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Epigenetic Alchemy for Reversion of Pluripotency and Blockage of Spontaneous Differentiation by Ectopic Expression of Telomerase

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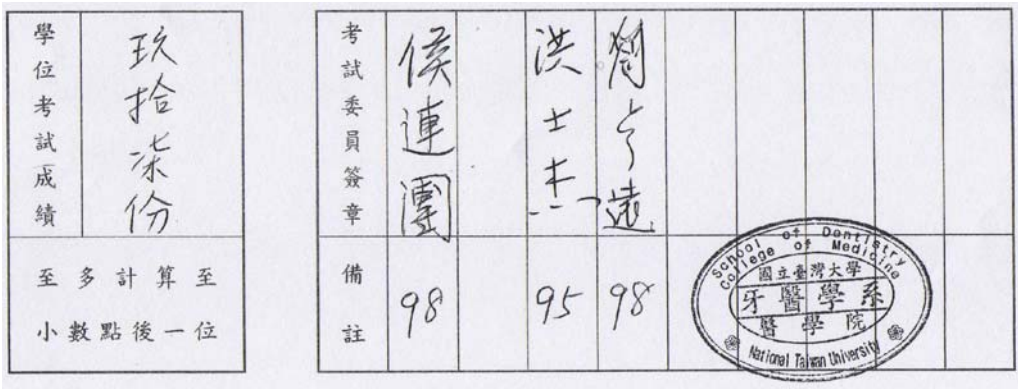
本論文係陳俊利君（R94422004）在國立臺灣大學醫學院臨床牙醫學研究所牙周病學組完成之碩士學位論文，於民國 97 年 01 月 25 日承下列考試委員審查通過及口試及格，特此證明。



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Abstract

Background: Cellular senescence and spontaneous differentiation are critical phenomena that impair long-tern study of cells in vitro. The same problems are similarly encountered in the investigation of human mesenchymal stem cells (hMSCs), despite its high proliferation rate and multipotency comparing to those of differentiated cells derived from different tissues. The present study was desigmed to overcome these problems and also explored to drive hBMSCs toward stemness.

Materials and Methods: Primary hBMSCs, E6E7-transfected hBMSCs (KP cells) and Htert transfected cells (3A6) were used in the present study under regular culture condition. Flow cytometry using many stem cell markers was applied to screen the similarity of studied cell types. Major targeted cells, 3A6, were generated by insertion of phTERT-IRES2-EGFP gene fragment using Nucleofector technology. Functional analysis on differentiation and dedifferentiation of all investigationed hBMSCs, KP and 3A6 were evaluated by RT-PCR of osteogenic, neural and adipogenic gene messages cytochemical staining and in vitro mineralization. Mater embryonic transcription gene markers, such as Oct4 and Nanog etc, and germline differentiation were further examined by RT-PCR, in unstimulated and stimulated BMP4 and RA condition.

Stemness related evidence and possible mechanism were explored by measurements of demethylation level of genomic DNA with CpG island microarray and level of various

demethylation enzymes, such as DNMT1, 3A, 3B and EZH2 by real-time PCR between 3A6 and KP cells. Further relative relashinship between 3A6 and ESC was investigated by microarray expression data sets and principal component analysis in Affymetrix™ U133 and principal compared with public accessible array databasesat Gene Expression Omnibus (GEO) (http: //www.ncbi.nlm.nih.gov/geo/).

Results: Ectopic expression of telomerase in a previously developed hMSC line was found to enhance pluripotency and block spontaneous differentiation of the cells. Surprisingly, the telomerase-transfected hMSCs (3A6) had a differentiation potential far beyond the normal hMSCs. They expressed trophoectoderm and germline specific markers in vitro, similar to those of embryonic stem cells, upon perturbations with BMP4 and retinoic acid, respectively. Furthermore, the telomerase-transfected hMSCs (3A6) displayed higher osteogenic and neural differentiation efficiency than their parental cells did, while there was a decrease in DNA methylation level as proved by a global CpG island methylation profile analysis. Possible underlying mechanisms were assayed by DNA methylation and its regulation enzymes. Notably, the demethylated CpG islands were found to be highly associated with development and differentiation associated genes. Principal component analysis further pointed out the expression profile of the cells converged toward embryonic stem cells.

Conclusions: In addition to the preservation of stem cells' characteristics in hTERT-transduced hMSCs, our present data also demonstrated the first pilot evidence that the reversion of pluripotency and blockage of spontaneous differentiation of hMSCs could be initiated in immortalized hBMSCs cell line by ectopic expression of telomerase.

Key words: Human mesenchymal stem cells; bone marrow stromal cells; telomerase; hTERT; pluripotency; spontaneous differentiation; CpG island; DNA methylation; DNMT; embryonic stem cells

Introduction

hBMSCs (human bone marrow stromal cells) based gene and cell therapy is one of the most promising and prospecting field of medicine because of their great self-renewal and versatile plasticity in vitro and in vivo (Gerson, 1999; Pittenger et al., 1999). Discovery of adult stem cell-derived pluripotent cells may bring a tantalizing castle into reality due to their ease of collection and versatility. Two important and interesting recent reports showed that Oct4 and Nanog characteristically expressed in embryonic stem cells (ESCs) could be detected in hBMSCs such as MAPCs (Jiang et al., 2002) and MIAMIs (D'Ippolito et al., 2004). These mesodermal originated cells were proven to be able to cross the germ layer boundaries and differentiated into ectoderm and endoderm successfully.

Though the nature plasticity of hBMSCs needs to be further elucidated in vivo, these amazing results have successfully challenged the traditional concept that the differentiation capacity of hBMSCs can be only limited to their resident local environment. Beyond the differentiation limitation, the pity comes from the rare distribution of hBMSCs in bone marrow (Friedenstein et al., 1982; Wexler et al., 2003) which hampers the clinical application and makes it necessary to expand the populations of hBMSCs in vitro. In addition, there are still two major difficulties encountered during in vitro expansion: cellular senescence and spontaneous differentiation.

Cellular senescence, also referred as cellular aging, could be defined as a cell's diminished replicative capacity and altered functionality (Beausejour, 2007). The phenomenon of cellular senescence causes changes of cells in view of physiological, functional, and molecular parameters (decreased transcription, decreased translation and decreased proteolysis; increased abnormal proteins and increased lipofucsin; increased number of both abnormal nuclei and size of lysosomes; elevated number of chromosomal abnormalities and reduced response to hormones) (Dice, 1993) during long-term cultures. These changes include typical Hayflick phenomenon of cellular aging, gradual decreasing proliferation potential, shortening telomere and impairment of functions (Bonab et al., 2006). Due to the nature cellular senescence, the expansion of hBMSCs would be limited up to 30~40 PDs (Banfi et al., 2000; Baxter et al., 2004; Stenderup et al., 2003) which severely impeded the application of these cells in clinical regenerative medicine. Meanwhile, literatures have demonstrated that decreased differentiation efficiency could be caused by cellular senescence in adipogenesis and osteogenesis (Bonab et al., 2006; Conget and Minguell, 1999; Digirolamo et al., 1999). Cellular senescence has been known to be generally associated with reduced expression of the human telomerase reverse transcriptase (hTERT) gene and shortened length of telomere (Bodnar et al., 1998). Most primary isolates of hBMSCs have deficient or limited detectable level of telomerase activity which contributes to limited replication life cycle

during in vitro expansion (Bonab et al., 2006).

Another important topic of great interest and clinical significance may be to keep the hBMSCs in a more quiescent state because their spontaneous differentiation overtly observed by several reports. Whether in rat or human BMSCs, several literatures have perceived spontaneous emergence of some lineage-specific genes or proteins expression in culture without any induced differentiation. The phenomenon included spontaneous adipogenesis (Seshi et al., 2000), osteogenesis (Seshi et al., 2000), myogenesis (Seshi et al., 2000) and neurogenesis (Li et al., 2007; Tondreau et al., 2004; Tseng et al., 2007). The most striking observation came from Woodbury (Woodbury et al., 2002) who found that in rat BMSCs, genes specific for all the three embryonic germ layers and germline cells were actively transcribed without any induction of differentiation. For exploring the possibility that if the gene expression of all three germ layers and germinal tissues were the consequence of mixed population of cells in primary BMSCs cultures, Woodbury (Woodbury et al., 2002) further assessed the expression of these representative genes in a clonal MSC line derived from a single cell. Interestingly, the clonally derived BMSCs recapitulated the gene expression pattern of primary BMSCs cultures, which suggested the spontaneous multidifferentiation capacity of individual BMSCs in cultures. In the present study, not only various lineage-specific genes, several developmental markers, such as Pax6, Gata4, Gata6, FoxA2 and Sox17, which designates early embryonic

development could also be detected in primary cultured hBMSCs. Enhanced expression of these genes from P3 to P10 hBMSCs confirmed the concept of spontaneous differentiation of hBMSCs during long term culture and the concurrent downregulation of embryonic markers, such as Oct-4 and Nanog. These data implicated the loss of pluripotency at the same time. Albeit important in clinical application, the potential problem of spontaneous differentiation is less, if any, investigated.

