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Differentiation potential of human suspension mononucleated peripheral blood cells

Ruzanna Ab Kadir¹, Shahrul Hisham Zainal Ariffin^{1*}, Rohaya Megat Abdul Wahab², Sahidan Senafi¹ and Fahrul Zaman Huyop³

¹School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Selangor, Malaysia.

²Department of Orthodontics, Faculty Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

³Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, University Technology Malaysia, Johor, Malaysia.

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Stem cells are known to have the ability to renew themselves and differentiate into a diverse range of specialized cell types. Currently, the differentiation potential of human peripheral blood mononucleated cells in suspension as stem cells is not well-understood. The aim of this study was to investigate the differentiation potential of suspension mononucleated cells from human peripheral blood to differentiate. The osteoblast and osteoclast differentiation potential of suspension peripheral blood mononucleated cells were examined by molecular, biochemical and cell morphology analyses. The expression of osteoblast marker (osteonectin, *SPARC*) and osteoclast marker (tartrate resistant acid phosphatase, *TRAP*) as well as high alkaline phosphate (ALP) and TRAP enzyme activity were observed at days 14 and 10 of osteoblast and osteoclast differentiation, respectively. Morphology analyses showed that mononucleated cells successfully differentiated into osteoblasts and osteoclasts. The existence of stem cells in mononucleated cells was evaluated by the expression of a stem cell factor (*KIT*) and a haematopoietic stem cell marker (signalling lymphocytic activation molecule family member 1, *SLAMF1*). This study has shown that these suspension mononucleated cells possess differentiation potential through *in vitro* study. Human peripheral blood suspension mononucleated cells that have multi-lineage differentiation potential may provide a new source of stem cells that may be used for bone regeneration and tissue engineering applications.

Key words: Peripheral blood, osteoblast cells, osteoclast cells, osteoblast differentiation, osteoclast differentiation.

INTRODUCTION

Stem cells are unspecialized cells that have two defining properties: The ability to differentiate into other cells and the ability to self-regenerate. Consequently, they are the most versatile and promising cell source for the regeneration of aged, injured and diseased tissues (Lutolf et al., 2009; Shahrul Hisham et al., 2005 a,b). There are two types of mammalian stem cells: embryonic stem cells that are isolated from the inner cell mass of blastocysts and adult stem cells that are found in adult tissues.

Embryonic stem cells can develop into virtually all cell types of the body because they are pluripotent. Adult stem cells are thought to be limited to differentiate into different cell types of their tissue of origin (multipotent). However, stem cell research has been highly controversial due to the ethical issues concerned with the culture and use of stem cells derived from human embryos. Nowadays, adult stem cells become the focus of stem cells research as they offer hope for cell therapy to treat diseases in the future and ethical issues do not impede their use.

In this study, mononucleated cells from peripheral blood as the source of adult stem cells were subjected to differentiate into osteoblast and osteoclast instead of the

*Corresponding author. E-mail: hisham@ukm.my or shahroy8@gmail.com. Tel: 603-89214361. Fax: 603-89252698.

more common source, bone marrow. Peripheral blood is well known as one of the main hematopoietic stem cell (HSC) sources other than bone marrow (Arslan and Moog, 2007). Peripheral blood can be widely used as a source of stem cells because these cells offer several advantages, such as being easier and commonly collected than bone marrow, which must be extracted from within the bone.

Previous work done by Wang et al. (2006) had only analysed adherent cells as peripheral blood stem cells. Therefore, in this study, we investigated differentiation potential of human mononucleated cells in suspension instead of adherent cells as source of stem cells. Adult stem cells are multipotent stem cells. To determine multipotency of suspension mononucleated cells, osteoblast and osteoclast differentiation were done as osteoblast originated from mesenchymal stem cells lineage and osteoclasts were originated from haematopoietic stem cells lineage. Our results demonstrated that mononucleated cells in suspension have potential to differentiate into multilineage bone cells (osteoblast and osteoclast). Thus, can be used as a source of cell-based therapy.

MATERIALS AND METHODS

Isolation and proliferation of suspension mononucleated cells from human peripheral blood

Peripheral blood mononucleated cells were isolated using the Ficoll-Paque density-gradient separation method. Human peripheral blood samples were obtained from 18 to 25-year-old healthy donors following the collection of consent from the donors and approval from the ethical committee of Faculty of Science and Technology, UKM. The blood samples were diluted three times with Hanks' balanced salt solution (Sigma, USA). The diluted blood samples were carefully layered 1:1.5 on Ficoll-Paque™ PLUS (GE Healthcare, Sweden) and centrifuged at 400 *g* for 20 min at room temperature. The mononucleated cells layer at the plasma-Ficoll interface were washed three times with phosphate buffer saline (PBS) and cultured in 6-well plates with complete medium containing alpha medium essential medium (AMEM), 2% (v/v) penicillin-streptomycin and 10% (v/v) new-born calf serum (NBCS) at 37°C in a humidified atmosphere containing 5% CO₂ for 4 days. After 4 days culture, non-adherent (suspension) mononucleated cells were transferred into new plates and maintained in the complete medium with twice weekly medium exchange for another 3 days to give a total of 7 days *in vitro* culture selection before being further used.

