

Research Report

Factors Influencing Osteogenic Differentiation of Human Embryonic Stem Cells

For a fulfillment of a one year sabbatical leave
April 2007 –March 2008

Of

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June 28, 2008

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Abstract

Problem: The study aims to investigate the factors influencing differentiation of hESCs into mesenchymal cell lineage with an emphasis on osteoblastic differentiation.

Methods: Human embryonic stem cells (hESCs) (BG01) were induced to form embryonic bodies (EBs) and were manually picked as cell clumps or enzymatically dissociated into single cells. Cells were then seeded on either fibronectin, gelatin or matrigel coated plates. These cells were cultivated in culture medium supplemented with fetal bovine serum (FBS) for mesenchymal differentiation, Group A and osteogenic medium for direct osteogenic differentiation, Group B. Differentiation was monitored by observing cell morphology and expressions of mesenchymal stem cell and mesenchymal cell phenotypes. To investigate growth of cells on three-dimensional matrixes, differentiated cells were transfected with a Lentivirus carrying Col2.3-GFP plasmid and seeded on a collagen composite scaffold (BD Bioscience) with human fibrin gel and cultured in osteogenic medium. Growth and osteogenic differentiation were monitored.

Results: It was found that hESCs could differentiate into mesenchymal stem cells and osteoblastic cells by bypassing embryonic body (EB) stage. Differentiation of hESCs through the single cell seeding method yielded a high degree of osteoblastic differentiation. Single cells had a low attachment rate and viability after seeding. Enriched matrix, such as matrigel, was crucial for initial attachment rate. Clumps of hESCs differentiated into multiple cell types in mesenchymal lineage, including mesenchymal stem cells, pre-osteoblasts, smooth muscle cells and fibroblasts, as they were demonstrated by expressions of CD105, CD73, STRO-1, Vimentin, Actin and collagen type I. A transition from mesoderm to mesenchymal cell types was indicated by co-expressions of Brachyury, a mesodermal marker, with those markers. Differentiations of these mixed populations of cells could be directed into osteogenic, adipogenic and chondrogenic in specialized condition mediums. Single cell seeding of hESCs showed markedly high osteogenic differentiation demonstrated by high ALP activity, osteocalcin and calcium levels. The differentiated cells were able to grow and differentiate on three-dimensional porous scaffold and fibrin matrix.

Conclusions: Extracellular matrix and culture medium played important roles in regulating initial attachment and growth of differentiated hESCs. Mesenchymal differentiation of hESCs could be induced directly from hESCs in clumps and single cell hESCs had a higher osteogenic potential.

Background of the study

Embryonic stem cells

Human embryonic stem cells were first derived from the inner cell mass of the blastocyst stage (100-200 cells) of embryo generated by *in vitro* fertilization (Thomson, *et al* 1998). Embryonic stem cells are pluripotent cells that are characterised by the ability to proliferate over prolonged periods of culture while remaining undifferentiated and maintaining a stable karyotype, but with the potential to differentiate into derivatives of all three germ layers. In order to maintain pluripotency, hESCs are cultured on mouse embryonic fibroblast (MEF) feeder layers and are grown under serum-free conditions using serum replacement with supplements of basic fibroblast growth factor. Embryonic stem cells are useful in studying early human embryonic development, cell differentiation, human genetic disorders and being unlimited supply of normal differentiated cells to engineer diseased tissues to regain normal function. There are seventy-one different hESC lines listed in the NIH Stem Cell Registry, with only 22 currently available to researchers (Hyslop, *et al* 2005).

Differentiation of hESCs into osteoblasts and mesenchymal stem cells

Highly proliferative and multi-lineage natures of hESCs enhance clinical application potential of cell transplantation technologies in regenerative medicine and bone tissue engineering. It is clearly shown that through embryonic body (EB) formation step, hESCs in osteogenic medium can be induced to differentiate into osteoblastic cells (Sottile, *et al* 2003). Later on it has been shown that efficiency of osteogenic differentiation can be enhanced by directly induced osteogenic differentiation of undifferentiated hESCs, either single cells or cell clumps, by bypassing EB formation step (Karner, *et al* 2007, Karp, *et al* 2006). The studies suggest that multiple cell types in EBs might delay osteogenic differentiation of hESCs and undifferentiated hESCs can spontaneously differentiate into osteoblastic cells (Karp, *et al* 2006).

