in freshly-collected RIA-W $CD45^{low}CD271^{high}$ cells relative to donor-matched IC-BM cells (**Figure 1, C**). Interestingly, RIA-W $CD45^{low}CD271^{high}$ cells had consistently greater levels of CellROX dye than donor-matched IC-BM cells (2.3-fold, *p*=0.0020; **Figure 1, D**). Similarly, RIA-W $CD45^{high}$ cells (hematopoietic lineage cells) had higher MFI levels of CellROX dye than donor-matched RIA-W $CD45^{high}$ cells (1.9-fold, *p*=0.0012), (**Figure 1, E**). Overall, RIA-W cells appeared to be more stressed than donor-matched IC-BM cells.

<u>Higher induced ROS and death susceptibility of freshly-collected RIA-W progenitor cells than IC-BM cells</u>

A further investigation of the stress status for freshly collected RIA-W cells, particularly in response to hypoxia, was conducted. Cell incubation under 1% oxygen for 1 hour was adequate to induce HIF levels in the RIA-W $CD45^{low}CD271^{high}$ cells (**Figure 2, A**). The oxidative stress reagent, TBHP was used a positive control, and its titration showed a dose-dependent increase of cellular ROS and death levels (**Figure 2, B & C**). When cells exposed to hypoxia, TBHP, or both, higher **ROS** level**s were** detected (an example is shown for RIA-W $CD45^{low}CD271^{high}$ cells, **Figure 2, D**). The comparison of donor-matched samples showed higher hypoxia-induced ROS levels in RIA-W $CD45^{low}CD271^{high}$ cells (mean of 1.6-fold, *p*=0.0312, Figure 2, E). The TBHP-induced ROS levels were consistently greater in RIA-W $CD45^{low}CD271^{high}$ cells than donor-matched IC-BM (mean of 3.5-fold, *p*=0.0078, **Figure 2, E**). Similarly, applying hypoxia and TBHP showed more ROS levels in RIA-W than IC-BM cells (mean of 1.5-fold, *p*=0.0312, Figure 2, E). Furthermore, exposure to 15mM TBHP for one hour induced a cell death (**Figure 2, F**). Importantly, The TBHP-induced dead cell

percentages of RIA-W CD45^{low}CD271^{high} were higher than IC-BM cells (p=0.0234, **Figure 2, G**) together confirming the apparent greater stress for RIA-W cells.

The RIA processing affected freshly-collected cell viability, but proliferation and osteogenic capacity appeared intact

An experimental simulation of the RIA procedure was performed using freshly-collected IC-BM cells. The CellROX MFI for CD45^{low}CD271^{high} cells diluted with saline, **then subsequently** treated by suction pressure or vortex were similar to the untreated control (**Figure 3, A**). However, the percentages of live cells (i.e. Calcein violet^{+}) among CD45^{low}CD271^{high} cells were significantly decreased after **subsequent** treatments (p=0.0158, p=0.0401 and p=0.0181 respectively, **Figure 3, B**). Furthermore, a significant positive correlation between dead cell percentages and ROS levels was detected for TBHP-treated RIA-W CD45^{low}CD271^{high} cells (p=0.0035, **Figure 3, C**) indicating that the RIA procedure could contribute to freshly-collected RIA-W cell stress.

RIA-W were seeded in the osteogenic media to assess if the high-stress level could have a further impact on the osteogenesis of colony-forming cells. **The average total colony-forming cell counts were 11,500 and 96,100 for IC-BM (15-ml) and RIA-W (650-ml) respectively, and all** colonies were positively stained for ALP (**Figure 3, D**). Of note, the ALP-stained **colony area**s were not significantly different for RIA-W and IC-BM samples (p=0.5293), (**Figure 3, E**). Furthermore, the integrated densities of ALP-stained colonies were comparable for RIA-W and IC-BM samples (p=0.5069), (**Figure 3, F**). Collectively, RIA-W and IC-BM-derived colonies had similar proliferation and **ALP expression**.

RIA-W MSCs had comparable surface phenotype, ROS levels and death susceptibility to IC-BM MSCs

Further investigations for longer-term fitness of RIA-W culture-expanded cells were performed. The RIA-W **and IC-BM** culture-expanded cell **identity** as MSCs was **similarly** confirmed as negatively expressed hematopoietic markers; CD45, CD19, CD14, and HLA-DR, and being **nearly** 100% positively expressed CD73, CD90, and CD105 (**Figures 4, A and B**).