Reverse-transcriptase polymerase chain reaction (RT-PCR) amplification

Total RNA was extracted using TRI Reagent™ (Sigma, USA) following the manufacturer's instructions. Mononucleated cells cultured in complete, osteoblast differentiation or osteoclast differentiation medium were harvested at day 7, 10 or 14, respectively. One microgram of total RNA was subjected to RT-PCR amplification using an Access RT-PCR system kit provided by Promega, USA. First-strand complementary DNA (cDNA) was synthesised by reverse transcription at 45°C for 45 min and

followed by Avian myeloblastosis virus reverse transcriptase inactivation at 94°C for 2 min. Second-strand cDNA synthesis and PCR amplification consisted of 40 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*), 54°C (signalling lymphocyte activation molecule family member 1, *SLAMF1*), 53°C (stem cell factor, *KIT*), 55°C (osteonection; *SPARC*) or 58°C (tartrate resistant acid phosphatase, *TRAP*) for 1 min and extension at 68°C for 2 min, with a final cycle at 68°C for 7 min. The specific primer sequences used were as follows: (*GAPDH*) [5'CCATGGAGAAGGCTGG³ (sense) and 5'CAAAGTTGTCAGGATGACC³ (antisense)], *SLAMF1* [5'CTCTGCGTTCTGCTCCTAC³ (sense) and 5'TTGGTCACTTCTG GGTCTG³ (antisense)], *KIT* [5'TCCTTGACCTTCGTGCTGT³ (sense) and 5'CCTTCCTGCTTTCCCGTAT³ (antisense)], *SPARC* [5'GCAGAGGAAACCGAAGAGG³ (sense) and 5'TTGTGGCAA GAAGTGGC³ (antisense)] and *TRAP* [5'GACCACCTTGGCAA TGTCTCTG³ (sense) and 5'TGG CTG AGG AAG TCA TCT GAG TTG³ (antisense)]. *GAPDH* was used as positive control. RT-PCR amplifications were performed in Mastercycler Gradient thermocycler (Eppendorf, Germany). The PCR products were separated by 1% (w/v) agarose gel electrophoresis. The separated DNA fragments were visualised by ethidium bromide (EtBr) staining and photographed using the alpha imaging system (Alpha Innotech, USA) under UV light.

The PCR products were gel-eluted using the Wizard SV gel and purification clean-up system (Promega, USA) before being ligated into the pGEM-T easy vector system (Promega, USA). The plasmids containing specific genes were identified by DNA sequencing using the BigDye terminator sequencing kit (Applied Biosystems, USA) and confirmed by comparison of the cloned human sequence to known sequences (GenBank/EML database) using the BLAST server at NCBI.

Differentiation of mononucleated cells into osteoblasts and osteoclasts

Suspension mononucleated cells were seeded at 1.0 x 10⁵ cells ml⁻¹ in 24-well plates (BD Bioscience, USA) for differentiation. Cells cultured in complete medium were supplemented with differentiation factors; 50 µg/ml ascorbic acid (Sigma, USA) and 10 mM β-glycerophosphate (Sigma, USA) for 14 days to induce differentiation into osteoblasts. For osteoclast differentiation, complete medium were supplemented with growth factors; 50 ng/ml recombinant soluble receptor activator of nuclear factor kappa B (NF-κB) ligand (sRANKL) and 25 ng/ml macrophage-colony stimulating factor (M-CSF) and then cultured for 10 days. Both cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells cultured in complete medium without differentiation/growth factors were used as control.

Alkaline phosphatase (ALP) enzyme activity assay

For ALP enzyme activity assays, the cultures were washed with PBS (Sigma, USA). The cells then were incubated in 0.1 M NaHCO₃-NaCO₃ buffer (pH 10), containing 0.1% (v/v) Triton X-100, 2 mM MgSO₄ and 6 mM p-nitrophenol phosphate (pNPP) (Sigma, USA) for 30 min at 37°C. The reaction was stopped by adding 1 ml 1.5 M NaOH (Sigma, USA) and the absorbance was measured at 405 nm. The ALP activity is represented as specific activity. The specific activity was determined by unit activity per total protein content (mg). Protein content was assessed by using Bradford method. One unit of ALP activity represents the hydrolysis of 1 µM pNPP per minute at 37°C. The ALP specific activity was presented in percentage value, which was compared with ALP specific activity in control cells (cells culture in complete medium) that act as basal

activity (100%).

Tartrate-resistant acid phosphatase (TRAP) enzyme activity assay

The cultures were washed with PBS and sodium acetate buffer (50 mM, pH 5.5) containing Triton X-100 (0.1% v/v) was added. The culture were then stored at -20°C. Cell extracts were collected and centrifuged after thawing. Protein content and TRAP enzyme activity were assayed in the supernatant of cell extracts. Protein content was assessed by using Bradford method. TRAP enzyme activity were assayed in cell extracts and in conditioned medium using pNPP as a substrate in an incubation medium (1 ml) containing the following components: 10 mM pNPP, 0.1 M Na-acetate (pH 5.8), 0.15 M KCl, 0.1% (v/v) Triton X-100, 10 mM Na-tartrate, 1 mM ascorbic acid and 0.1 mM FeCl₃. The mixture was incubated for 1 h and the reaction was stopped by adding 0.3 M NaOH. The absorbance was immediately read at a wavelength of 405 nm. One unit of TRAP activity hydrolyses 1 μM pNPP per minute at 37°C. The TRAP specific activity was presented in percentage value, which was compared with TRAP specific activity in control cells (cells culture in standard growth medium) that act as basal activity (100%).

Morphology analyses

Approximately 1 × 10⁵ cells/well in 24-well plates (BD Labware, England) were washed with PBS and centrifuged at 76 g for 5 min, 25°C. Von Kossa and May Grunwald-Giemsa staining was done for observing osteoblast and osteoclast cells, respectively.

Von Kossa staining was carried out by fixing the cells with 10% (v/v) formalin in PBS for 30 min and washed with deionised distilled water (ddH₂O) three times. Then, the cells were stained with freshly prepared 5% (v/v) silver nitrate (Sigma, USA) solution for 30 min and washed well with ddH₂O three times. Next, the cells were developed with fresh 5% (v/v) sodium carbonate (Sigma, USA) in 25% (v/v) formalin more than 5 min for mineral and matrix staining. After three washes with ddH₂O, the cells were finally fixed with 5% (v/v) sodium thiosulfate (Sigma, USA) for 2 min to remove unreacted silver nitrate. Finally, the cells were washed well with ddH₂O three times and air-dried.