To apply hESCs in clinical application, it is essential that differentiation pathway of the cells is clearly defined to prevent teratoma formation *in vivo* (Ahn, *et*

al 2006). In tissue regeneration, interaction of multiple cell types creates functional tissue. Thus directing differentiation potential of highly plasticity cells as hESCs into pluripotential precursor cells such as mesenchymal stem cells (MSCs) will be a great advantage for tissue repair in terms of better control of differentiation potential and creating precursor of multiple cells types, including adipocytes, chondrocyte, myocyte and osteoblasts.

Previous studies clearly show differentiation of hESCs into mesenchymal stem cells which possess similar functional differentiation potential and share partial similarity of gene expression as hMSCs from bone marrow and cell lines (*Barberi, et al* 2005, *Olivier, et al* 2006), *but function of cell signaling and molecular characteristics of the differentiated MSCs are needed to be further investigated.*

hESCs can differentiate into MSCs with and without EB formation steps and the differentiation process is clearly influenced by condition of culture medium (*Barberi, et al* 2005, *Olivier, et al* 2006, *Trivedi and Hematti* 2007, *Xu, et al* 2006). *Olivier et al., 2006* induce MSC differentiation by cultivated cell clumps in culture medium with 10% FBS for 50 – 180 days. *Barberi et al., 2005* and *Trivedi et al., 2007* reported that co-culture of hESCs with OP9 induced MSC differentiation of single cell hESCs and commented that OP-9 co-culture enhance MSC differentiation rate of hESCs (*Barberi, et al* 2005, *Olivier, et al* 2006). These studies clearly showed that the differentiated cells expressed cell surface markers of MSCs and were able to differentiate into cells in mesenchymal lineage, adipocytes, osteoblasts and myocytes (*Barberi, et al* 2005, *Olivier, et al* 2006, *Trivedi and Hematti* 2007, *Xu, et al* 2006). However, numbers of positive cells are low, 5% and the differentiation is feeder layer dependent and require a long culture time of more than 40 days (*Olivier, et al* 2006). These disadvantages hinder high efficiency of MSC induction and require cell surface sorting to enrich MSCs population.

Fetal bovine serum (FBS) is a common factor playing an essential role in mesenchymal cell culture medium of all studies. Culture medium with FBS supplemented with condition medium from hepatocarcinoma cell line (HepG2) (*Hwang, et al* 2006) and co-culture with mouse OP9 cells, respectively (*Barberi, et al* 2005, *Trivedi and Hematti* 2007) are reported to promote mesodermal and mesenchymal differentiation of hESCs, H1 and H9 cell lines *Xu et al., 2007* report relation between degree of mesodermal differentiation in EBS and osteogenic differentiation. The authors reported that differentiated cells deriving from EBS,

which were incubated in a high concentration of knock out serum replacement (KOSR), 80 – 90% and low concentration of 10-20% FBS for 5 days, differentiated into fibroblast-like cells with osteoblastic differentiation potential.

There is no clear evidence demonstrating sequential differentiation of cells from mesoderm to mesenchymal and osteoblastic cells. Only Kaerner et al. 2007 showed a brief expression of Bracyury and FTL-1, markers of mesodermal differentiation, during osteoblastic differentiation of hESCs in mineralizing medium. The study also showed the differences of patterns of gene expression in differentiation process among different cell lines (Kaerner, et al 2007). Thus, there is no evidence demonstrating molecular transitional sequences of hESCs into osteoblastic cells, consequently, factors regulate and control osteogenic differentiation can not be identified.

Rational of the study

hESCs may serve as a model to study molecular mechanism regulating differentiation of cells in mesenchymal lineage and may be a potential source for cell transplantation in skeletal repair. Despite it is clearly shown that MSCs can differentiate into different cells types, pattern of gene expressions and regulation signals have not been clearly defined. Creating common features of regulating gene and expression patterns are more complicated when differences among cell lines and conditions do exist.

Gene expression profile and MSC differentiation potential of hESCs – BG01 are needed to be further investigated. There is no evidence to demonstrate a role of mesodermal differentiation and specific genes or molecular signal on mesenchymal differentiation of hESCs. Molecular signaling controlling growth and differentiation of the differentiated MSCs are needed to be further studied.