There were no significant differences in the positive marker expression levels between donor-matched RIA-W and IC-BM MSCs (**Figure 4, C**) indicating similar surface phenotype.

The culture-expanded RIA-W MSCs showed similar basal CellROX levels to IC-BM MSCs (MFI: 1104 and 1048 respectively), with no significant difference (p=0.6455), (**Figure 4, D**). Additionally, the hypoxia, TBHP, or both-induced CellROX levels were comparable for cultured RIA-W MSCs and IC-BM MSCs (p=0.3408, p=0.8278, and p=0.8297 respectively), (**Figure 4, E**). Furthermore, there was no significant difference of the TBHP-induced dead cell percentages between culture-expanded RIA-W MSCs and IC-BM MSCs (p=0.1875, medians 36 and 41% respectively), (**Figure 4, F**). Altogether, the higher stress and death susceptibility of RIA-W cells seemed to normalise after culture-expansion.

<u>The osteogenesis and proliferation of RIA-W MSCs were similar to IC-BM MSCs</u>

As similar ROS levels were detected for culture-expanded RIA-W and IC-BM MSCs, we next compared their **surface ALP** levels **using flow-cytometry** (**Figure 5, A**). **T**he **ALP** levels were significantly induced **in osteogenic cultures** compared to the expansion **cultures** for RIA-W and IC-BM MSCs (p=0.0213, p=0.0029 respectively), (**Figure 5, B**). However, no difference was noted for **ALP** levels between differentiating RIA-W and IC-BM MSCs (p=0.6056), (**Figure 5, B**).

A gradual increase of the XTT absorbance levels from the lowest to the highest **MSC** concentration were comparably noted for **1%** and **21% O_2** (**Figure 5, C**). However, a **lower XTT level** trend under **1% O_2** was observed for the lowest cell concentration (2-fold less). These proliferation patterns were equally detected for RIA-W and IC-BM MSCs (**Figure 5, C**). Finally, β-gal levels were measured to assess if these RIA-W MSCs were going into senescence earlier than donor-matched IC-BM MSCs. Interestingly, the β-gal levels were equal for both RIA-W and IC-BM MSCs under **21% or 1% O_2** (**Figure 5, D**) confirming **the** similar proliferative potential for RIA-W and IC-BM MSCs.

Discussion

For bone regenerative therapies, the need for accessible and abundant sources for bone progenitor cells is essential. **Our data confirmed a high yield of bone progenitor cells in**

RIA-W consistently with previous studies **(12, 13). The RIA-W bag (650ml) contained on average 96,1000 progenitor cells, which is potentially adequate for improving of non-union fracture healing** (8). **We also uniquely reported the proliferative and osteogenic fitness of these progenitor cells.** Noteworthy, this is the first study measuring the stress levels in progenitor cells using a flow-cytometry based method, and detecting higher stress levels for RIA-W freshly-collected progenitor cells ($CD45^{low}CD271^{high}$ cells) than donor-matched IC-BM at the basal and induced levels.

We hypothesized that the RIA procedure, including irrigation, suction and dilution/storage in saline could **trigger** this RIA-W cell stress as donor-matched IC-BM aspirate cells were exposed only to suction pressure. As anticipated, the experimental **RIA** mimicking caused significant **MSC** death. Although no difference was detected in ROS levels, the **cell stress** coupling **with** pro-death susceptibility has been noted in our study and other previous reports (19, 20) as excess ROS can disturb cell signalling and apoptosis pathways (20). Together, the RIA procedure most likely **caused the** apparent RIA-W cell stress.

Our results indicated that RIA-W-derived colonies or MSCs maintained their ALP expression. Furthermore, when culture-expanded under **21% or 1% O_2 (hypoxia),** RIA-W MSCs retained comparable ROS levels and being able to proliferate without early senescence thus can adapt to *in vitro* culture conditions (21). These results demonstrated a temporary and reversible **RIA-W cell** stress status and confirmed the key role of the external environment in biological cell fitness.