For May Grunwald-Giemsa staining, the slides were immersed in 100% (v/v) May-Grunwald for 2.5 min. The slides were then transferred directly to 4% (v/v) Giemsa for 2.5 min and briefly rinsed with distilled water. Excess dye was wiped and air-dried. The von-Kossa and MayGrunwald-Giemsa stained areas were viewed by light microscopy. The percentages of differentiated cells were calculated based on approximately 100 cells counted randomly from each slide. Each photomicrograph was representative of 3 independent experiments with actual magnifications of 400x.

RESULTS

Stemness of human peripheral blood mononucleated cells

The stemness of isolated mononucleated cells was determined by expression of a stem cell factor (*KIT* gene) and a haematopoietic stem cell marker (*SLAMF1*). As shown in Figure 1b, the control mononucleated cells were positive for the presence of *KIT* and *SLAMF1* genes, whereas these genes were not expressed in mononucleated cells cultured in osteoblast and osteoclast

differentiation medium for 14 and 10 days, respectively. The RT-PCR showed amplification of products of the expected size for *SLAMF1* (403 bp) and *KIT* (316 bp). The *GAPDH* gene was expressed in both mononucleated cells cultured in complete (undifferentiated mononucleated cells) osteoblast and osteoclast differentiation (differentiated mononucleated cells) mediums, as shown by the RT-PCR product of 195 bp (Figure 1a).

Expression of osteoblast and osteoclast molecular marker in differentiated mononucleated cells

Mature osteoblasts and osteoclast cells are characterised by the expression of specific gene markers. SPARC and TRAP has been considered an important marker in osteoblastic (Ishigaki et al., 2002) and osteoclastic differentiation (Shi et al., 2004) respectively. RT-PCR was performed to confirm the expression profiles of these genes before and after differentiation. By RT-PCR analysis, we observed that mononucleated cells cultured in osteoblast differentiation medium for 14 days were positive for SPARC activation with a size of 211 bp, in contrast to mononucleated cells cultured in complete medium (Figure 1c). For the osteoclast differentiated cells, the expression of TRAP (176 bp) was detected only in mononucleated cells after being cultured in osteoclast differentiation medium (Figure 1d). All experiments were controlled by GAPDH expression.

Biochemical analysis of differentiated mononucleated cells

ALP enzyme activity assay was carried out to measure osteoblast differentiation while for osteoclast differentiation; TRAP enzyme activity assay was done. All biochemical assays were conducted simultaneously on days 0, 3, 5, 7, 10 and 14 for osteoblast differentiation and 0, 3, 5, 7 and 10 for osteoclast differentiation. Figure 2 shows the percentage of ALP activity during osteoblast differentiation. Mononucleated cells cultured in osteoblast differentiation medium were positive for ALP enzyme as the enzyme activity gradually increased from day 3 to day 14 of osteoblast differentiation. A significant ($p < 0.05$) increase were observed at day 7, 10 and 14 when compared with control cells at the same day which indicate that mononucleated cells were successfully differentiated to osteoblasts. However, ALP and TRAP activities were observed in osteoblast differentiation. TRAP enzyme activity shows an increment from day 3 to 10 of during osteoclast differentiation (Figure 3). There was a significant increased of TRAP activity at days 7 and 10 of osteoclast differentiation, indicating that the mononucleated cells differentiated into osteoclasts. In contrast, control cells proved to lack detectable of TRAP activity. Control cells were mononucleated cells cultured in complete medium without differentiation factors or growth

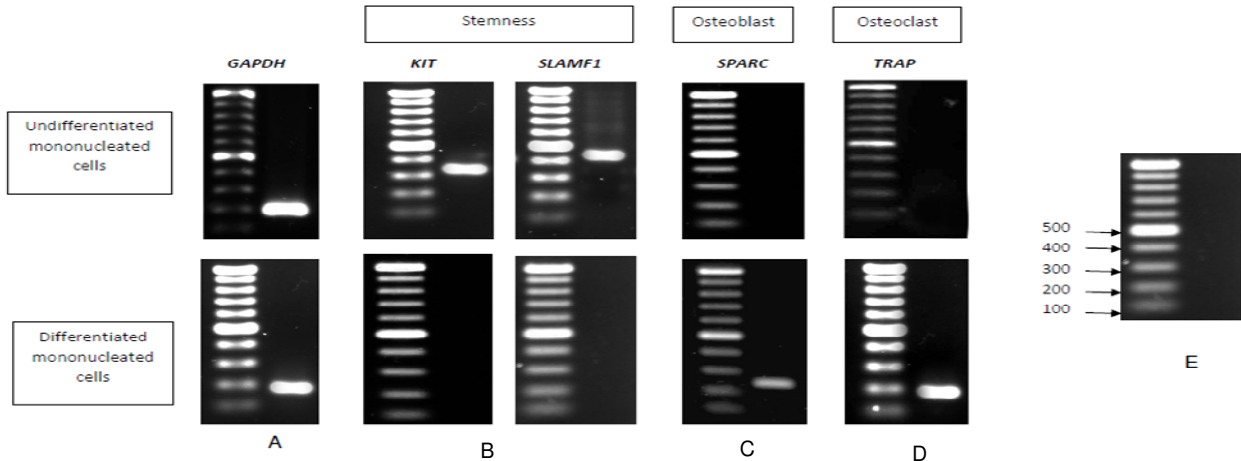


Figure 1. RT-PCR analysis on undifferentiated mononucleated and differentiated mononucleated cells. Total RNA was isolated from mononucleated cells cultured in complete (undifferentiated mononucleated cells), osteoblast and osteoclast differentiation medium (differentiated mononucleated cells). (A) The expression of *GAPDH* (195 bp), the housekeeping gene, was used as positive control for RT-PCR analyses; (B) the expression of *KIT* (316 bp) and *SLAMF1* (403 bp) of undifferentiated cells indicate the stemness of the cells; (C) the expression of *SPARC* (211 bp) and *TRAP* (176) as osteoblast and osteoclast marker respectively; (D) the labelled 100 bp DNA ladder to identify the approximate size of RT-PCR products.