In need of great amount of cell source for stem cell-based tissue engineering, it seems remarkably significant to crave for resolutions dealing with cellular senescence and spontaneous differentiation of hBMSC, that is, to keep the stemness of hBMSCs during long term culture. In previous study, we attempted to conquer the obstacle mentioned above by transferring of HPV16 *E6/E7* genes into hMSCs isolated from bone marrow (Hung et al., 2002), and established a cell line-KP. Establishment of KP did successfully overcome the drawback of its cellular senescence in that it could be passaged over 100 PDs. Unfortunately, the phenomenon of spontaneous differentiation could not be avoided, which still caused the loss of stemness of KP during long term culture.

It has been mentioned for a long time that the role of telomerase in maintaining telomere length and immortality of embryonic stem cells (Amit et al., 2000; Thomson et al., 1998). But in hBMSCs, with exception of MAPCs (Jiang et al., 2002) and MIAMIs (D'Ippolito et al., 2004), no telomerase activity could be detected (Zimmermann et al.,

2003), which contributed to the shortening of telomere at a rate similar to that of non-stem cells (30–120 bp/ PD) (Fehrer and Lepperdinger, 2005). hBMSCs are found to cease dividing at telomere length about 10 kb (Baxter et al., 2004). Ectopic expression of hTERT in normal human cells, however, has been documented to be a strategy to bypass cellular senescence and to extend life span of cells (Bodnar et al., 1998; Broccoli et al., 1995). In addition to cell replication, current interest and attention seems to focus on the role of hTERT in regulation stem cell biology. Overexpression of hTERT in ES cells was reported to enhance differentiation capacity toward hematopoietic lineage (Armstrong et al., 2005), and ectopic expression of hTERT in hBMSCs were described to enhance osteogenic differentiation as well (Gronthos et al., 2003a; Shi et al., 2002; Simonsen et al., 2002). Notably, a recent report revealed an interesting phenomenon that with increasing expression of lineage-determining gene, such as cbfa1, expression level of hTERT decreased upon osteogenic differentiation due to an inhibition effect of cbfa1 on hTERT transcription (Isenmann et al., 2007). The inverse relationship between the presence of hTERT and differentiation-related genes was also observed by other groups (Wang et al., 2007). An interesting question is that if this inverse relationship is still valid when the expression level of hTERT is intentionally elevated by ectopic introduction of hTERT gene to hBMSCs? In our pilot study, we did observe downregulation of a variety of lineage specific genes after hTERT transduction, and we postulated that this phenomenon

might contribute to the reversion of stemness of hBMSCs that was elicited by ectopic expression of hTERT. The recovery of stemness by ectopic expression of hTERT, in our hypothesis, would be represented by conversion of hBMSCs from a commitment to a quiescent state with preservation of versatile differentiation capacity and enforced differentiation efficiency. In an attempt to clarify the hypothesized dedifferentiated effect on BMSCs by the insertion of hTERT, the establishment of hTERT-expressed hBMSCs is necessary. In our study, hTERT gene was not transferring into primary culture hBMSCs, but rather, to E6E7 expressed cell line-KP, due to the current evidence that hTERT alone might be insufficient to bypass the cellular senescence (Okamoto et al., 2002) and that extreme difficulty in selecting and expanding successfully transduced single cell clone in primary cultured hBMSCs. Furthermore, transducing hBMSCs with both hTERT and E6E7 had been suggested as a better strategy to circumvent cellular senescence (Okamoto et al., 2002).

With progressing of knowledge about gene regulation through epigenetic mechanism, including DNA methylation and chromatin modification, the globalized gene silencing phenomenon observed after ectopic expression of hTERT needed to be elucidated. In mammals, methylation of cytosines in cytosine guanine dinucleotide (CpG) island had been well-known to play a crucial role in mediating epigenetic gene silencing (Boyes and Bird, 1991; Watt and Molloy, 1988). Four DNA methyltransferases (DNMTs)

have been identified so far in mammals. Among them, DNMT1 has been known to maintain the pre-existing methylation state of genome during DNA replication (Leonhardt et al., 1992). DNMT3a and DNMT3b are de novo methyltransferases which would target unmethylated CpG sites (Okano et al., 1999). DNMT2, which has been shown to exhibit weak methyltransferaseactivity in vitro (Hermann et al., 2003), would be responsible to methylate tRNA(Goll et al., 2006). In addition to DNMTs, in this study, we try to prove our hypothesis that in hBMSCs, through some mechanism, ectopic expression of hTERT may modulate stemness genes and down-regulate development-associated and lineage-determining genes, and thus increases stemness of hBMSCs by shifting from a tissue-committed state to a more quiescent state.

Meanwhile, we also try to confirm the notion that after reversion from a commitment to a more quiescent and primitive state of the hTERT-transduced hBMSCs, these cells still retain versatile differentiation potential, even shifting toward germline and trophoectoderm differentiation, which were characteristics merely to hESCs in previous studies.

Finally, we explore to verify underlying mechanism that whether alterations mentioned above after hTERT transduction are attributed to the influence of hTERT on the methylation state of the whole genome, especially the genes responsible for early development and lineage-determining. If this postulation was true, the hypermethylation

of these genes would explain the phenomenon of gene silencing and keep hBMSCs in pluripotent status after hTERT transduction in the present study.

I. Review of Literature

1. Cell Biology of Bone Marrow Stromal Stem Cells (BMSCs)

A. Definition of Stem Cells in Adult Bone Marrow (BM)

The basic definition of the term "stem" refers to the ability of progenitor cells to divide and produce an undifferentiated daughter cell, which is designed as "self-renewal", and meanwhile, to produce another daughter cell that is differentiated or divides then differentiate (Sell, 2004). It is extremely hard, however, to prove that the so called "stem cells" to be able to self-renew. For this reason, the expandable population of mesenchymal cells with differentiation capacity is termed mesenchymal progenitor cells by some groups while others utilize traditional terminology based on their field of study (Sell, 2004).

Friedenstein and Owen et al. were the first to characterize cells that compose the physical stroma of bone marrow (Friedenstein et al., 1970; Friedenstein et al., 1966; Owen, 1988). Friedenstein et al.(Friedenstein et al., 1966) observed the formation of cartilage and bone within the diffuse chamber and demonstrated that bone marrow had the potential to form bone and cartilage. Furthermore, he noticed that the osteogenic potential of bone marrow derived from a specific subgroup of cells, and when these cells were plated at low density, they rapidly adhere and could be easily separated from the

nonadherent hematopoietic cells by repeated washing, and accordingly, these cells were termed colony-forming unit-fibroblast (CFU-f) (Friedenstein 1973). Friedenstein later illustrated that approx 30% of isolated CFU-f colonies were able to form bone alone or bone with the microenvironment necessary for the formation of hematopoietic element (Friedenstein, 1980). The first concept of "stem cells" residing in bone marrow was presented by Owen (Owen, 1978). He (Owen, 1978), based on his hypothesis, expanded and proposed a model for the stromal lineage that contained "stem cells", "committed progenitors" and "maturing cells" compartments, and included a lineage diagram for "stromal stem cells" that included "reticular", "fibroblastic", "adipocytic," and "osteogenic" cells as end-stage phenotypes (Sell, 2004).

The main source of MSCs is in the bone marrow, which has been conventionally regarded as an organ composed of two main systems according to distinct resided cell lineages; the hematopoietic tissue proper and the associated supporting stroma (Bianco et al., 2001). Even though, the MSCs only constitute a small percentage of whole population of bone marrow cells. Only 0.01% to 0.001% of mononuclear cells isolated on density gradient (ficoll/percoll) medium give rise to plastic adherent fibroblastic-like colonies (Pittenger et al., 1999). Even in the same donor, the numbers of bone marrow MSCs are different upon repeated puncture in view of the yield and the quality (Phinney et al., 1999).

The BMSCs are postulated to arrive in bone marrow by three different mechanisms (Sell, 2004). First, they can enter bone marrow along with vasculature. Second, they can migrate into the marrow space after vascularization along the vessel paths (Nakahara et al., 1992; Yoo and Johnstone, 1998). In fact, the first two mechanisms differ only in the timing of the entrance of BMSCs into bone marrow, but not paths of arrival or migration. Third, they can arrive through the blood proper, suggesting the existence of MSCs in circulation, and meanwhile the circulating MSCs endows the possibility of delivering reparative MSCs to repair adult tissue (Sell, 2004).

Besides bone marrow, MSCs are also reported to be located in other tissues of the human body (Bobis et al., 2006), such as adipose tissue (Gronthos et al., 2001), umbilical cord blood, chronionic villi of the placenta (Igura et al., 2004), amnionic fluid (Tsai et al., 2004), peripheral blood (Zvaifler et al., 2000), fetal liver (Campagnoli et al., 2001; in 't Anker et al., 2003), lung (in 't Anker et al., 2003), and even in exfoliated deciduous teeth (Miura et al., 2003). Althrough MSCs are widly distributed in the whole body, the amount of MSCs decreases with aging (Fibbe and Noort, 2003) and infirmity (Inoue et al., 1997). Their presence reaches greatest amount in neonate and reduces in number during the later lifespan, which is about one-half of the neonate stage at the age of 80 (Fibbe and Noort, 2003). The circulating fetal MSCs also reach highest number in the first trimester

and declines during the second trimester to about 0.0001% and 0.00003% of the nucleated cells in cord blood (Campagnoli et al., 2001).