We modelled here potentially hostile environments (hypoxia) that MSCs could face following their delivery/application into the disadvantaged bone healing site (22). Also, using a control oxidative-stressing agent, TBHP and the flow cytometry-based method quantifying ROS levels can be beneficial for future investigations of the stress in other bone healing-related cells, e.g. endothelial cells and immune cells (23, 24). Our data suggested that hypoxia did not alter the culture-expanded MSC **proliferation** except for low-density seeded cells. This note can be explained, as suggested before, by a hypoxia-related quiescent state for low-cell numbers to protect against apoptosis (25, 26). Also, hypoxia has been shown to reduce the high passaged MSC **senescence** (i.e. ≥ 6 passages) (27), but we aimed was to check if early-passaged RIA-W MSCs undergo senescence limiting the comparison with these data.

As a limitation of our study, we used CD73, CD90 and CD105 as MSC markers, which have not been shown to correlate with *in vitro* or *in vivo* proliferation or differentiation assays. Moreover, culture-expanded MSCs were reported as widely variable in expressing pluripotent markers and other markers, e.g. CD146 (28). Therefore, future in vivo assessment of culture-expanded MSCs will be needed.

In summary, **besides having adequate progenitor cell quantities, RIA-W** cells **showed** higher stress signs soon after collection. However, this stress seemed to be temporary, reversible and related to the RIA procedure. Compared to IC-BM, RIA-W cells can efficiently survive, proliferate and are fit for bone formation even when minimally-cultured. For therapeutic exploitation, these RIA-W cells could be concentrated/enriched using low-stress procedures without centrifugation, e.g. dielectrophoretic cell separation (29). Also, the RIA-W cell **storage could** be improved by including nutritious media in the collection bag as recommended for bone grafts (30, 31) and at low temperature (32). Considering our findings, the wasted RIA-W bag in routine surgical practice could be of great clinical value as an **abundant** source of functionally-competent bone progenitor cells.

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Figure legends

Figure 1: The basal ROS levels of freshly-collected in RIA-W and IC-BM cells.

A. The gating strategy on flow-cytometry plots. The cell scatters were used on FSC/SSC plot to exclude debris then live cells were identified as SYTOX negative cells, then $CD45^{low}CD271^{high}$ cells were used as a surrogate of the freshly-collected bone progenitor cells.

B. **The total live cells after processing of RIA-W and IC-BM were counted using trypan blue.**

C. The histograms for mean fluorescence intensity (MFI) of CellROX in $CD45^{low}CD271^{high}$ cells of donor-matched IC-BM aspirates and RIA-W samples as an indicator of cellular ROS levels.

D. The comparison of MFI levels of CellROX for $CD45^{low}CD271^{high}$ cells between donor-matched RIA-W and IC-BM samples (n=10). *: *p* value<0.05, Wilcoxon matched-pairs signed rank test.

E. The comparison of MFI levels of CellROX for $CD45^{high}$ cells between donor-matched RIA-W and IC-BM samples (n=10). *: *p* value<0.05, Wilcoxon matched-pairs signed rank test.

Figure 2: The induced ROS levels and death susceptibility of freshly-collected CD45lowCD271high cells in RIA-W bag compared to IC-BM samples.

A. The MFI levels of HIF in RIA-W $CD45^{low}CD271^{high}$ cells under 1-hour 21% O_2 or hypoxia (1% O_2). The bars represent the mean of 3 samples with the standard error of the mean.

B. The bars represent the mean percentage **(with the standard error)** of dead **cells** (SYTOX+) among RIA-W $CD45^{low}CD271^{high}$ cells following treatment with different doses of TBHP, **n=5**.
C. The MFI levels of CellROX RIA-W $CD45^{low}CD271^{high}$ cells following treatment with different doses of TBHP. The bars represent the mean of 5 samples with the standard error of the mean.
D. The overly histograms showing the MFI of CellROX expressed in freshly-collected $CD45^{low}CD271^{high}$ cells without or with exposure to hypoxia (1% O_2) and/or 800µM TBHP.
E. The bars present the mean with the standard error of the mean of CellROX MFI for donor-matched samples (n=6) from IC-BM aspirate and RIA-W bag. *: *p* value<0.05, Wilcoxon matched-pairs signed rank test.
F. The flow cytometry plots for gating strategy of measuring the percentages of dead cells among freshly-collected $CD45^{low}CD271^{high}$ RIA-W cells exposed to 15mM TBHP for 1 hour.
G. The comparison of the percentages of dead cells among $CD45^{low}CD271^{high}$ cells between freshly-collected and donor-matched RIA-W and IC-BM after treatment with TBHP. *: *p* value<0.05, Paired t-test.