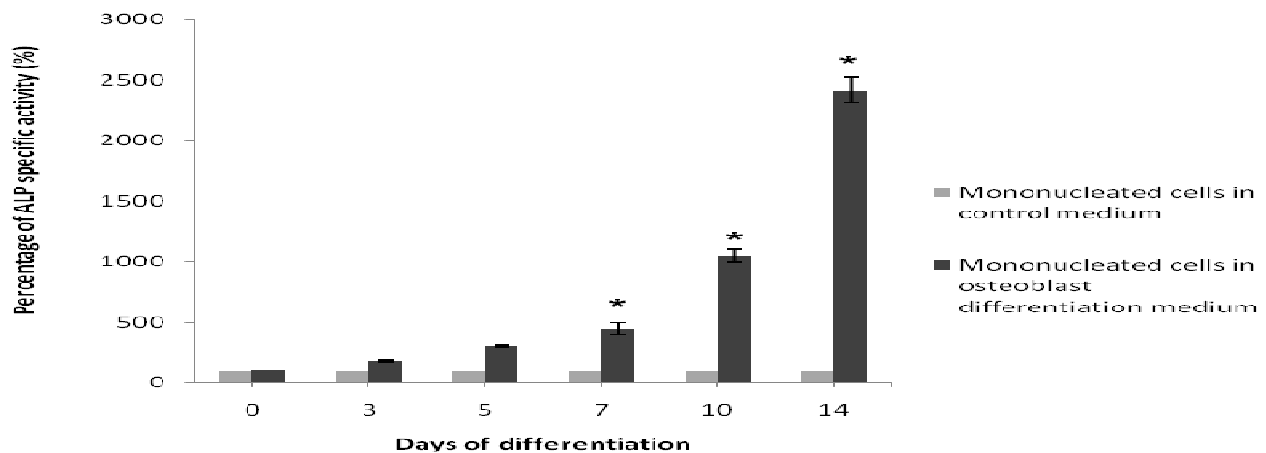


Figure 2. Percentage of ALP specific enzyme activities during osteoblast differentiation. ALP specific enzyme activity was assayed during 0, 3, 5, 7, 10 and 14 days of osteoblast differentiation. (*) indicates a significant ($p < 0.05$) increase of ALP enzyme activity when compared with control cells using a paired t-test. Results are presented as mean \pm SD ($n = 3$).

factors. Each experiment was done on triplicate measurements.

Morphology analysis of differentiated cells

An osteoblastic morphology was acquired by the deposition of mineralized extracellular matrix as demonstrated with Von Kossa staining (Figure 4a). The bone nodules containing mineralized extracellular matrix will be stained black or dark brown from the von Kossa staining procedure. When fewer developed nodules are present,

the matrix will be stained yellow to brown in colour. As shown in Figure 4a, cells that were stained dark brown or black were increased upon time of differentiation. No mineralized matrix could be detected in the undifferentiated mononucleated cells at day 0 of osteoblast differentiation. The osteoblastic morphology was further assessed by calculating the percentage of mononucleated cells that successfully differentiate into osteoblast cells. Figure 5 shows the percentage of mineralized cells. At day 7, ~35% of the mononucleated cells were differentiated into osteoblasts, while at day 14 of differentiation, the percentage increased to ~78%.

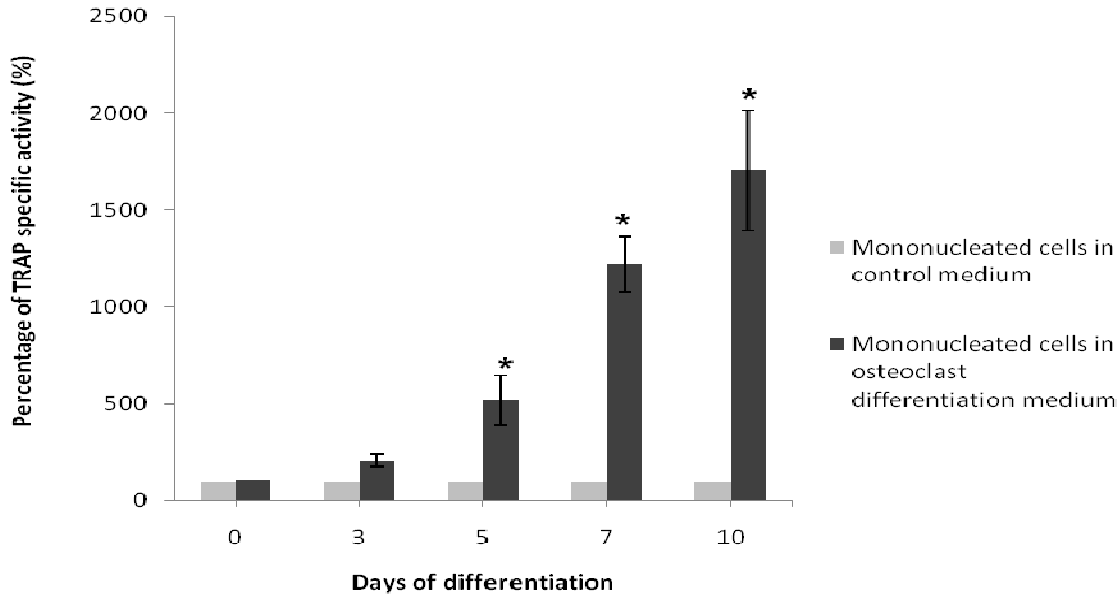


Figure 3. Percentage of TRAP specific enzyme activities during osteoclast differentiation. TRAP specific enzyme activity was assayed during 0, 3, 5, 7 and 10 days of osteoclast differentiation. (*) indicates a significant ($p < 0.05$) increase of TRAP enzyme activity when compared with control cells using a paired t-test. Results are presented as mean \pm SD ($n = 3$).