B. Heterogeneity of the BMSCs

Some evidences clearly indicated a heterogeneous nature of the BMSCs population (Kucia et al., 2005a; Kucia et al., 2005c; Ratajczak et al., 2004), which was clarified by the presence of different properties of individual colonies, such as colony sizes, growth rates and cell morphologies ranging from fibroblast-like spindle-shaped cells to large flat cells (Bianco et al., 2001). In addition, if the cultures are allowed to develop for more than 20 days, some colonies are positive for alkaline phosphatase (ALP) stain, while others are either negative or positive in the central region and negative in the periphery (Friedenstein et al., 1982). Some colonies formed nodules which can be identified by Alizarin Red S or von Kossa staining for calcium (Bianco et al., 2001). Some colonies could still accumulate fat and were identified by oil red O staining (Herbertson and Aubin, 1997) or formed cartilage which can be stained with alcian blue (Berry et al., 1992).

C. Surface Markers on BMSCs

Besides cell morphology and physiology, expression of cell surface antigens is another evidence of BMSC's heterogeneity, and to date, no single specific marker which

designates BMSCs has been identified (Bobis et al., 2006). BMSCs express a large number of adhesion molecules, extracellular matrix proteins, cytokines and growth factor receptors associated with their function and cell interactions within the bone marrow stroma (Devine and Hoffman, 2000). The population of BMSCs isolated from bone marrow express: CD44, CD105 (SH2; endoglin), CD106 (vascular cell adhesion molecule; VCAM-1), CD166, CD29, CD73 (SH3 and SH4), CD90 (Thy-1), CD117, STRO-1 and Sca-1 (Boiret et al., 2005; Conget and Minguell, 1999; Dennis et al., 2002; Gronthos et al., 2003b). To another aspect, BMSCs do not possess markers typical for hematopoietic and endothelial cell lineages: CD11b, CD14, CD31, CD33, CD34, CD133 and CD45 (Pittenger et al., 1999). The absence of CD14, CD34 and CD45 enable us to distinguish BMSCs from the hematopoietic precursors (Baddoo et al., 2003). BMSCs are also known to express a set of receptors associated with matrix- and cell-to-cell adhesive interactions, like integrins αß3 and αß5, ICAM-1, ICAM-2, LFA-3 and L-selectin (Boiret et al., 2005; Conget and Minguell, 1999; Pittenger et al., 1999).

Some surface antigens, however, may change during the culturing process due to specific culture conditions, components (especially growth factors added) in the medium and the duration prior to individual passages (Dazzi et al., 2006). The alteration of chemokine receptor expression could also occur during passage of human BMSCs (Honczarenko et al., 2006). CCR1, CCR7, CCR9, CXCR4, CXCR5 and CXCR6 were

expressed at the second passage of BMSCs but all of these molecules disappeared at the 12-16th passages accompanied with the disability of the cells to migrate towards specific chemokine attractants (Honczarenko et al., 2006). Meanwhile, loss of the expression of chemokine receptors was accompanied with a decrease in the expression of adhesion molecules, such as ICAM-1, ICAM-2, VCAM-1 and CD157 (Honczarenko et al., 2006). Moreover, the changes of BMSCs' phenotype were relevant to increasing cell cycle arrest and induction of apoptotic pathway (Honczarenko et al., 2006). With the progression of cellular differentiation of BMSCs, the alteration of one surface antigen has also been observed that CD166 (activated leukocyte cell adhesion molecule) presented on undifferentiated BMSCs is absent from the cells that differentiate toward osteogenic lineage (Bruder et al., 1998).

The antibody STRO-1 (Simmons and Torok-Storb, 1991) has, so far, been the most useful antibody for identifying and selecting for positively MSCs in bone marrow (Sell, 2004). The STRO-1 marker was successfully used to isolate the CFU-f cells from marrow (Simmons et al., 1994), and STRO-1-selected cells had been shown to be osteogenic (Gronthos et al., 1994), chondrogenic, adipogenic, and hematopoiesis supportive (Dennis et al., 2002). The STRO-1 staining could be used to characterize BMSCs, and sort cells by magnetic activated cell sorting (Dennis et al., 2002; Gronthos et al., 1994; Tamayo et al., 1994).

D. Morphology, Growth and Expansion of BMSCs

BMSCs that initially adhere to plastic culture dish have fibroblastic appearance and shift to symmetrical colony after plating for 5-7 days (Sell, 2004). Although various culture protocols of BMSCs have been published, these cells displayed most rapid proliferation and retained their optimal multipotency when cultured at low density (Sekiya et al., 2001). Culture density not only affects growth but also cell morphology of BMSCs. Tropel et al. observed that BMSCs displayed a spindle-like shape at low density, but they started to grow in several layers and become flat in cell shape with torn ends at confluence (Tropel et al., 2004). Growth of BMSCs could be characterized by the occurrence of three phases (Bruder et al., 1997; Colter et al., 2001): (1) an opening lag phase of 3-4 days; (2) a rapid expansion (log phase); (3) a stationary phase (growth plateau).

BMSCs have been known to maintain in culture for 20-30 population doublings with their capacity of differentiation under proper conditions (Friedenstein et al., 1970). Recent studies even showed that BMSCs could be maintained in culture for more than 50 population doublings (Ramalho-Santos et al., 2002) which indicates the great proliferative potential of these cells. According to a study examining the profile of BMSCs cell cycle, major part of these cells remained in the G0/G1 phase and only 10% in phase S (Conget and Minguell, 1999). Notwithstanding a report had observed that

BMSCs could maintain normal karyotype and telomerase activity even at passage 12 (Pittenger et al., 1999), extensive subcultiavation of BMSCs impaired their functionality and confronted them with the crisis of senescence and apoptosis (Conget and Minguell, 1999).

A number of cytokines, growth factors, hormones and other molecules have been known to impact on proliferation of BMSCs (Bobis et al., 2006). PDGF, FGF-2 and EGF have been proved to be potent mitogens for BMSCs (Bruder et al., 1997). In contrast, the addition of interferon-alpha and interleukin 4 to the culture could raise opposite results (Bruder et al., 1997; Jeong et al., 2005). The proliferative activity of BMSCs has been shown to be in proportion to their increased potential of late differentiation potential (Prockop et al., 2003).

E. Cross-over Plasticity of BMSCs

According to the previous study results, it was believed that BMSCs could differentiate only to mesodermal tissues (Bobis et al., 2006). Many recent data, however, has revealed that these cells could differentiate into various kind of lineages rather than only mesodermal origin, such as hepatocytes and even neurons (Jiang et al., 2002; Pittenger et al., 1999). The former hypothesis claimed that the plasticity of BMSCs was attributed to the stochastic repression/induction model that various sets of BMSCs occurred upon a

series of gene silencing events during development (Dennis and Charbord, 2002). Whatsoever, the stochastic repression/induction model was not generally accepted due to different data from other researchers, and there were alternative explanations of the phenomenon of stem cell plasticity. Based on the previous data of transdifferentiation, three important explanations have been proposed (Kucia et al., 2005a). First, some authors assumed that the alteration of BMSCs phenotype was caused by cell fusion with other lineages of cells (Alvarez-Dolado et al., 2003; Terada et al., 2002; Ying et al., 2002), and the fused cells retained signatures of both parental cells. The drawbacks of this hypothesis, however, come from the facts that cell fusion is an extremely rare phenomenon and preferentially to cells with polyploidity, such as hepatocytes, Purkinje cells and skeletal muscle cells (Kucia et al., 2005a). Additionally, several recent publications demonstrating plasticity of BMSCs also excluded the possibility that cell fusion was a cause of cell transdifferentiation (Almeida-Porada et al., 2004; Harris et al., 2004; Jang and Sharkis, 2004; Shefer et al., 2004; Wurmser et al., 2004).

Second, epigenetic alterations might occur in BMSCs in response to external stimuli under some circumstances, and thus modulate the expression of some early development and lineage-determining genes. Such a mechanism could be happened during reproductive/therapeutic cloning because some studies observed that nuclei isolated from differentiated somatic cells were plastic and might be reprogrammed and trans-

differentiated when injected into cytoplasm of enucleated oocyte (Hochedlinger and Jaenisch, 2003; Kucia et al., 2005a). Accordingly, when stem cells isolated from original physiological environment are exposed to stress factors associated to culture condition in vivo, epigenetic alteration may possibly take placed (Kucia et al., 2005a).