Aims of the study

A main goal of this study is to induce MSC differentiation of hESC, BG01 cell line, from individual hESC and compare molecular and functional behaviors of cells with hMSCs. Pattern of gene expression will be investigated to determine regulating genes and compare with hMSC cell line and hMSC from bone marrow stromal cells.

In this first part of the study, the study aimed to investigate factors influencing differentiation of hESCs into mesenchymal cell lineage with an emphasis on osteoblastic differentiation.

Materials and methods

hESC Cell culture

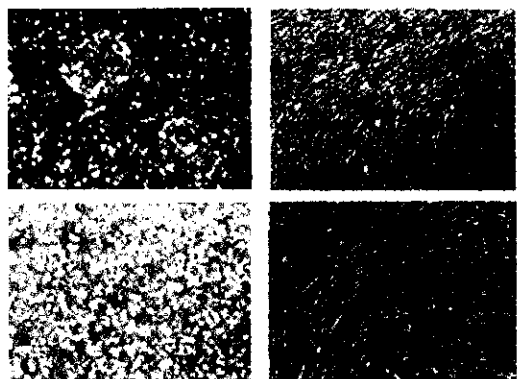
Human embryonic stem cells (hESCs) (BG01, Bresagen, Inc. Atlanta, GA) were cultivated on irradiated mouse embryonic fibroblast (MEF) feeder layer at a density of 4×10^5 cells/60 mm plates (0.1% gelatin-coated plates). The hESC culture medium consists of 80% DMEM-F12, 20% (v/v) knockout serum replacement (KOSR), 200 mM L-glutamine, 10 mM nonessential amino acids, 14.3M β -mercaptoethanol and 4 ng/ml bFGF. Cell cultures were incubated at 37°C in 5% CO₂ at 95% humidity. Culture medium was changed everyday and manually passaged every 7 day.

Effects of matrixes on cell attachment and growth

hESCs in undifferentiated stage in passages 40-50 were manually picked as cell aggregate or enzymatically dissociated into single cells. Cells aggregates were seeded on fibronectin coated plate (20 μ g/ml, 2 ml/60 mm plate), while single cells were seeded on fibronectin, matrigel (1 mg/ml, 2 ml/60 mm plate) or gelatin (0.1% gelatin, 2 ml/60 mm plate) coated plates (1×10^5 cells/cm²). Attachment and growth of cells on the matrixes were monitored under light microscope. At confluence, they were passaged on to cell culture plate surface and cultivated in differentiation mediums according to study design (*Fig 1*).

Fig. 1: Demonstrating initial cell seeding in osteogenic medium, Group B of

- hESCs in aggregate on day 1 and (B) confluent cells in passage 5 and
- (C) hESCs in single cells on day 1 and (D) confluent cells in passage 5



Mesenchymal and osteogenic differentiation

The study was categorized into two groups. In Group A, hESCs in aggregates were continuously cultivated in medium supplemented with 10% fetal bovine serum (FBS) for mesenchymal differentiation for 5 passages, and then in passages 6-7, they were further cultivated in osteogenic medium to induce osteogenic differentiation. .

In Group B, hESCs in aggregates and single cells were continuously cultivated in osteogenic medium for a direct osteogenic differentiation (*Fig. 1*).

Differentiation mediums

Mesenchymal differentiation medium comprised of 80% DMEM-F12, 10% fetal bovine serum, 200 mM L-glutamine and 10 mM nonessential amino acids (Olivier et al., 2006).

Osteogenic differentiation medium comprises of alpha-Minimum Essential Medium (α -MEM), 50 μ g/ml ascorbic acid, 5 mM β -glycerophosphate and 100 nM dexamethasone (Sottile et al., 2003).

Adipogenic medium: DMEM high glucose, 10% FBS, 1 μ M dexamethasone, 10 μ g/ml insulin, 0.5mM 3-Isobutyl-1-methyl-xanthine and 0.2mM indomethacin (Olivier et al., 2006).