These results showed that the percentages of differentiated osteoblasts cells that were stained dark brown or black were increased upon differentiation.

Osteoclastic morphology was illustrated by the presence of multinucleus cells. May Grunwald-Giemsa staining will revealed the large cells containing many nuclei in their cytoplasm which is characteristic of bone-resorbing cells or osteoclast. The nucleus will stained as dark purple while the cytoplasm will stained as light purple with Grunwald-Giemsa staining. From Figure 4b, the morphology of multinucleated osteoclast cells were observed at day 10 of mononucleated cells cultured in osteoclast differentiation medium. The percentage of fully differentiated osteoclasts increased with time (Figure 6). Approximately 19 and 50% fully differentiated osteoclasts were observed at day 5 and 10 of osteoclast differentiation, respectively.

DISCUSSION

In many different adult tissues, stem cells generate new cells either continuously or in response to injury (Clarke and Frisen, 2001). Adult stem cells are multipotent stem cells that can give rise to different types of cells. To fulfil the criteria of multipotency, stem cells should be capable of self-regenerate and differentiate into several lineages *in vitro* (Hows, 2005). Throughout this study, the differentiation potential of mononucleated in suspension has been demonstrated.

The existence of stem cells in isolated mononucleated cells were determined by activation of *KIT* and *SLAMF1*.

GAPDH, a well-known housekeeping gene was used as positive control for RT-PCR analysis because it is constitutively expressed at the same level in mammalian cells and tissues (Barber et al., 2005). As shown in Figure 1b, *KIT* and *SLAMF1* genes only activate in primitive undifferentiated mononucleated cells. *KIT* was previously reported to be expressed in both human and mouse undifferentiated embryonic stem cells, with a role in maintaining their undifferentiated state and correlated with functional measures of their pluripotency (Basahmboo et al., 2006) and with the self-renewal function of foetal and adult stem cells (Hassan, 2009; Abdulrazak et al., 2010). In addition, the activation of the *SLAMF1* gene that we observed from RT-PCR amplification demonstrated that the mononucleated cells were HSC. *SLAMF1* (CD150) is the founding member of the SLAM family of cell surface receptors (Engel et al., 2003) and SLAM family receptors are differentially expressed among haematopoietic pro-genitors in a way that correlates with progenitor primitiveness. The expression of *SLAMF1* is unique because it is only expressed by HSC, not by non-self-renewing haematopoietic progenitors or restricted haematopoietic progenitors (Mark et al., 2005). Thus, the suspension mononucleated cells we obtained from peripheral blood were demonstrated to exhibit the existence of stem cells, specifically HSC.

The potentiality of mononucleated cells to differentiate into other specialized cells was examined by *in vitro* differentiation of mononucleated cells towards osteoblast and osteoclast cells using specific induction mediums. Mononucleated cells were subjected to differentiate into