The most convincing explanation of BMSCs, plasticity may originate from the concept that in addition to hematopoietic stem cells, other scarce subpopulations of versatile tissue-committed stem cells (TCSCs) and even more primitive pluripotent stem cells (PSCs) perhaps accumulate in bone marrow during ontogenesis (Kucia et al., 2006a; Kucia et al., 2005a; Kucia et al., 2005b; Kucia et al., 2006b; Kucia et al., 2005c; Kucia et al., 2007; Kucia et al., 2006d; Ratajczak et al., 2007). The basis of this model emphasizes the presence of heterogeneous population of stem cells at different differentiation level from PSCs to TCSCs, and these cells are reservoir of primitive cells for tissue repair and organ regeneration, which can migrate from BM to peripheral blood after tissue injury or organ damage (Kucia et al., 2005a). The establishment of this model challenges the former concept that pluripotent stem cell is characteristic only to embryonic stem cell during embryogenesis. Recently observations of several studies support the presence of positive embryonic markers, such as Oct-4 (Niwa et al., 2000), Nanog (Mitsui et al., 2003) and SSEA (Muramatsu and Muramatsu, 2004) in the PSCs of BM. Among these embryonic markers, Oct-4 is the most pivot in that it is an essential embryonic

transcription factor that plays a critital role in specification of ES cells (Boiani and Scholer, 2005; Hay et al., 2004) and is downregulated during development (Kucia et al., 2007). To date, various groups have identified Oct-4 positive stem cells in BM, such as very small embryonic-like (VSEL) cells, multipotent adult progenitor (MAPC) cells, multipotent mesenchymal stromal (MSC) cells and marrow-isolated adult multilineage inducible (MIAMI) cells. These later cell types derived from BM not only display potential in vitro and in vivo to differentiate into cells from all three germ layers (D'Ippolito et al., 2004; Jiang et al., 2002); mesoderm, ectoderm and endoderm. Unexpectedly and strikingly, BM has also been recently identified as a potential sources of precursors of germ cells (Johnson et al., 2005; Nayernia et al., 2006). In addition to bone marrow, Oct-4 positive stem cells have also been discovered in many other tissues (Kucia et al., 2007; Ratajczak et al., 2007) such as epidermis (Dyce et al., 2004; Yu et al., 2006), bronchial epithelium (Ling et al., 2006), myocardium (Mendez-Ferrer et al. 2006), pancreas (Danner et al., 2007; Kruse et al., 2006), testes (Guan et al., 2006; Kanatsu-Shinohara et al., 2004), retina (Koso et al., 2006) and amniotic fluid (De Coppi et al., 2007). These findings not only consolidate the theory of developmental deposition of Oct-4 positive PSCs in developing organs (Ratajczak et al., 2007), but also suggest that these cells might circulate in the peripheral blood and shuttle between BM and other organs during tissue injury or organ damage (Eghbali-Fatourechi et al., 2005; Gomperts

et al., 2006; Kucia et al., 2004; Kucia et al., 2006c; Palermo et al., 2005; Togel et al., 2005).

2. Aging and Senescence of MSCs

A. Definition of aging mesenchymal stem cells (MSCs)

Due to the aforementioned rare distribution of adult mesenchymal stem cells (MSCs), in vitro cultivation and expansion of MSCs cannot be avoided for the purposes of experimental study. Although much promise of clinical applications of MSCs has been proposed, the senescence, or cellular aging of MSCs during long-term culture significantly impeded the propagation of their therapeutic use. Consequently, the resulting loss of proliferation and functionality of MSCs caused by cellular aging contrasts with those of the immortal embryonic stem cells, which results in the major hindrance of clinical utilization of adult MSCs. The aging discussed here is restricted to "in vitro aging", which happens during prolonged cultivation in vitro. Cellular aging, also referred as cellular senescence, is generally defined as a cell's diminished replicative capacity and altered functionality (Beausejour, 2007). The phenomenon of cellular senescence was firstly described as replicative senescence by Hayflick (Hayflick and Moorhead, 1961) and defined as "an essentially irreversible arrest of cell division". Notably, arrest of growth in senescent cells to over time would negatively influence the ability of renewable tissues to replace damaged or dysfunctional cells and thus reduced their capacity for tissue repair (Beausejour, 2007; Smith and Pereira-Smith, 1996; Stanulis-Praeger, 1987). In addition, recent reports also revealed that these metabolically

active senescent cells might accumulate over time (Herbig et al., 2006) and secret biologically active molecules which would affect environment of normal tissue and behavior of neighboring cells (Krtolica et al., 2001).

B. Influence of aging on MSCs

Various changes of physiological, functional, and molecular parameters have been described in senescent stem cells, and these changes included typical Hayflick phenomenon of cellular aging, gradual decreasing proliferation potential, telomere shortening and impairment of functions (Bonab et al., 2006). It has been reported that several characteristics of senescent stem cells could be regarded as markers of aged MSCs. Firstly, some authors noticed that aged MSCs exhibited larger size (Baxter et al., 2004; Mauney et al., 2004; Stenderup et al., 2003) with more pseudopodia (Mauney et al., 2004), further spreading and marked intracellular actin stress fibers than non-senescent parts (Stenderup et al., 2003). In fact, it has been long discovered that increase in cell size is often associated with senescence (Hayflick and Moorhead, 1961). The MSCs from older patients or young patients do display different morphology in culture that MSCs from young donors exhibit spindle-type morphology and this characteristic fades over long-term cultivation (Baxter et al., 2004). In contrast, when MSCs are immortalized by either SV40 (Negishi et al., 2000) or telomerase (Kobune et al., 2003), these cells shift considerably to a smaller size than original cells they derived from.

Secondly, another significant phenomenon occurs in senescent MSCs is a gradual decreasing proliferation potential. When actual age of a culture is recorded by population doublings (PDs), it has been reported that a single-cell-derived colonies of MSCs can be expanded up to as many as 30~50 PDs in about 10~18 weeks (Bonab et al., 2006; Colter et al., 2000; Stenderup et al., 2003). Moreover, it has also been revealed that growth curve relationship between cumulative PDs and duration of culture demonstrates a relatively linear decreasing rate of PDs with the progression of time. An appreciable decrease in the number of PDs was seen in the latter period of culture (more than 130 days in culture), implicating that proliferative potential of MSCs decreased remarkedly after 120 days in vitro expansion (Bonab et al., 2006).

Thirdly, when it comes to aging MSCs, one of the most puzzling issues might be the differentiation potential of aged MSCs. Differentiation into various lineages has been used as a marker for the multipotential nature of these cells (Pittenger et al., 1999), and such multipotency of MSCs seems to change with age. Alteration of osteogenic and adipogenic differentiation capacity of MSCs with age, especially the osteogenic potential of aged MSCs have been extensively investigated. Although there is conflicting evidence with some groups reporting no change, majority of studied disclose an age-related decreased potential of osteogenic differentiation potential of MSCs in their late passages (Bonab et al., 2006; Conget and Minguell, 1999; Digirolamo et al., 1999). Regarding to

adipogenetic differentiation, it has been revealed early (Meunier et al., 1971) that aged MSCs lose its osteogenic potential and gain adipogenetic potential which termed “ adipogenetic switch” (Ross et al., 2000). Some recent reports, however, do not observe such changes, and even others observe a decreased adipogenesis of aged MSCs (Bonab et al., 2006). Although no definite statement regarding age-related effects on differentiation potential could be made in spite of some considerable researches (Sethe et al., 2006), a recent study indicated that the efficiency of differentiation into local tissue (homing) of transplanted MSCs was found to be severely decreased following culture (Rombouts and Ploemacher, 2003). These data elicited a fundamental question that if in vitro differentiation data could be unequivocally applied in vivo.

Fourthly, besides aforementioned alteration of aged MSCs, some senescent markers in vitro were also utilized to detect the aging process of MSCs. Among these markers, bata-galactosidase (beta-GAL) activity at pH 6 was reported to be associated with cellular senescence in vitro (Sethe et al., 2006), and one theory suggests that beta-GAL activity is associated with the RAS pathway (Minamino et al., 2003) and with lysosomal dysfunction (Kurz et al., 2000). One further study (Stenderup et al., 2003) demonstrated that beta-GAL activity increased in late-passage MSCs, but there was no difference between MSCs from young and aged donors. Another group (Park et al., 2005) also found that not only beta-GAL, but also p53 and p16/RB increased in prolonged

cultivation of human MSCs. While beta-GAL was regarded as a reliable marker for senescence in low-density culture and seemed to be correlated with aging in vivo (Dimri et al., 1995; Sethe et al., 2006), its wide application in vivo was limited (Severino et al., 2000).

3. Role of Telomere Length and Telomerase in MSCs

A. The telomere structure and telomerase

Telomeres are specialized chromatin structures at the ends of eukaryotic chromosomes that prevent the ends of chromosomes from being recognized as a DNA break, which cap and protect every eukaryotic chromosome end against chromosomal fusion, recombination, and terminal degradation (Blasco, 2007; Chan and Blackburn, 2002; Hiyama and Hiyama, 2007). Telomeric DNA consists of short guanine-rich repeat sequences in all eukaryotes with linear chromosomes. In vertebrate, telomeres are composed of TTAGGG repeats bound by a protein complex called shelterin (Blasco, 2007; de Lange, 2005; Liu et al., 2004a) which have roles in chromosome protection and in the regulation of telomere length. Telomere length in human somatic cells is remarkably heterogeneous among individuals, ranging from 5 to 20 kb, according to age, organ, and the proliferative history of each cell (Hiyama and Hiyama, 2007; Wright and Shay, 2005). During the process of DNA synthesis and cell division, telomere becomes shortened as results of incomplete replication of linear chromosomes, that is, the "end-replication problem" (Hiyama and Hiyama, 2007). The progressive shortening of telomere is one of the molecular mechanisms of the aging because when telomeres reach a certain length, cells generally stop dividing and enter chromosome senescence and loss of cell viability (Blasco, 2005; Collins and Mitchell, 2002; Harley et al., 1990; Wright

and Shay, 2005). A theory suggest the possibility that telomere dysfunction could be viewed as a specialized form of DNA damage and thus contributes to the aging of human stem cells (Sharpless and Depinho, 2007). Telomere shortening caused by the absence of adequate telomerase activity occurs with cell proliferation and finally results in an alteration of telomere structure, which is sensed by the cells as a DNA double-strand break (Sharpless and Depinho, 2007). To prevent degradation by exonucleases or processing as damaged DNA, the G-strand overhang can fold back and invade the double-stranded region of the telomere, thereby generating a looped structure known as the telomere loop or T-loop (Griffith et al., 1999), which is reinforced with TRF2 and other telomeric DNA-binding proteins named shelterin (de Lange, 2005; Hiyama and Hiyama, 2007). Telomere repeats are generated by a cellular reverse transcriptase known as telomerase (Blasco, 2007; Chan and Blackburn, 2002). A recent study with highly purified telomerase extracts has demonstrated that the telomerase enzyme contains two molecules. These compose of the telomerase reverse transcriptase subunit (Tert) and the telomere-associated RNA molecule (Terc), as well as one molecule of dyskeratin (Blasco, 2007; Cohen et al., 2007), a protein known to stabilize the telomerase complex (Collins and Mitchell, 2002). Telomerase can add telomeric repeats onto the chromosome ends, and prevents the replication-dependent loss of telomere and cellular senescence in highly proloferative cells of the germline and in the majority of cancers (Blasco, 2005).