Figure 3: The effect of the RIA procedure and the proliferation and osteogenesis of RIA-W and IC-BM colonies.

A. The MFI levels of CellROX in IC-BM $CD45^{low}CD271^{high}$ cells either not processed (control) or diluted with 1:10 saline, **then additionally processed with** suction or by vortex (n=5). *: *p* value<0.05, Paired t-test.
B. The percentage of live cells (**Calcein** $violet^{+}$ cells) among $CD45^{low}CD271^{high}$ cells following dilution with saline, **then with additional** processing with suction or **by** vortex compared to the control sample (n=5).
C. The correlation between the percentage of dead cells (SYTOX+) of $CD45^{low}CD271^{high}$ cells and the MFI of CellROX in live cells when freshly-collected RIA-W cells (n=5) were treated with different concentrations of TBHP (400µM, 800µM, 8mM, 15mM). *: *p* value<0.05, r=0.5608, Spearman r test (n=20).
D. The RIA-W and IC-BM colonies showing ALP staining after culture in the osteogenic medium.
E. The bars represent the mean of the area of colonies (with **the** error of means) when compared between RIA-W and IC-BM (n=6) using Image J software.

F. The bars represent the mean of **the** integrated density of colonies (with **the** error of means) when compared between RIA-W and IC-BM (n=6) using Image J software.

Figure 4: The phenotype, ROS levels and death susceptibility of culture-expanded RIA-W and IC-BM MSCs.

A. The flow cytometry histograms showing the surface phenotype of passage 3 culture-expanded MSCs from RIA-W and IC-BM samples. Both types of cells were tested using antibodies for hemopoietic cell markers (CD45, CD19, CD14, and HLA-DR) and the positive MSC markers (CD73, CD90, and CD105). Grey histograms represent the isotype controls, and the black **and dark grey** histograms represent the **RIA-W and IC-BM** test samples.

B. **The bars represent the mean percentage of passage 3 culture-expanded RIA-W and IC-BM cells expressing surface markers (n=3).**

C. The bars represent the mean of MFI (with **the** standard error of means) for the positive markers when compared between passage 3 culture-expanded RIA-W and IC-BM cells (n=3).

D. The comparison of the basal the CellROX MFI between passage 3 cultured RIA-W and IC-BM MSCs (n=4).

E. The comparison of the induced the CellROX MFI between passage 3 cultured RIA-W and IC-BM MSCs with exposure to hypoxia and/or TBHP (n=4). The bars represent the mean of the CellROX MFI with **the** standard error of means.

F. The dot plot shows the mean percentages of dead cells (SYTOX+) of culture-expanded RIA-W and IC-BM MSCs after treatment with TBHP (n=6).

Figure 5: The osteogenesis, proliferation, and senescence of cultured RIA-W MSCs and IC-BM MSCs.

A. **The** flow cytometry plots showed the gating strategy for the cells based on FCS and SSC scatters, then MSCs were identified as $CD73^{+}CD90^{+}$ **$CD105^{+}CD45^{-}$**cells. The grey histogram represents **ALP** expression in MSCs cultured in the osteogenic medium compared to ones cultured in the expansion medium (black histogram).

B. A comparison of **surface ALP** levels between donor-matched RIA-W and IC-BM MSCs cultured in either the expansion or the osteogenic milieu. The figure shows the mean levels with the standard error of means (n=3) *: p value<0.05, Paired t-test.

C. The proliferation of culture-expanded donor-matched RIA-W and IC-BM MSCs was compared under **21%** and **1% O_2** conditions. The bars present the mean **of** the XTT absorbance levels with the standard error of the mean (n=6). Three cell concentrations were seeded (250, 500 and 1000 cells, black, white and grey bars respectively) per well of 96-well plate.

D. The senescence of donor-matched culture-expanded RIA-W MSCs and IC-BM MSCs was compared under **21% and 1% O_2** conditions. The figure presents the mean of β-gal levels (with the standard error of the mean) for donor-matched samples (n=8).