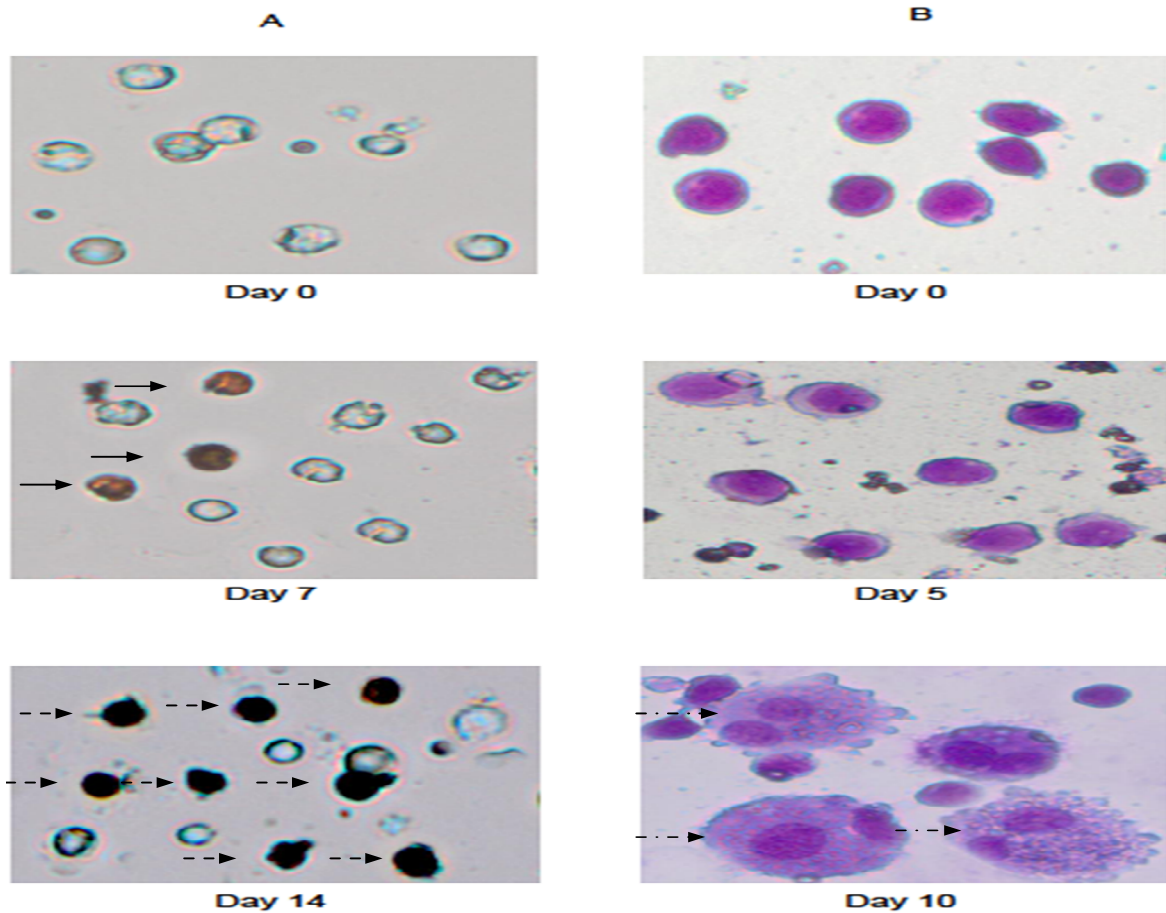


Figure 4. Morphology of differentiated osteoblast by Von Kossa staining and osteoclast cells stained by May-Gruwald-Giemsa. (A) and (B) were morphology of mononucleated cells cultured in osteoblast and osteoclast differentiation medium respectively. For osteoblast cells, arrows represent the cell nodules containing calcium mineral stained brown (-->) or black (->); while for osteoclast cells arrows represent the multinucleated cells (- · · ·>). Each photomicrograph above was representative of independent experiments with actual magnifications of 400x.

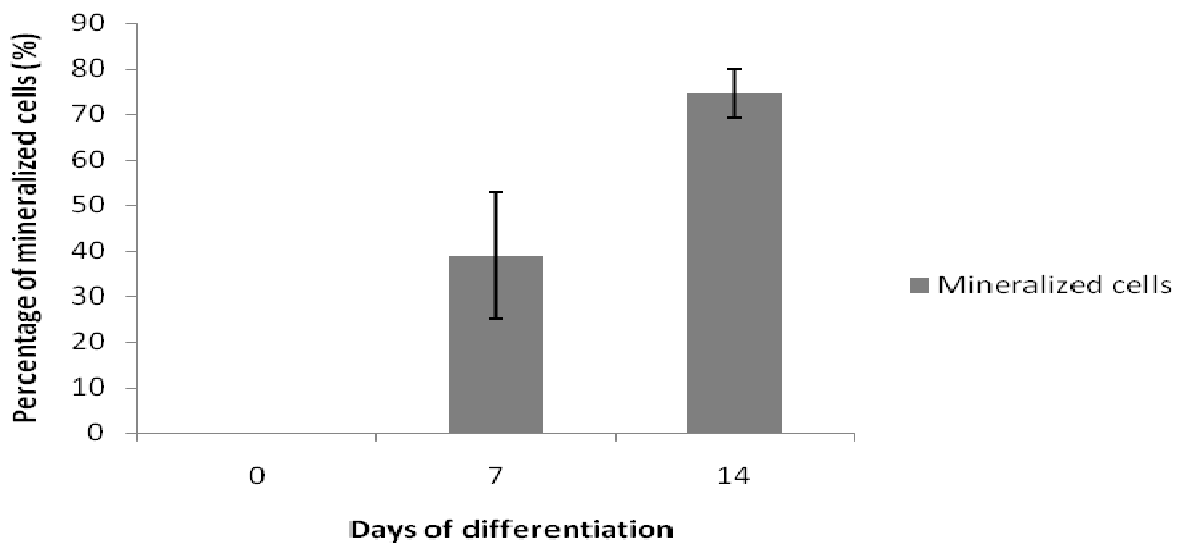


Figure 5. Percentage of mineralized cells. Mineralized cells indicate osteoblast cells that were stained as brown or black by Von Kossa staining after 0, 7 and 14 days of osteoblast differentiation.

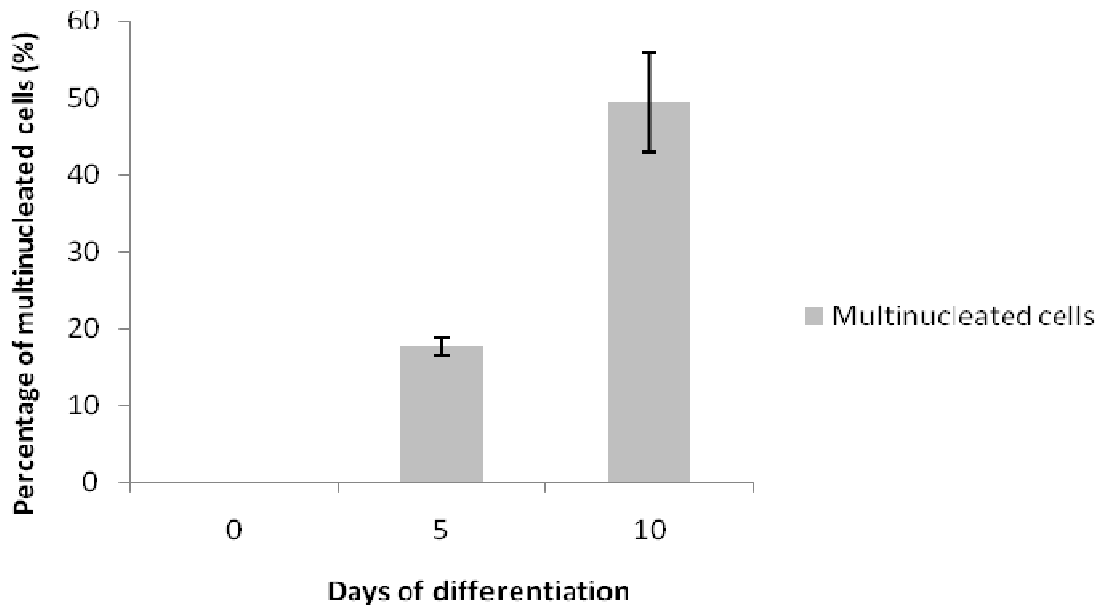


Figure 6. Percentage of multinucleated cells stained by May-Gruwald-Giemsa. Percentages of multinucleated osteoclast cells were calculated after 0, 5 and 10 days of osteoclast differentiation