Therefore, the immortality of cancer cells, germ-line cells and embryonic stem cells are postulated to be possibly associated with telomerase activity and telomere maintenance (Hiyama and Hiyama, 2007). Except for stem cells and lymphocyte, the telomerase activity of most human somatic cells is gradually diminished after birth and thus, telomere length shortens with each cell division (Hiyama and Hiyama, 2007). To ensure proper telomere function and avoid the activation of DNA damage pathways which may result in replicative senescence or cell death, a critical length of telomere repeats is required (Hiyama and Hiyama, 2007). Low levels of telomerase activity had been detected in human adult stem cells including haematopoietic and non-haematopoietic stem cells such as neuronal, skin, intestinal crypt, mammary epithelial, pancreas, adrenal cortex, kidney and mesenchymal stem cells, and the telomerase activity explained the prolonged poliferative capacity and the mechanism that maintained telomere length through many cell divisions (Hiyama and Hiyama, 2007). Different from the majority of human stem cells, cells that undergo rapid expansion, such as committed haematopoietic progenitor cells, activated lymphocytes, or keratinocytes, and in tissues with a low cell turnover such as the brain, displayed an upregulated telomerase activity (Haik et al., 2000).

B. Telomere elongation mechanisms

The majority of tumors and immortal cell lines has high levels of telomerase to sustain their immortal growth by preventing telomere shortening and bypassing senescence and apoptosis (Blasco, 2005). In contrast, the adult organism has only limited amount of telomerase activity, which results in the attrition of telomeric DNA during aging and also disability to compensate for the progressive telomere shortening that occurs as cells divide during tissue regeneration (Blasco, 2005; Blasco, 2007; Collins and Mitchell, 2002; Harley et al., 1990). Even though, in the lack of telomerase activity, some immortal cell lines and tumors are still able to maintain or elongate their telomeres through activation of another mechanism known as alternative lengthening of telomeres (ALT) (Dunham et al., 2000; Muntoni and Reddel, 2005). In yeast and mammals, ALT has been shown to involve homologous recombination events between telomeric sequences (Blasco, 2007; Dunham et al., 2000; Lundblad, 2002; Muntoni and Reddel, 2005). Heterogeneous telomere lengths are characteristics of ALT-positive cells that both very short and very long telomeres are present at the same time (Blasco, 2007; Dunham et al., 2000; Lundblad, 2002; Muntoni and Reddel, 2005). Mechanisms of ALT, however, cannot rescue the viability of Terc-deficient mice, which suggests that this later mechanism do not function to overcome the crisis of telomere shortening of most multicellular organisms (Blasco, 2007). In addition, it is noticed that ALT is mostly restricted to Terc-

deficient mice, as well as immortal cell lines and tumors, which suggests the existence of a mechanism that actively represses ALT in normal cells (Blasco, 2007). Several recent reports revealed that Pot1 and TRF2 or TRF2-interacting proteins such as WRN, which are components of shelterin complex, could influence telomere recombination and thus are potential regulator of ALT (Blanco et al., 2007; Blasco, 2007; Laud et al., 2005; Wu et al., 2006). Other reports also implicated that both subtelomeric DNA methylation (Benetti et al., 2007; Gonzalo et al., 2006) and histone methylation at telomeres (Benetti et al., 2007) were potent repressors of telomere recombination and ALT activation.

C. Telomere and Telomerase in MSCs

The phenotype of replicative senescence in cultured MSCs is dependent on the studied species (Hiyama and Hiyama, 2007). In regard to human MSCs, they are reported to cease dividing early at around 30~50 population doublings (Bonab et al., 2006; Colter et al., 2000; Stenderup et al., 2003). On the contrary, murine MSCs that have high telomerase activity can be passaged for more than 100 population doublings (Meirelles Lda and Nardi, 2003). The rate of telomere shortening of hMSCs has been reported to be about 30-120 bp/ PD (Fehrer and Lepperdinger, 2005; Stenderup et al., 2003). Interestingly, one study, at least, reported that bone marrow-derived hMSCs maintained long telomeres without the upregulation of telomerase activity for more than 100

population doublings undr culture with basic FGF (Yanada et al., 2006). Moreover, the relationship between ectopic expression of telomerase and differentiation efficiency of hMSCs had been discussed. It was implicated that forced telomerase expression in hMSCs led to an extend life span and enhanced differentiation potential that the efficiency of telomerase-overexpressing cells to form bone in vivo was greatly enhanced (Shi et al., 2002; Simonsen et al., 2002). On the other hand, mMSCs knocked-down of their telomerase activity completely failed to differentiate into adipocyte or chondrocyte, even in early passages (Hiyama and Hiyama, 2007; Liu et al., 2004b). Recently, a report unraveled that subtelomeric DNA hypomethylation would facilitate telomere elongation in mammalian cells, and this result also suggested that such epigenetic modification of chromatin might occur in hMSCs (Gonzalo et al., 2006). It is highly possible that telomerase is required for both cell replication and differentiation, and thus the differentiation potential and regenerative capacity of hMSCs may be attributed to the minimum level of telomerase activity expressed in hMSCs (Hiyama and Hiyama, 2007).

4. Epigenetic Regulation of Development and Stem Cell Differentiation

A. Epigenetic signature of pluripotency

As the progression of biotechnology, it is capable of elucidating the profile of gene expression and their relative abundance in a particular cell type, but this information provides us little about the genes that are not actively transcribed in cells (Spivakov and Fisher, 2007). What is more importantly, it is hard for using expression profiling directly to discriminate between genes that are subject to active repression and those that are not transcribed simply due to the absence or limitation of activating proteins (Spivakov and Fisher, 2007). For this reason, we would not figure out how tissue-specific genes, that will be required for executing later stages in development, are prevented from expression by ES cells, although the potential for their expression is retained (Spivakov and Fisher, 2007). To solve this problem, some underlying mechanisms of gene control in stem cells have been proposed, and they are collectively referred to as epigenetic regulation. These control mechanisms encompass a range of different properties that have been shown to affect gene expression without changes in DNA sequence (Spivakov and Fisher, 2007). Major epigenetic mechanisms include DNA cytosine methylation, histone modifications such as acetylation and methylation of histone tails, and small non-coding RNA controlled pre- and post-transcriptional regulation of gene expression (Wu and Sun, 2006). Moreover, epigenetic information is known to be able to transmitted through

sequential rounds of cell division (Nakatani et al., 2006; Richards, 2006) because epigenetic marks, including methylated DNA (Jaenisch and Bird, 2003) and modified histones (Henikoff et al., 2004) are propagated at S phase. It is the feature of epigenetic inheritance that lead to the postulation that chromatin has a central role in maintaining transcriptional patterns during development (Spivakov and Fisher, 2007).

B. The roles of chromatin modifications on self-renewal and differentiation of stem cells

Post-translational modification of core histones and methylation of genomic DNA have been revealed to be associated with both chromatin and transcriptional status of genes (Fischle et al., 2003; Hsieh, 2000; Meshorer and Misteli, 2006). During ES-cell differentiation, besides changes in the global genome activity, alterations of histone-modification patterns also occur (Lee et al., 2004). For example, during differentiation, an increase in the silenced chromatin mark tri-methylated residue K9 of histone H3 (H3-triMeK9) and a decrease in the global levels of acetylated histone H3 and H4 (Keohane et al., 1996; Lee et al., 2004; Meshorer et al., 2006), which is usually associated with active chromatin regions, are noted (Meshorer and Misteli, 2006). These findings all suggest that the chromatin of ES-cell is overall either in a more active state or marked with activity-associated histone modifications, and that when differentiation of ES-cell occurs,

the chromatin is transitioned to a transcriptionally less-permissive status (Meshorer and Misteli, 2006). Several repressed heterochromatin marks, such as H3-triMeK9, H3-MeK27, H3-diMeK27, H4-diMeK20 and H4-triMeK20, are found to elevate during RA-induced mouse ES-cell differentiation (Martens et al., 2005). Moreover, the inhibition of mouse ES-cell differentiation after treatment with histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), also indicates the functional relevance to global histone deacetylation during ES-cell differentiation (Lee et al., 2004). Local histone modifications, along with global changes, are believed to be important for the proper control of differentiation-specific genes (Meshorer and Misteli, 2006). Representative example is the promoter of the ES-cell marker, Oct4, which is observed to be enriched for the active mark, H3-triMeK4, in undifferentiated rather than in differentiating ES cells (Lee et al., 2004). Similar findings are also shown for *λ5-VpreB1* (Szutorisz et al., 2005), B-cell differentiation determining gene, and *NFM* (Kimura et al., 2004), neuronal differentiation determining gene, which all contain active chromatin marks in undifferentiated ES cells despite their inactivity. These observations do indicate that the maintenance of transcriptionally competent chromatin is an active process mediated by histone modifications. The histone modifications help to preserve the pluripotent state of ES cells and mark the transcriptionally competent loci expressed later in the differentiating process (Szutorisz and Dillon, 2005). In addition, there is current evidence