Chondrogenic medium DMEM high glucose, 10%FBS, 100 mM sodium pyruvate, 40 μ g/ml proline, 100 nM dexamethasone and 200 μ M ascorbic acid and 10 ng/ml TGF- β 3 (Mackay, *et al* 1998)

Functional differentiation

To investigate potential of mesenchymal stem cells to differentiate into three different cell types in mesenchymal lineage, cells in Group A were undergone functional differentiation into osteogenic, adipogenic and chondrogenic differentiation. Cells in osteogenic and adipogenic mediums were cultivated on two-dimensional cell culture plate, 5×10^3 cells/cm² and chondrogenic medium as cell pellet culture, 2×10^6 cells/pellet. For cell pellet culture, 2×10^6 cells in chondrogenic medium were centrifuged at 500 g for 5 min. Rounding up of cell pellet could be observed on the next 24 hours. Culture medium was changed every 3 days and cells were incubated at 37°C in 5% CO₂ at 95% humidity.

Differentiation was monitored by observing cell morphology and expressions of mesenchymal stem cells (MSCs) (cell surface markers) and osteogenic (alkaline phosphatase activity (ALP), osteocalcin levels and mineralized bone matrix), adipogenic (lipid deposit in cytoplasm) and chondrogenic cell phenotypes (alcian blue and safranin O stainings of glycoproteinous matrix) (Mackay AM et al., 1998; Olivier et al., 2006; Sottile et al., 2003; Karp et al., 2006).

Characterization of cell surface markers

Differentiated cells in mesenchymal medium were undergone immunohistochemical staining to determine expression of MSC cell surface markers, CD105 (eBioscience), CD166 (BD PharmingenTM), CD73 (eBioscience) and STRO-1 (Santa Cruz Biotechnology, Inc.), mesenchymal cells, vimentin (Santa Cruz Biotechnology, Inc.), smooth muscle alpha actin (Santa Cruz Biotechnology, Inc.) and collagen type I (Santa Cruz Biotechnology, Inc.). Cells were seeded on 2-well chamber slide (Lab-Tek, NUNC), 1×10^4 cells/cm² for 24 hour, then they were fixed with cold methanol for 5 min and undergone standard procedure for immunohistochemical staining using R.T.U. Vectastain Universal, ABC kit (Vector lab, California) with AEC substrate for peroxidase (Vector lab, California).

Expression of CD105 and STRO-1 were also monitored using flow cytometrical analysis (FCM) (Lian, *et al* 2007).

Growth and differentiation of cells on three-dimensional matrixes

Col2.3-GFP lentivirus is an osteoblast lineage specific. GFP expression increased overtime and was intense after 24 days which correlated with osteoblast differentiation (Kalajzic, *et al* 2002a, Kalajzic, *et al* 2002b). To investigate osteoblastic differentiation of cells on three-dimensional matrixes, cells in Group A in passage 6 were transfected with a lentivirus carrying Col 2.3-GFP plasmid and seeded on a collagen composite scaffold (BD Biosciences, MA) with human fibrin gel (20 mg/ml) and cultured in osteogenic medium for 21 days.

Before examining under confocal laser scanning microscope (CLSM), the cell constructs, cells on scaffolds, were lively labeled with red live-cell tracker (Molecular Probe, USA). Growth and osteogenic differentiation on three-dimensional matrixes were monitored under confocal laser scanning microscope (CLSM) at culture-day 21.

Results

Effects of matrixes:

Single cells had a low attachment rate and viability after seeding. Enriched matrix, such as matrigel, was crucial for initial attachment rate (*Fig 2*) and culture medium supplemented with FBS enhanced growth of the differentiated hESCs, particularly hESCs in single cells..

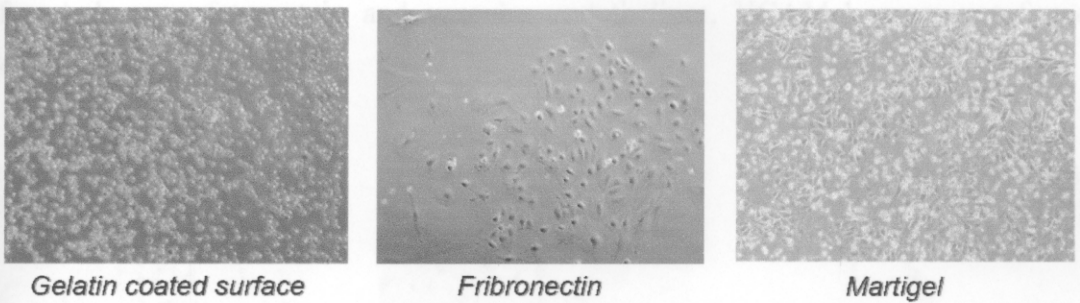


Fig 2: Initial single cell seeding of hESCs in single cell suspension on gelatin, fibronectin and matrigel coated plates, 24 hrs after seeding

