

# **ADULT MESENCHYMAL STEM CELLS DERIVED FROM EMBRYONIC STEM CELLS**

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## **ABSTRACT**

Mesenchymal stem cells (MSC) are useful in cell therapy and stem cell research but they have a limited lifespan in culture. Our goal was to develop a unique and limitless supply of mesenchymal stem cells derived from human embryonic stem cells, opening up new uses and enhance existing uses of these important cell types. For any application that uses large quantities of MSC, such as high throughput drug compound screening, eventually MSC will have to be rederived, likely from a different genetic background, thus assay responses may differ among genetic backgrounds and could reduce assay consistency and robustness. Using the NIH approved cell lines BG01 and WA09, respectively, we have developed a method for the derivation of MSC-like cell lines that can differentiate into mesenchymal lineages such as bone and cartilage. The cells will be of use by researchers developing human and veterinary applications in cell therapy

## **INTRODUCTION**

MSC are one of the most extensively studied adult stem cells and have broad potential uses. A few of their uses include, studying developmental pathways, disease modeling, in vitro toxicology models, compound screening, humanized animal models and cellular therapy. Although MSC are proliferative, a major drawback is that unlike ESC, MSC are not immortal stem cells. Mesenchymal stem cells reside in tissues of mesenchymal origin particularly in the bone marrow but they have also been found in many other tissues. The MSC is extremely rare, being only 1 in every 100,000 cells in bone marrow (Pittenger et al., 1999). Under both in vivo and in vitro conditions, these cells can differentiate down the adipogenic, chondrogenic, osteogenic lineages. MSCs have a role in hematopoiesis since they form part of the bone marrow microenvironment that promotes hematopoietic stem cell proliferation and differentiation. In addition to the common reports of MSC plasticity, several laboratories have reported a broader differentiation spectrum for MSCs including cell phenotypes from other embryonic germ layers such as neuron-like cells. Due to their stem cell nature, MSCs exhibit proliferative potential in vitro (50 doublings) while retaining multipotential differentiation capacity (Bianchi et al., 2003).

Despite their longer in vitro lifespan, MSCs eventually senesce in culture like other primary cells (Banfi et al., 2002). For instance, multi-colony derived (non-clonal) MSC lines cultured under optimal conditions can undergo about 25 passages representing more than 50 cell doublings before senescence (Bianchi et al., 2003). In another study, human MSC clonal lines completely stopped growing at about 22 cell doublings after approximately 80 days in culture (Muraglia et al., 2000), showing a significant, but limited life span in vitro. Strategies to prolong MSC replication without impairing their multipotentiality have been attempted. Several studies have shown that forced expression of human telomerase reverse transcriptase (hTERT) in MSCs can dramatically extend their lifespan to > 260 population doublings, while maintaining their osteogenic,

chondrogenic, adipogenic, neurogenic and stromal differentiation potential (Kobune et al., 2002; Shi et al., 2003). However over expression of hTERT is not normally seen in MSC and their function may be altered. Further, immortalized MSCs can express higher levels of osteogenic lineage-specific genes, such as Cbfa1/Runx2, osterix and osteocalcin, compared with non-transduced MSCs, which could potentially compromise their ability to commit to other cell lineages and utility as a normal human cell source for research and drug discovery (Gronthos et al., 2003). Therefore, there are limits to MSC proliferation and immortalizing MSC has drawbacks.

For any application that uses large quantities of cells, such as high throughput drug compound screening, eventually MSC will have to be rederived, likely from a different genetic background, thus assay responses may differ among genotypes backgrounds and reduce assay consistency and robustness. We propose to eliminate these problematic variables by producing a highly uniform source of human MSC like cells from a human ESC master source eliminating genetic background variability and eliminating the need to reintroduce gene reporter assays since the human ESC are immortal and will have the original genetic modifications.