of epigenetic regulation which depicts the existence of the temporary inactivation of differentiation-specific genes in pluripotent cell types (Reik, 2007). These observations unravel that genes required during development and differentiation are those in the homeobox (*Hox*), distal-less homeobox (*Dlx*), paired box (Pax) and sine-oculis-related homeobox (Six) gene families. These genes are held repressed in pluripotent ES cells by the Polycomb group (PcG) protein repressive system in mice and humans (Reik, 2007). PcG protein repressive system marks the histones associated with these genes by inducing methylation of the lysine residue at position 27 of the histone H3 (H3K27) (Azuara et al., 2006; Boyer et al., 2006; Lee et al., 2006). It is also found that when ES cells lose the expression of EED (embryonic ectoderm development), a component of the PcG-protein repressive complex (PRC), the developmental genes are partly derepressed and ES cells are prone to spontaneous differentiation (Azuara et al., 2006; Boyer et al., 2006).

PcG-protein repressive complexes (PRCs) are a subclass of histone modification enzymes that are highly conserved throughout the evolution (Valk-Lingbeek et al., 2004). Polycomb repressive complex 2 (PRC2) is found to contain both histone deacetylase (HDAC) and histone methyltransferase (HMT) activity, which link hypoacetylation and H3-K9/K27 methylation (Valk-Lingbeek et al., 2004). In another aspect, PRC1 recognizes the H3-K27 methylation mark established by PRC2 through its conserved

chromodomain and takes part in stable maintenance of PRCs mediated gene silencing effect (Wu and Sun, 2006). Chromatin modifying activities opposite to epigenetic control mediated through PRCs also drawed much attention. Testis specific TAF (TBP-associated factor) associated trithorax (trx) action (tri-methylation of H3-K4) had been demonstrated to counteract PcG-mediated repression to allow terminal differentiation of Drosophila male germ cell precursors (Chen et al., 2005; Wu and Sun, 2006).

Some developmental genes, however, were present within bivalent chromatin regions which contain both inactivating marks (methylated H3K27) and activating marks (H3K4) (Bernstein et al., 2006; Szutorisz et al., 2005). The bivalent chromatin marks were demonstrated that when the PRCs expressions were downregulated during differentiation and the repressive marks had been removed, these genes were automaticaly poised for transcriptional activation through the H3K4 methylation mark (Reik, 2007). Although epigenetic silencing by PRCs could be mitotically heritable (Ringrose and Paro, 2004), these marks could also be rapidly removed by enzymatic demethylation of H3K27 (Klose et al., 2006). Therefore, in contrast to the terminal silencing achieved by the DNA methylation, developmental genes that were silenced by PRCs in pluripotent tissues required repressive marks to be rapidly and flexibly moved when differentiation begain (Reik, 2007). Another group of genes which encoded pluripotency-sustaining transcription factors, such as Oct4 and Nanog, were required for

early development or for germ-cell development only. These pluripotency-associated genes expressed by ES cells but silent during the differentiation of these cells were also known to be mediated by epigenetic regulation with a defined kinetics of acquiring repressive histone modifications and DNA methylation (Feldman et al., 2006; Reik, 2007).

Albeit silence of genetic element could be achieved through histone modifications, these easily reversible modifications were not good gatekeeper for long-term silence (Shi et al., 2004; Takeuchi et al., 2006). Thus, prolonged silence of genetic element must be mediated by an additional epigenetic mechanism. An important component of this process is DNA methylation (Miranda and Jones, 2007). It was noticed that within gene promoters, even when the repressive marks were removed, DNA methylation still prevented the reactivation of silent genes (McGarvey et al., 2007). It is also found that DNA methylation was important for many cellular processes including the silence of repetitive elements, X-inactivation, imprinting and development. The roles of DNA methylation in these processes ensured the daughter cells to retain the same expression pattern as the precursor cells (Miranda and Jones, 2007). DNA methylation is a covalent modification in which the 5' position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor (Miranda and Jones, 2007). In mammals, methylation of cytosines in cytosine guanine

dinucleotide (CpG) island has been known to play a crucial role in mediating epigenetic gene silencing through two possible mechanisms. First, it has been proved that cytosine methylation can directly silence gene expression by inhibiting DNA binding factors to its recognition gene area (Watt and Molloy, 1988). Second, some authors (Boyes and Bird, 1991) and other investigators also prove that methylated-CpG can recruit Methyl-CpG-binding proteins (MBPs), which in turn function to silence transcription and modify the surrounding chromatin structure. In mammals, four DNA methyltransferases (DNMTs) have been identified. Among them, DNMT1 has been known to maintain the pre-existing methylation state of genome during DNA replication (Leonhardt et al., 1992). DNMT3a and DNMT3b are de novo methyltransferases which will target unmethylated CpG sites (Okano et al., 1999). DNMT2, which has been proved to have weak methyltransferase activity in vitro (Hermann et al., 2003), is responsible to methylate tRNA (Goll et al., 2006).

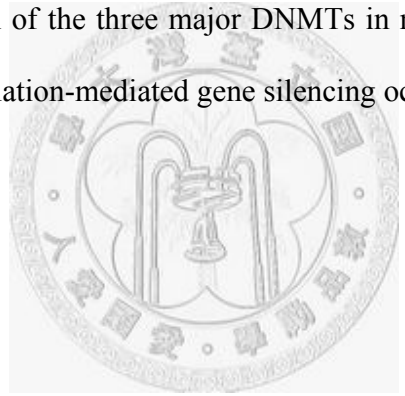
In differentiating ES cells, increase of CpG island DNA methylation with enhanced expression of DNMTs was also noticed (Kremenskoy et al., 2003; Shen et al., 2006), and the deletion of three major DNMTs would cause hypomethylation and thorough blockage of differentiation of ES cells (Carlone et al., 2005; Jackson et al., 2004). These findings plus the fact that global methylation marks are erased during early embryogenesis and then increase during in vitro expansion (Maitra et al., 2005) suggest that differential

changes in CpG island DNA methylation profile may serve as an indicator of "primitiveness" or un-commiment status of stem cells. What's noteworthy, several studies (Hattori et al., 2004; Taylor and Jones, 1979; Tsuji-Takayama et al., 2004) even observed the phenomenon of dedifferentiation caused by treating cells with demethylation agent 5-azacytidine (5-AzaC), which implied that DNA demethylation might cause a reversion of cells to a more pluripotent state (Meshorer and Misteli, 2006). In 1979, Taylor and Jones (Taylor and Jones, 1979) firstly described the phenomenon that by treating C3H/10T1/2 cells with the demethylating agent 5-azacytidine (5-AzaC), differentiate into striated muscle cells, adipocytes and chondrocytes was found to be induced. The authors explained that the conversion of these cells to new phenotypes was caused by a reversion of cells toward a primitive pluripotent state and subsequently gave rise to other lineages. In consistent with this viewpoint, both partially differentiated ES cells (Tsuji-Takayama et al., 2004) and trophoblast stem cells (Hattori et al., 2004) treated with 5-AzaC were found to be induced toward trend of dedifferentiation as well.

C. hTERT and DNMTs

In literatures, only two interesting reports significantly observed the relationship between the expression of hTERT and DNMTs. Young (Young et al., 2003) discovered a previously unknown function of hTERT that its ectopic expression in normal human fibroblasts would activate the DNMT1 promoter activity and maintain the DNMT1

activity even during serial subcultivation. Although the underlying mechanism is still unknown that whether through direct effect of telomerase protein or through its telomerase activity. Results of this report revealed that hTERT could inhibit the expression of genes related to cellular aging. Another study observed similar findings but with some different phenomenon (Casillas et al., 2003). The authors (Casillas et al., 2003) found that upregulation of mRNA and protein expression level of DNMT1, DNMT3a and DNMT3b did occur after cellular tansformation by SV40 T/t-antigen, hTERT and H-ras. They also observed a significant increase of three major DNMTs enzyme activity in transformed human fetal lung fibroblast following extended population doubling. They suggested that up-regulation of the three major DNMTs in neoplastic cell lines could in part explain the hypermethylation-mediated gene silencing occurs in human cancers.



5. Summaries and Conclusions

Although an infinite life span and absolute pluripotency are merely characteristics to embryonic stem cells (ESCs), the ethical considerations almost obstruct their possible clinical application of ESCs. Hence it seems that human mesenchymal stem cells (hMSCs) derived from bone marrow are significant sources of patient and disease-specific stem cells. Furthermore, cellular senescence and spontaneous differentiation of hMSCs, however, are often encountered, which severely impede expansion and wide application of hMSCs clinically. For circumventing these drawbacks found in ESCs or hBMSCs, a new approach should be explored. Based on deficiency in the issue discussed above, we developed a human telomerase reverse transcriptase (hTERT) overexpressed model in a previously immortalized hMSC line. In our prelimary study, we found some interesting observations that ectopic expression of hTERT elicited dedifferentiation phenomenon of hMSCs. Therefore in the current study, we try to unravel relationships between dedifferentiation effect of hTERT, and reverse pluripotency and block spontaneous differentiation in hMSCs. Underlying epigenetic mechanisms involved in their gene regulation and dedifferentiation are also investigated. The results of current study hopefully would take a great step forward in establishing the feasibility and applicability of adult stem cells in future clinical applications.