Mesenchymal cell differentiation:

It was found that the culture condition promoted differentiation of hESCs into cells in mesenchymal lineage. hESCs could differentiate into MSCs and osteoblasts by bypassing EBs stage. Morphologies of cells became homogenous with fibroblast-like appearance in a higher passage number. In passages 5-7, shapes of cells were homogenous with fibroblast-like appearance (*Fig 3A&B*).

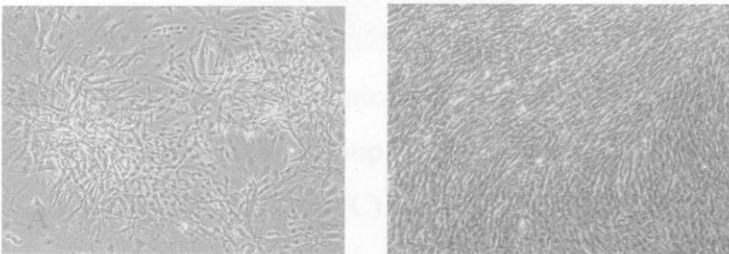


Fig 3: Differentiated hESCs in MSC culture medium in the first passage on day 7 (A) and in the seventh passage on day 25 (B) showing homogeneity of size and shape of cells in a higher passage.

Differentiation of hESCs from cell aggregate in Group A

CELL SURFACE EXPRESSION

hESCs in aggregate differentiated into MSCs and different cell types in mesenchymal lineage which were demonstrated by expressions of CD105, CD166, STRO-1 and vimentin, smooth muscle alpha-actin and collagen type I ((Lian, *et al* 2007). A transition from mesoderm to mesenchymal cell types was indicated by a co-expression of Brachyuru, a mesodermal marker with those MSC markers. Expression of endoderm, α -fetoprotein and vascular endothelium, VCAM-1, were not found (Karner, *et al* 2007) (Fig 4).