## MATERIALS AND METHODS

**Media: WA09 human ESC media:** DMEM/F12 medium (Gibco) supplemented with 20% KSR (Gibco), 0.1 mM MEM, 50 U/ml Penicillin, 50 µg/ml Streptomycin, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 4 ng/ml of bFGF; **MEF media:** DMEM (Gibco) supplemented with 20% fetal bovine serum (FBS, Hyclone), 0.1mM MEM, 0.1 mM β-mercaptoethanol (Gibco), and 4 ng/ml bFGF. **human MSC Derivation media:** EGM2-MV (Lonza) is a proprietary media mix containing 5% FBS, VEGF, FGF-2, EGF, R3-IGF-1, hydrocortisone and ascorbic acid. **Adipogenic media: Derivation:** DMEM (High Glucose), Pen/Strep (Gibco), 1µM dexamethasone, 10µg/ml insulin, 200µM indomethacin, 500µM 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma), 10% FBS (Hyclone); **Maintenance:** DMEM (HG), Pen/Strep, 10µg/ml insulin and 10% FBS. **Chondrogenic derivation media:** DMEM (HG), 100nM dexamethasone, Pen/Strep, 50µg/ml ascorbic acid, 40µg/ml L-proline, 1X ITS+1 supplement, 1mM sodium pyruvate (Sigma), 10ng/ml TGFβ-3 (R&D Systems). **Osteogenic derivation media:** DMEM (Low Glucose), 100nM dexamethasone, 50µM ascorbic acid, 10mM β-glycerophosphate (Sigma), 10% FBS and Pen/Strep.

**Mouse embryonic fibroblast (MEF) feeder layers:** MEFs used for maintaining the WA09 line are derived from E13.5 random bred mouse fetuses by standard procedures in MEF media and culture dishes are treated with 0.2% gelatin, cells harvested, quantified and cryopreserved in aliquots. For preparation of feeder layers, aliquots are thawed, and MEFs are proliferated to confluence before inactivation with growth in 10 µg/mL mitomycin C (Sigma) in MEF media for 2 hrs. The procedure is approved by the IACUC.

**Flow Cytometry:** Flow cytometry were utilized to track the expression of the MSC markers CD90 and CD105 positive and negative CD34 (BD Bioscience) and the

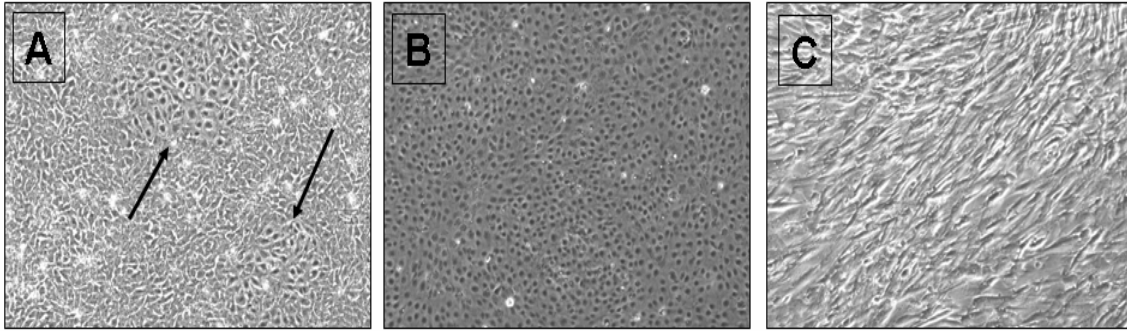
pluripotent marker Oct 4 (R&D systems). For the analysis,  $10^5$  cells for each condition is extracted for antibody incubation or control for the first 10 passages then every 5 succeeding passages. Cultures will be trypsin passaged, washed in PBS<sup>++</sup> and fixed for 10 min in 4% paraformaldehyde (PFA), washed 2x with PBS<sup>++</sup> then blocked for 30 min with ice cold FACS buffer (PBS<sup>++</sup> + 5% FBS) on ice. After blocking cells are pelleted and resuspended in 100  $\mu$ l of FACS buffer then incubated with either a fluorophor conjugated antibody or isotype control for 30 min on ice in the dark. Cells are washed and resuspended in 0.5 ml FACS buffer. Flow cytometry was performed on a FACSCaliber cytometer (Becton Dickinson) and analyzed using FloJo software (Treestar, Inc) at the UGA flow cytometry core facility.