osteoblast by adding differentiation factors: ascorbic acid and β -glycerophosphate. Osteoblast differentiation was assessed by ALP activity, an early marker of osteoblastic cell differentiation (Shen et al., 2009). As observed, ALP activity and abundance of mineralised deposits were significantly higher on day 14 of osteoblast differentiation in the presence of these two differentiation factors. Ascorbic acid enhances osteoblastic differentiation by increasing collagen accumulation, which results in increased ALP expression in some osteogenic cells (Shiga et al., 2003), while β -glycerophosphate is routinely added to bone cell cultures to induce osteo-genesis and promote calcium phosphate deposition (Coelho and Fernandes, 2000). The mechanism by which β -glycerophosphate induces mineralisation is closely linked to the high ALP activity of bone cell cultures; this compound is rapidly hydrolysed by ALP to produce high levels of local phosphate ions providing the chemical conditions for mineral deposition (Coelho and Fernandes, 2000). Following the period of matrix maturation, nodule cells begin to mineralize the extracellular matrix (Porter et al., 2003, Ariffin et al., 2010) and these nodules will stain dark brown or black with Von Kossa staining (Wang et al., 2006; Yazid et al., 2010). Osteonectin, also called secreted protein acidic and rich in cysteine (*SPARC*) or BM-40, is a matricellular glycoprotein expressed in a variety of mammalian tissues including muscle, brain, adipose, testes, kidney, skin, bone and cartilage (Kapinas et al., 2009). Its expression is increased in areas of extracellular matrix remodeling such as wound healing, angiogenesis, tumor growth and metastasis (Bradshaw and Sage, 2001). The membrane-bound enzyme alkaline phosphatase, together with other glycoproteins (oste-

onectin, trombospondins, fibronectin, osteopontin, bone sialoprotein) and proteoglycans (decorin, biglycan, fibromodulin), are proportionally the most abundant noncollagenous proteins of the bone extracellular matrix and are considered as pertinent markers of osteoblastic differentiation (Garcia et al., 2002). Thus, the presence of calcium nodules (Figure 4) related with the increase of ALP activity (Figure 2) and the activation of the *SPARC* (Figure 1) gene showed that the respective cells had differentiated into osteoblasts.

Mononuclear monocytic precursor cells become committed to osteoclast lineage under the influence of M-CSF and RANKL (Tanaka et al., 2005). These stimulated precursors express the TRAP, which acts as a biological marker for osteoclasts activity during all stages of development and maturation. A number of genes such as *TRAP* and *CTSK* are activated during osteoclastogenesis (Shi et al., 2004). All of these genes have been functionally implicated in osteoclastic bone resorption (Feng et al., 2001). *TRAP* is important during osteoclast differentiation as it is required for bone matrix resorption and collagen turnover (Matsuo and Irie, 2008; Siti Afeefah et al., 2011). Expression of *TRAP* gene as well as significant increased of TRAP enzyme activity were observed in mono-nucleated cells cultured in osteoclast differentiation medium which contain M-CSF and recombinant sRANKL. Present results also show that undifferentiated mono-nucleated cell culture was negative for the presence of *TRAP* gene and TRAP enzyme activity. This finding is in agreement with previous findings that *TRAP* is specific osteoclast markers that are highly expressed during osteoclast differentiation (Intan Zarina et al., 2008, 2010). Osteoclast cell is a large multi-

nucleated cell. RANKL stimulates the development and fusion of mononuclear monocytic precursor cells to become large multinuclear cells. When these differentiated cells reach a critical size they become activated mature osteoclasts and adhere to bone surfaces, forming a lacuna underneath in which to resorb the bone (Gardner, 2007). The morphological analysis in this study proved that upon time of osteoclast differentiation, percentage of multinucleated cells was highly increased. This shows that the morphology of the osteoclast induced cells exhibit the characteristics of osteoclasts.

CONCLUSION

In conclusion, this study has provided biochemical; molecular biology and morphological evidence that suspension mononucleated cells from human peripheral blood have fulfil the criteria of multipotency stem cells as these cells are capable of self-regenerate and underwent multi-lineage differentiation. With the increasing demand of peripheral blood samples for stem cell research, we have demonstrated that suspension mononucleated adult peripheral blood can provide additional source of stem cells for cell-based therapy.