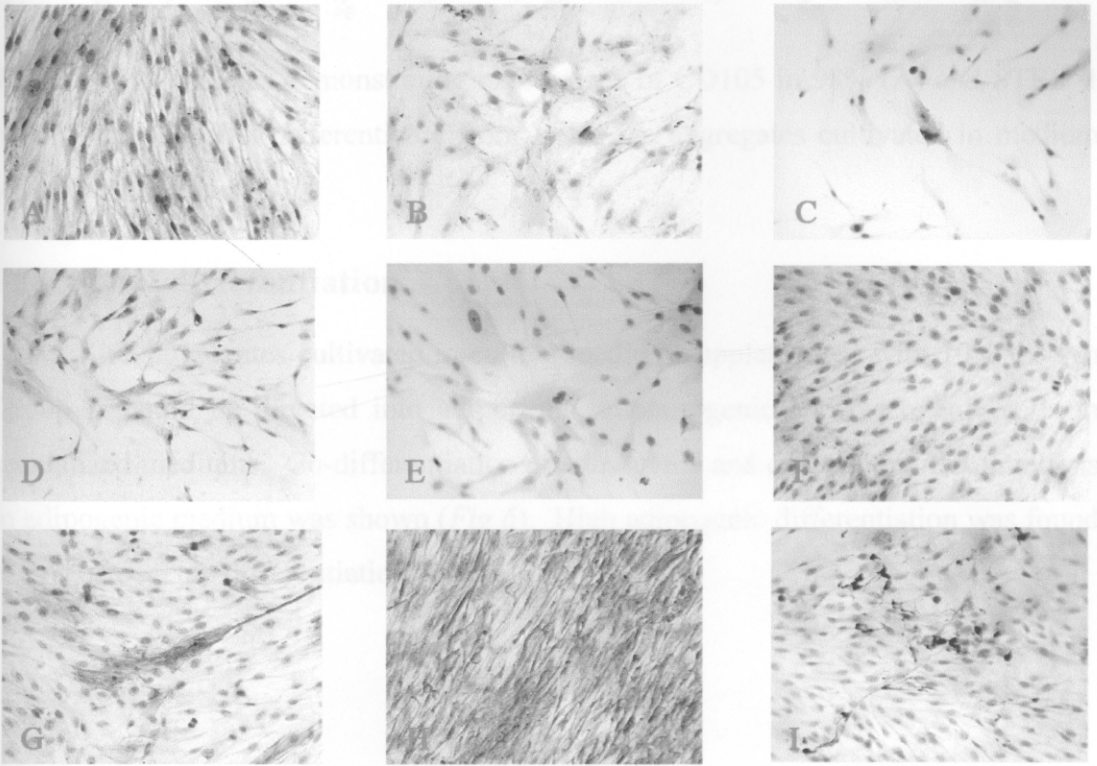


Fig 4: Demonstrating immunohistochemical staining of cell aggregate cultivated in medium supplemented with FBS, Group A. Brown to red staining indicates expression of (A) CD105, (B) CD166, (C) CD73, (D) STRO-1, (E) Brachyury, (F) alpha fetoprotein, (G) alpha actin, (H) vimentin and (I) collagen type I (x20, AEC substrate with hematoxylin counter staining)

FLOW CYTOMETRICAL ANALYSIS

Flow cytometrical analysis (FCM) exhibited that more than 80% of cells in Group A expressed CD105 on their cell surface and less than 5% were positive for STRO-1 (*Fig 5*).

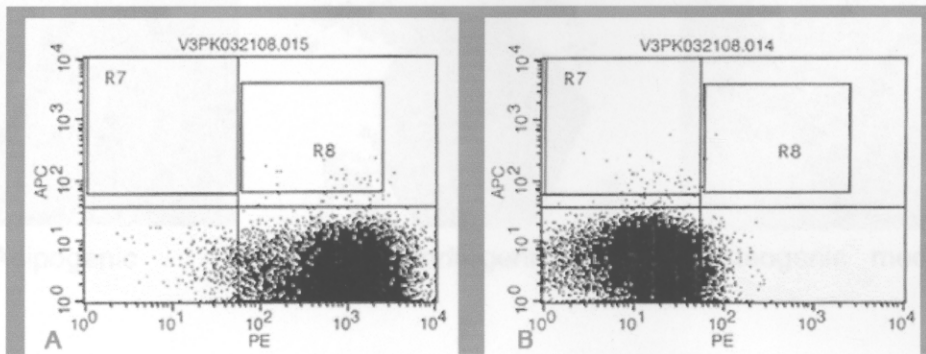


Fig 5: FCM analysis demonstrating expressions of CD105 in 98% (A) and STRO-1 in 3.5% (B) of cells differentiated from hESCs in aggregates cultivated in medium with FBS in Group A.

Functional differentiation

Cell aggregates cultivated in culture medium supplemented with 10% FBS in Group A could be directed into adipogenic, chondrogenic and osteogenic cells in specialized mediums. Co-differentiation of adipogenic and osteogenic differentiations in adipogenic medium was shown (*Fig 6*). High adipogenic differentiation was found in low osteogenic differentiation culture.