### **Gene Transcription and Quantitative Real-Time PCR**

hESC were grown as described over an 30 day period in 6-well plates. Samples were collected for RNA analysis on day 0 (Control) and every 5 days thereafter. Total RNA was isolated using a Qiagen RNeasy kit according to the manufacturer's instructions and quantified using RNA 600 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA was reverse transcribed using Superscript II (Invitrogen). For quantitative Real-Time PCR, a low-density array was custom designed by Applied Biosystems (ABI, Foster City, CA) for 47 primer sets plus an 18S endogenous control. The 47 primers sets included gene markers for pluripotency (10), the three germ layers: ectoderm (5), mesoderm (14) and endoderm (3), endothelial (6), trophoblast (2) and miscellaneous markers (7). The qRT-PCR low-density array was processed using an ABI 7900HT. Relative quantification of the gene expression output was performed using Sequence Detection System software (SDS v2.2.1, ABI). The SDS utilizes relative quantification of gene expression by way of the comparative  $C_T$  method where the relative quantity (RQ) =  $2^{-\Delta\Delta C_T}$ ,  $\Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{Time\ x} - (C_{T,Target} - C_{T,Actin})_{Time\ 0}$  and  $C_T$  is defined as the threshold cycle where the target gene surpasses a defined amplification. All genes are normalized to 18S as a loading control and day 0 as the base line expression.

**Functional assays: von Kossa staining:** Wash with PBS<sup>++</sup> and fix wells with 4% PFA, add 5% silver nitrate solution and expose to UV light for 60 min, wash with dH<sub>2</sub>O, incubate in 5% sodium thiosulfate solution for 5 min, wash with dH<sub>2</sub>O and acquire bright field and phase contrast images.

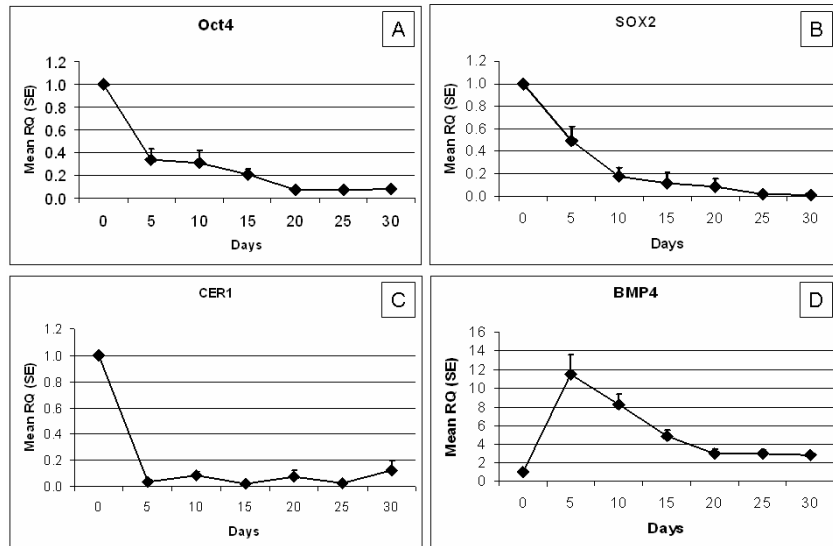
## RESULTS AND DISCUSSION



**Figure 1 MSC-like cells can be derived from human ESC in monolayer culture.** human ESC (BG01 and WA09) grown on laminin in a monolayer will initiate epithelial foci (arrows) when cultured in EGM-2-MV media (A). Over a 20 to 30 day period the foci expand throughout the dish forming a confluent monolayer with a uniform epithelial morphology (B). When the cells are passaged as a single cells suspension onto plastic tissue culture ware without a substrate the epithelial cells transition to a mesenchymal phenotype within two passages (C). Phase contrast images were acquired at 40x magnification.

### Human ESC to a MSC adherent differentiation morphology.

Using the NIH approved cell lines BG01 and WA09, we have developed a method for the derivation of MSC-like cell lines. Human ESC are grown without feeder cells on laminin in human ESC proliferation media until ~90% confluent. At this point the media is changed and the cells are grown in the proprietary media EGM-2-MV (Lonza) for 20-30 days. During this time the stem cells begin to differentiate as epithelial foci (**Fig. 1A**,