II. Hypothsis and Specific aims of this study

Hypothesis of this study

After successful transfection of hTERT gene segment, hMSCs would bypass cellular senescence, block spontaneous differentiation, and shift back to ESCs' characteristics. The latter changes were associated with alterations in DNA methylation patterns.

Aims of this study

1. To characterize the nature of hBMSCs after gene transfection with hTERT regarding to osteogenic, neurogenic gene expression.
2. To explore transcriptional gene markers associated with ESCs and test whether hTERT-transfected hMSCs regarding to osteogenic, neurogenic gene expression display similar gene markers by RT-PCR and real time PCR.
3. To investigate differentiation potential and potency of hTERT-transfected hMSCs by functional gene expressions, histochemical staining and in vitro mineralization.
4. To evaluate whether hTERT-transfected hMSCs possess the capacity similar to germline and trophoectoderm differentiation.
5. The roles of DNA methylation-modification factors, such as DNA methyltransferases (DNMTs) responsible in the reversion of hMSCs to a pluipotency state, would also be explored.

III. Research experiments

A. Introduction

Bone marrow mesenchymal stem cells (MSCs) are considered one of the most promising and prospective resources for cell and gene therapy because of their great self-renewal and versatile plasticity in vitro and in vivo (Pittenger et al., 1999). However, there are still two major hindrances, cellular senescence and spontaneous differentiation, encountered during in vitro expansion of MSCs (Woodbury et al., 2002). Cellular senescence could be defined as diminished replication, altered functionality (Beausejour, 2007), and deteriorated potential for differentiation (Bonab et al., 2006). Spontaneous differentiation, known as the emergence of lineage-specific markers without any directed differentiation, would diminish the proportion of undifferentiated stem cells, and therefore compromised the benefit of human MSCs (hMSCs) for clinical application. Thus, identifying methods for inhibiting senescence and spontaneous differentiation, and reversing hMSCs to a more primitive state has attracted great research interest.

In a previous attempt to immortalize hMSCs with increased life span, we have established a cell line-KP by transferring HPV16 E6E7 genes into hMSCs (Hung et al., 2002). Though KP successfully overcomes the drawback of cellular senescence and could be passaged over 100 population doublings (PDs), the phenomenon of spontaneous differentiation could not be avoided (Hung et al., 2004). Telomerase, known to maintain

the telomere length, has been indicated to play a role in self-renewal and pluripotency of embryonic stem cells (ESCs) (Amit et al., 2000). However, hMSCs express no telomerase activity with telomere shortening in a rate similar to non-stem cells (30–120 bp/ PD), and cease to divide when the telomere length is less than 10 kb (Baxter et al., 2004). Besides, ectopic expression of human telomerase reverse transcriptase (hTERT), the catalytic component of telomerase**,** has been proved not only to bypass cellular senescence and extend life span (Bodnar et al., 1998), but also to influence differentiation potential (Shi et al., 2002).

In mammals, DNA methylation of cytosines in cytosine guanine dinucleotide (CpG) islands, known to mediate epigenetic gene silencing (Boyes and Bird, 1991; Watt and Molloy, 1988), plays pivotal roles in embryonic development (Hashimshony et al., 2003; Siegmund et al., 2007; Weber et al., 2007) and ESC differentiation (Meshorer and Misteli, 2006). For example, treating ESCs or somatic cells with demethylation agent such as 5-azacytidine (5-AzaC) resulted in dedifferentiation, thereby pointing out the association of DNA methylation with the differentiation state (Hattori et al., 2004; Taylor and Jones, 1979; Tsuji-Takayama et al., 2004). These results also imply methods that reverse the differentiation state of stem or progenitor cells will induce changes in DNA methylation patterns (Meshorer and Misteli, 2006).

In this study, we hypothesized, after ectopic expression of hTERT, hMSCs would bypass senescence and block spontaneous differentiation with changes in DNA methylation patterns. Meanwhile, we also tried to prove the heightened differentiation potential of hTERT-transfected hMSCs by directing germline and trophoectoderm differentiation. Finally, the roles of DNA methylation-modification factors, such as DNA methyltransferases (DNMTs) in the reversion of hMSCs to a pluipotency state would be explored.

B. Materials and Methods

1. Cell Cultures

Primary hMSCs were obtained from the Tulane Center for Preparation and Distribution of Adult Stem Cells (**http://www.som.tulane.edu/gene_therapy/distribut.shtml**). The cells were grown in alpha minimal essential medium (αMEM; GIBCO/BRL, Carlsbad, CA; **http://www.invitrogen.com**) supplemented with 16.6% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (GIBCO/BRL) at 37°C under 5% CO2 atmosphere. The medium was changed twice per week and a subculture was performed after they reached about 80 % confluency.

The hMSC strain (KP) was developed by transfection with the type 16 human papilloma virus proteins E6E7 as described previously (Hung et al., 2004). This strain is grown in DMEM-LG (Gibco, Grand Island, NY; http://www.invitrogen.com) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The medium was changed twice per week and a subculture was performed at 1:3 to 1:5 split every week. Using flow cytometry, cells express CD29, CD44, CD73, CD90, CD105, SH2, and SH3, but otherwise, they lack expression of CD34 and CD166.

2. DNA Delivery Methods.

KP cells were transfected with phTERT-IRES2-EGFP, which was generated by inserting a 3.45-kb *EcoRI-EcoRI* fragment containing the hTERT cDNA into pIRSE2-EGFP (BD company, USA) using Nucleofector technology as recommended by the manufacturer (AMAXA Biosystems, Cologne, Germany). The efficiency of transfection as evaluated by the expression of EGFP was around 70%. The cells were then suspended in an appropriate volume of 20% FBS-supplemented DMEM-LG medium, seeded in 96well plate for selecting single cell clone by neomycin (400μg/ml).

3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Total RNA was extracted using the Tri Reagent (Sigma) according to the manufacturer's specifications. First strand cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen), Random primer (Invitrogen), 10 mM dNTPs (Invitrogen), 5× First Strand synthesis buffer, 0.1M DTT, and RNaseOUT ribonuclease RNase inhibitor (Invitrogen). PCR was performed using cDNA as the template in a 50 μl reaction mixture containing a specific primer pair of each cDNA according to the published sequences. The reaction products were resolved by electrophoresis on a 1.5% agarose gel and

visualized with ethidium bromide. Sequences of PCR primers and PCR conditions can be provided on request.

4. Real-Time RT PCR.

Real-Time PCR was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems) and the TaqMan Universal Master Mix (Applied Biosystems). Analysis of the results was carried out using the software supplied with the machine. The software calculates each gene expression relative to the β-actin housekeeper gene (delta CT) and then relative to controls (delta delta CT) using the fluorescence threshold of the amplification reaction and the comparative CT method. Sequences of PCR primers, probe and PCR conditions can be provided on request.

5.Differentiation Protocols

Trophoectoderm differentiation protocol was modified from a previous method (Xu et al., 2002). Cells at 50% of confluence were treated with 100 ng/mL BMP4 (R&D Systems, Minneapolis, MN) in DMEM-LG supplemented with 10% FBS. Medium was changed twice per week. Germline differentiation protocol was performed with a protocol modified from previous report (Nayernia et al., 2006). In brief, cells were plated at a

density of 1~2 x 10^4 cells/cm^2 in DMEM-LG supplemented with 10% FBS and 2μM retinoic acid (RA, Sigma) with medium change twice per week. For osteogenic differentiation, cells were seeded at a density of 10^4 cells/cm^2 and induced in DMEM-LG supplemented with 10% FBS, 50 μg/ml ascorbate-2 phosphate (Nacalai, Kyoto, Japan), 10^{-8} M dexamethasone (Sigma) and 10 mM β-glycerophosphate (Sigma) with medium change twice per week. For neurogenic differentiation (Pera et al., 2004), 100ng/ ml recombinant human Noggin (R&D Systems) was added into the serum-free DMEM-LG culture medium.

6. Histochemical Studies

Cells were fixed in 2% paraformaldehyde for 10 min and stained for alkaline phosphatase activity and in vitro mineralization by Alizarin red-S (Hung et al., 2002) to reveal osteogenic differentiation. After washing 5 times with PBS, stained cultures were photographed.