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REFERENCES

- Abdulrazak H, Moschidou D, Jones G, Guillot PV (2010). Biological characteristics of stem cells from foetal, cord blood and extraembryonic tissues. *J. R. Soc. Interface*, 7: S689-S706.
- Ariffin SH, Abidin IZ, Yazid MD, Wahab RM (2010). Differentiation analyses of adult suspension mononucleated peripheral blood cells of *Mus musculus*. *Cell Commun. Signal*, pp. 8: p. 29.
- Arslan O, Moog R (2007). Mobilization of peripheral blood stem cells. *Transfus. Apher. Sci.*, 37(2): 179-185.
- Barber RD, Harmer DW, Colemann RA, Clark BJ (2005). GAPDH as a housekeeping gene; Analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol. Genomics*, 21(3): 389-395.
- Basahmboo A, Taylor AH, Samuel K, Panther JJ, Whetton AD, Forrester LM (2006). The survival of differentiating embryonic stem cells is dependent on the SCF-Kit pathway. *J. Cell Sci.*, 119(15): 039-46.
- Bradshaw AD, Sage EH (2001). SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. *J. Clin. Invest.*, 107(9): 1049-1054.
- Clarke D, Frisén J (2001). Differentiation potential of adult stem cells. *Curr. Opin. Genet. Dev.*, 11(5): 575-80.
- Coelho MJ, Fernandes MH (2000). Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, β -glycerophosphate and dexamethasone on osteoblastic differentiation. *Biomaterials*, 21(11): 1095-1102.
- Engel P, Eck MJ, Terhorst C (2003). The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat. Rev. Immunol.*, 3(10): 813-821.
- Feng X, Novack DV, Faccio R, Ory DS, Aya K, Boyer MI, McHugh KP, Ross FP, Teitelbaum SL (2001). A Glanzmann's mutation in beta 3 integrin specifically impairs osteoclast function. *J. Clin. Invest.*, 107(9): 1137-1144.
- Garcia T, Roman-Roman S, Jackson A, Theilhaber J, Connolly T, Spinella-Jaegle S, Kawai S, Courtois B, Bushnell S, Auberval M, Call K, Baron R (2002). Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of mouse calvaria primary osteoblasts *in vitro*. *Bone*, 31(1): 205-211.
- Gardner CR (2007). Morphological analysis of osteoclastogenesis induced by RANKL in mouse bone marrow cell cultures. *Cell Biol. Int.*, 31(7): 672-682.
- Hassan HT (2009). KIT expression in human normal and malignant stem cells. *Leuk. Res.*, 33(1): 5-10.
- Hows J (2005). Adult stem cell therapy beyond haemopoietic stem cell transplantation? An update. *Transpl. Immunol.*, 14(3-4): 221-223.
- Ishigaki R, Takagi M, Igarashi M, Ito K (2002). Gene expression and immunohistochemical localization of osteonectin in association with early bone formation in the developing mandible. *Histochem. J.*, 34(1-2): 57-66.
- Intan Zarina ZA, Shahrul Hisham ZA, Rohaya MAW, Sahidan S, Zaidah ZA (2008). Osteoclast and osteoblast development of *Mus musculus* haemopoietic mononucleated cells. *J. Biol. Sci.*, 8(3): 506-516.
- Intan Zarina ZA, Shahrul Hisham ZA, Zaidah ZA, Rohaya MAW (2010). Potential differentiation of three types of primitive cells originated from different proliferation term of mouse blood. *Sains Malaysiana*, 39(2): 305-313.
- Kapinas K, Kessler CB, Delany AM (2009). miR-29 suppression of osteonectin in osteoblasts: regulation during differentiation and by canonical Wnt signaling. *J. Cell Biochem.*, 108(1): 216-224.
- Lutolf MP, Gilbert PM, Blau HM (2009). Designing materials to direct stem-cell fate. *Nature*, 462(7272): 433-441.
- Mark JK, Omer HY, Toshihide I, Osman HY, Cox T, Sean JM (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*, 121(7): 1109-1121.
- Matsuo K, Irie N (2008). Osteoclast-osteoblast communication. *Arch. Biochem. Biophys.*, 473(2): 201-209.
- Porter RM, Huckle WR, Goldstein AS (2003). Effect of dexamethasone withdrawal on osteoblastic differentiation of bone marrow stromal cells. *J. Cell Biochem.*, 90(1): 13-22.
- Shahrul Hisham ZA, Intan Zarina ZA, Sahidan S, Nor Muhammad M, Rohaya MAW, Zaidah ZA (2005a). Stem Cells in Blood Development. *Sains Malaysiana*, 34(1): 21-26.
- Shahrul Hisham ZA, Rohaya MAW, Ismanizan I, Nor Muhammad M, Zaidah ZA (2005b). Stem Cells, Cytokines And Their Receptors. *AsPac J. Mol. Biol. Biotechnol.*, 13(1): 1-13.
- Shen Q, Zhu S, Hu J, Geng N, Zou S (2009). Recombinant human bone morphogenetic protein-4 (BMP-4)-stimulated cell differentiation and bone formation within the expanding calvarial suture in rats. *J. Craniofac. Surg.*, 20(5): 1561-1565.
- Shiga M, Kapila YL, Zhang Q, Hayami T, Kapila S (2003). Ascorbic acid induces collagenase-1 in human periodontal ligament cells but not in MC3T3-E1 osteoblast-like cells: Potential association between collagenase expression and changes in alkaline phosphatase phenotype. *J. Bone Miner. Res.*, 18(1): 67-77.
- Shi Z, Silveira A, Patel P, Feng X (2004). YY1 is involved in RANKL-induced transcription of the tartrate-resistant acid phosphatase gene in osteoclast differentiation. *Gene*, 343(1): 117-126.
- Siti Afeefah MY, Rohaya MAW, Sahidan S, Zaidah ZA, Razak MA, Shahrul Hisham ZA (2011). Proliferation and Biochemical Analyses of Osteoblast/Osteoclast Differentiation from Human Mononucleated Cells. *Sains Malaysiana*, 40(4): 305-309.
- Tanaka S, Nakamura K, Takahashi N, Suda T (2005). Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL-RANK signaling system. *Immunol. Rev.*, 208: 30-49.
- Wang XS, Sam SW, Yip KH, Lau HYA (2006). Functional characteriza-

tion of human mast cells cultured from adult peripheral blood. *Int. Immunopharmacol.*, 6(5): 839-847.

Wang YH, Liu Y, Maye P, Rowe DW (2006). Examination of Mineralized Nodule Formation in Living Osteoblastic Cultures Using Fluorescent Dyes. *Biotechnol. Prog.*, 22(6): 1697-1701.

Yazid MD, Ariffin SH, Senafi S, Razak MA, Wahab RM (2010). Determination of the differentiation capacities of murines' primary mononucleated cells and MC3T3-E1 cells. *Cancer Cell Int.*, 10: p. 42.