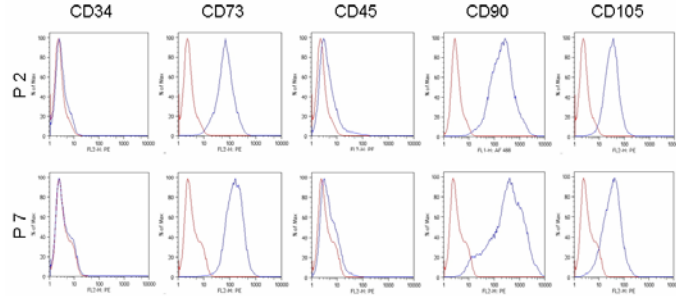


**Figure 2. Gene expression profiles suggest mesoderm formation.** human ESC cell line WA09 was grown as described over a 30d period with samples taken every five days and processed for RNA. The samples were tested for gene expression by quantitative real-time PCR and normalized for loading to 18S with gene expression quantified using  $\Delta\Delta C^T$  relative quantification method then expressed as fold change with respect to day 0  $\pm$  SE. The presented data represents four independent experiments.

arrow) eventually forming a confluent epithelial sheet (**Fig. 1B**) that upon passaging as a single cell suspension onto tissue culture plastic dishes transitions to a mesenchymal phenotype (**Fig. 1C**). These cells exhibit a “finger-print” morphology seen in bone marrow derived MSC in culture. The derived cells, referred to as hES-MSC, have maintained their phenotype and marker expression up to 7 passages with experiments on-going.

To determine the potential pathways involved in the derivation of the hES-MSC we have performed quantitative real-time PCR time course studies over 30 days with samples taken every 5 days and pluripotent human ESC used for baseline gene expression. As expected, the differentiation process leads to a down-regulation of the pluripotent marker OCT4 (**Fig. 2A**). The ectoderm and endoderm germ layer markers SOX2 (**Fig. 2B**) and CER1 (**Fig. 2C**) both indicated a rapid down-regulation of gene expression while the mesoderm marker BMP4 showed a transient up-regulation and gradual decrease to a maintained elevated level (**Fig. 2D**).

This could indicate the culture system is preferentially producing mesoderm and its derivatives without cell contamination from other germ layers. When examining the profiles of MSC there isn't one marker that defines the phenotype, therefore, it is necessary to look at a battery of markers (**Fig. 3**). We have investigated the expression of multiple proteins at two time points, the second passage after approximately 20 days of differentiation (**Fig. 3, upper panel**) and the seventh passage (**Fig. 3, lower panel**). As expected, the derived cells were negative for the hematopoietic markers CD34 and CD45, but were positive for the commonly used MSC markers CD73, CD90 and CD105. This suggests the cells formed under the current differentiation conditions bear a molecular signature that parallels what is seen in adult MSC isolated from bone marrow. To test the



**Figure 3. Flow cytometry marker profile suggests mesenchymal lineage.** Human ESC derived cells were dispersed into a single cell suspension and prepared for analysis by flow cytometry at passages 2 and 7. The cells were sorted using FACScaliber and analyzed with FloJo software. The derived cells were consistently negative for the hematopoietic markers CD34 and CD45 while being positive for mesenchymal markers CD73, CD90 and CD105.



**Figure 4. Derived cells show osteogenic potential.** Derived cells were cultured in either growth medium (Control) or osteogenic media (Osteogenic) for 20d. Cells were fixed, and calcium production detected with von Kossa staining. Bright field images were acquired for both conditions at 40x magnification.

differentiation capacity the derived cells were grown for 20 days in either growth media (negative control) or osteogenic medium. The wells were assayed for calcium production by von Kossa staining and bright field images acquired (**Fig. 4**). The derived cells cultured under osteogenic conditions show positive von Kossa staining indicating they have the capacity to differentiate into cells with osteogenic potential analogous to bone marrow derived human MSC.

## CONCLUSIONS

Our MSC can be propagated and robust enough to yield consistent quantitative results for drug discovery and cell therapy. This same procedure will be applicable in several species including livestock and horses. These robust MSC could be used in many ways by the research community. For example an investigator can quantify the yield of specific cell types following differentiation of our MSC. Further, an investigator can obtain single cells for FACS, genetically engineered cells, or any other applications in biomedical and pharmaceutical research that require single cell populations. As a direct result, investigators will obtain uniform, quantifiable data and measure dose-response relationships in screens for therapeutic compounds or for basic research.

## LITERATURE CITED

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