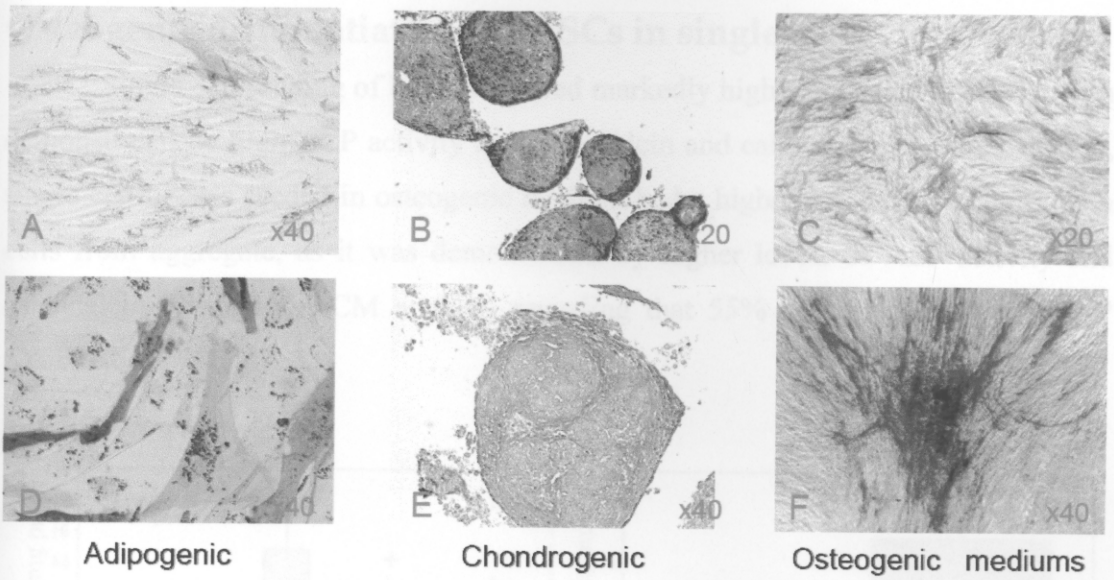


Fig 6: Functional differentiation of MSCs from cell aggregate, Group A, in specialized medium for 21 days, A & D in adipogenic with oil red o (in red) and ALP (in blue) double staining, B&E, pellet culture in chondrogenic with safranin O (in red and alcian blue (in blue) stainings and C & F, in osteogenic mediums with ALP staining and ALP & Von Kossa (in black) co-staining.

Osteogenic differentiation on three-dimensional matrix

The differentiated cells were able to grow and differentiate on three-dimensional porous scaffold and fibrin matrix. Osteogenic differentiation of MSCs from cell aggregate in Group A was further demonstrated by expressing Col 2.3-GFP of cells growing in fibrin gel on 3D-collagen scaffolds and cell culture plate (Fig 7).

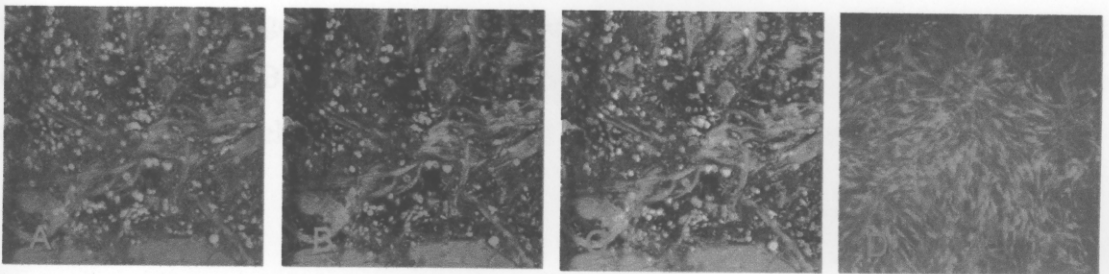


Fig 7: Images from CLSM displaying growth and expression of Col2.3-GFP of MSCs derived from hESC aggregates in Group A differentiating in osteogenic medium in fibrin gel on collagen scaffolds for 28 days *in vitro*. (A) GFP expressing cells, (B) live cell labeling (C) superimposition of A and B and (D) growth of cells on cell culture plate (x20 magnification).

Osteogenic differentiation of hESCs in single cells

Single cell seeding of hESCs showed markedly high osteogenic differentiation demonstrated by high ALP activity and osteocalcin and calcium levels. In Group B, single cell hESCs seeded in osteogenic medium had a higher osteogenic activity than cells from aggregate, as it was demonstrated by higher levels of ALP activity and osteocalcin levels and FCM analysis revealing that 55% of these cells were ALP positive cells (*Fig 8*).

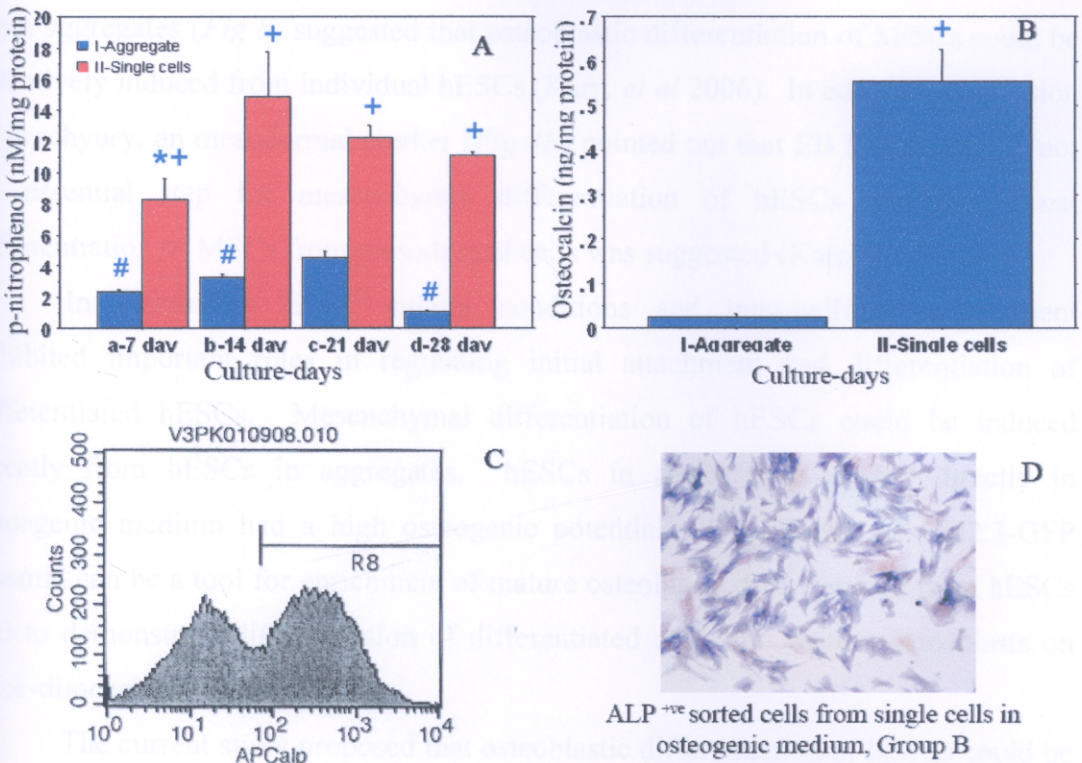


Fig 8 Demonstrating expression of osteogenic phenotypes of hESC aggregates and single cells in osteogenic medium in Group B (A) levels of ALP activity during 28 days cell culture. (B) levels of osteocalcin in culture medium on day 28 (C) FCM analysis of ALP⁺ cells in R8 and (D) ALP staining (in blue) of the sorted cells

Discussion and Conclusion

The presence culture condition promoted differentiation of hESCs into MSCs and osteoblasts demonstrated by functional differentiation of MSCs and high levels of expressions of MSCs and osteoblastic phenotypes (*Fig 4-8*) (Karner, *et al* 2007, Karp, *et al* 2006, Sottile, *et al* 2003). An expression of Col 2.3-GFP, a maker of a late stage osteoblastic differentiation revealed that MSCs from cell aggregate could differentiate into mature osteoblasts on three-dimensional matrix (*Fig 7*). Higher levels of osteoblastic phenotypes of cells from hESCS in single cells in comparison to cells from aggregates (*Fig 8*) suggested that osteoblastic differentiation of hESCs could be effectively induced from individual hESCs (Karp, *et al* 2006). In addition, expression of brachyury, an mesodermal marker (*Fig 4E*) pointed out that EB formation was not an essential step for mesenchymal differentiation of hESCs and transitional differentiation of MSCs from mesodermal cells was suggested (Karp, *et al* 2006).

In conclusion ECM, culture conditions and inter-cellular environment exhibited important roles in regulating initial attachment and differentiation of differentiated hESCs. Mesenchymal differentiation of hESCs could be induced directly from hESCs in aggregates. hESCs in single cells seeded directly in osteogenic medium had a high osteogenic potential. Transfection of Col2.3-GFP plasmid can be a tool for enrichment of mature osteoblasts differentiated from hESCs and to demonstrate differentiation of differentiated cells into mature osteoblasts on three-dimensional matrix.

The current study proposed that osteoblastic differentiation of hESCs could be achieved through direct osteoblastic differentiation of hESCs as single cells suspension in osteogenic medium.

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