7. DNA Methylation Array

a. DNA preparation

Genomic DNA was extracted from samples using QIAamp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

b. aPRIMES

1 μg genomic DNA was restricted to completion with 10U *Mse*I at 37°C in a final volume of 10 μl in the buffer prepared with the 10 × One-Phor-All Buffer *PLUS* (GE Healthcare). Heat inactivation was carried out at 65°C for 20 min. *Mse*I fragments were then subjected to ligation with PCR linkers, *Mse*I linker-S (5'-TAA CTA GCA TGC-3') and *Mse*I linker-L (5'-AGT GGG ATT CCG CAT GCT AGT-3') overnight. Half of the resulting ligated *Mse*I fragments were digested with the restriction enzyme McrBC (New England Biolabs, Beverly, MA, USA) for 3 h following the conditions recommended by the supplier. The other half of the *Mse*I fragments were digested with the three methylation-sensitive endonucleases *Hpa*II (New England Biolabs; recognition site CCGG, 3 h, 37°C), *Hha*I (New England Biolabs; recognition site CGCG, 3 h, 37°C) and *Bst*UI (New England Biolabs; recognition site CGCG, 3 h, 60°C) according to the recommendations of the supplier. Digested DNA fragments were then treated with 1 μl Proteinase K (Invitrogen, Karlsruhe, Germany) for 1 h at 37°C with subsequent heat inactivation at 80°C for 10 min. For the LM-PCR steps, 2X PCR Master Mix (Promega,

Madison, Wisconsin, USA) was added to a final volume of 50 μl. A MJ thermocycler was programmed to 68°C for 10 min, followed by 27 cycle loops at 94°C (40 s), 57°C (30 s) and 68°C (75 s). Final elongation was carried out at 72°C for 10 min. PCR products were purified by ethanol precipitation. DNA was eluted in 50 μl nuclease free H_2O.

c. Labeling and hybridization to microarrays

Both the *Hpa*II/*Hha*I/*Bstu*I-digested and the McrBC-digested samples were differentially labeled with Cy5- or Cy3-conjugated dUTP by use of an Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies, USA). Labeled targets were subsequently cleanup by the use of a Centricon YM-30 column (Millipore), pooled and mixed in a 500-μl hybridization mixtures with 50 μg of human Cot-1 DNA (Invitrogen) in 1X hybridization buffer (Agilent Technologies). Before hybridization to the array, the hybridization mixtures were denatured at 95°C for 3 min and incubated at 37°C for 30 min. To remove any precipitate, the mixture was centrifuged at ≥14,000 ×*g* for 5min and the supernatant was transferred to a new tube. The labeled and denatured DNA target was then hybridized to human CpG island microarray (G4492A, Agilent Technologies, USA) at 65°C for 40 h. The arrays were washed with 0.5 × SSC/0.005% Triton X-102 (wash 1) at room temperature for 5min, and then with 0.1 × SSC/0.005% Triton X-102 (wash 2) at 37℃ for 5min.

d. Image and microarray data analysis

After drying by nitrogen gun blowing, microarrays were scanned with an Agilent microarray scanner (Agilent Technologies, USA) at 535 nm and 625 nm for Cy3 and Cy5, respectively. Scanned images were analyzed by Feature extraction 9.1 software (Agilent Technologies, USA) to quantify signal and background intensity for each feature. Microarray data were firstly normalized with print-tip loess, followed by background-correction, normalization and analysis by the limma package within the R environment (version 2.1.0). The methylation level was determined as the ratio of Cy5/Cy3 in each spot. The raw data from the array experiments is available from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) under the series accession number GSE (pending number). For Gene Ontology (GO) analysis of the genes decreased in CpG island methylation, we determined the statistically significant GO terms using the hypergeometric probability distribution. For each GO term, a p-value was calculated representing the probability that the number of genes that are annotated at the term could have been found by chance.

8. Microarray expression data sets and principal component analyses

The expression profile of hTERT-transfected hMSCs was implemented by using the Affymetrix™ HG U133 Plus 2.0. To determine the similarity of the expression profiles between hTERT-transfected hMSCs and various normal human tissues, MSCs, and ESCs, PCA was performed in 31 Affymetrix™ U133 Plus 2.0 array data produced by us or from public accessible array databases using the Partek® Genomics Suite™ software (Partek Incorporated, St. Louis, Missouri). All microarray datasets in this paper are available at Gene Expression Ominbus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under the accession no. of GSE7234 and GSE9520.

C. Results

Downregulation of Oct-4 and Nanog in late-passage primary mesenchymal stem cells

Embryonic transcription factors, such as Oct4 and Nanog, normally expressed in early embryos and ESCs, inhibit tissue-specific genes and enhance self-renewal and pluripotency (Boiani and Scholer, 2005). To evaluate whether loss of pluripotency occurred during normal passage of hMSCs, we examined the expression of Oct4 and Nanog in primary hMSCs. Semiquantative RT-PCR and real-time RT-PCR analysis revealed higher mRNA levels of Oct4 and Nanog at passage 3 (P3) than at passage 10 (P10) (Fig. 1A), indicating loss of pluripotency during expansion of primary hMSCs.

ESCs, a powerful tool to study mammalian development, form embryoid bodies (EBs) and express a panel of developmental markers upon removal of feeder layer or leukemia inhibitory factor. To evaluate whether spontaneous differentiation with the expression of developmental markers occurred during normal passage of primary hMSCs, we examined the expression levels of ectoderm (Pax6 (Hill et al., 1991)), primitive endoderm (Gata4 and Gata6 (Fujikura et al., 2002)) and definitive endoderm (Sox17 and FoxA2 (Kubo et al., 2004)) markers by RT-PCR. The expression levels of Pax6, Gata4 and FoxA2 were higher at P10 than at P3 (Fig. 1Ba). We next looked at the expression of germline markers (Clark et al., 2004), and found the expression levels of Stella, Dazl,

Vasa and Scp3 were higher at P10 (Fig. 1Bb). Finally, we examined two lineage-specific markers expressed in EBs, the neural (Nestin) and cardiac specific genes (Nkx 2.5 and cTn1) and found P10 had higher expression of Nestin and cTn-1 (Fig. 1Bc). These results point to upregulation of developmental markers and lineage-specific genes in late-passage primary hMSCs.

Transient upregulation of Oct4 and Nanog during early differentiation of hTERT-transfected hMSC

Since differentiation and cellular senescence are associated with loss of telomere length (Baxter et al., 2004; Sharpless and DePinho, 2004), and telomerase is highly expressed in ESCs (Amit et al., 2000; Thomson et al., 1998), we hypothesized overexpression of hTERT would enhance pluripotency and overcome spontaneous differentiation. Because of the difficulties in bypassing cellular senescence (Akimov et al., 2005; Okamoto et al., 2002), selecting and expanding single cell clones by expressing hTERT alone in primary hMSCs and the fact both hTERT and E6E7 are necessary to circumvent cellular senescence thoroughly (Akimov et al., 2005; Okamoto et al., 2002), we avoided transferring hTERT to primary hMSCs, but rather to E6E7-transfected hMSCs- the KP cells(Hung et al., 2004) and monitored transgene expression by a fluorescent reporter-

green fluorescence protein (eGFP). Several single-cell derived clones were isolated and one of the clones, 3A6 was used for further analysis. 3A6 grown in monolayer in DMEM-LG supplemented with 10% FBS had a remarkably shorter population doubling time (1.9 days) compared with the parental KP cells (3.0 days). RT- PCR revealed the expression of hTERT and eGFP in 3A6. Flow cytometry also demonstrated 3A6 has a normal surface protein profile like the normal hMSCs (Supplementary Fig. 1).

Besides the increase in proliferation rate, the ectopic expression of hTERT might also affect pluripotency. Therefore, we compared the expression levels of Oct4 and Nanog between KP and 3A6. Unexpectedly, RT-PCR and real-time RT-PCR unraveled the downregulation of both Oct4 and Nanog in 3A6 compared with KP (Fig. 2A). Downregulation of the embryonic transcription factors such as Oct4 and Nanog is associated with differentiation of neural stem cells, hematopoietic stem cells and MSCs. However, an increase in Oct4 expression in ESCs causes differentiation into primitive endoderm (Niwa et al., 2000), mesoderm (Niwa et al., 2000) and early cardiac lienage (Zeineddine et al., 2006). Overexpression of Nanog also drives the expression of ectoderm markers (Zeineddine et al., 2006). The expression pattern of Oct4 and Nanog during differentiation is completely different between ESCs and adult stem cells such as MSCs, and should serve as an indicator to discriminate ESCs from MSCs (Darr et al., 2006; Niwa et al., 2000; Zeineddine et al., 2006). We therefore induced 3A6 to undergo

osteogenic and neural differentiation and examined the expression of Oct4 and Nanog. During osteogenic differentiation, we noticed a continuous upregulation of Oct4 and Nanog until day 7 followed by downregulation of both genes at day 14 (Fig. 2Ba). Similarly, during neural differentiation, the upregulation of Oct4 and Nanog was observed during early differentiation (Fig. 2Bb). These results indicated 3A6 has a differential gene expression of embryonic markers similar to the early differentiation of ESCs, suggesting the most primitive state of hTERT-transfected hMSCs

Downregulation of developmental markers and lineage-specific genes in hTERT-transfected hMSCs

To clarify the blocking of spontaneous differentiation by ectopic expression of hTERT in hMSCs, we compared the expression of developmental markers and lineage-specific genes between 3A6 and KP by performing RT-PCR for trophoectoderm (CDX2 and CGß), germline (Dazl, Vasa and Scp3), osteogenic (BSP, Bone Sialoprotein and OCN, Osteocalcin) and neural (Pax6 and Nestin) specific markers. We noted a general downregulation of expression for all these genes at 3A6 compared with KP (Fig. 2C), indicating the ability of hTERT to block spontaneous differentiation and maintain 3A6 in an undifferentiated state.

Transient upregulation of Oct-4 and Nanog upon early differentiation of hTERT-expressed hBMSC

Downregulation of the embryonic markers such as Oct-4 and Nanog is associated with differentiation of neural stem cells, haematopoietic stem cells and MSCs, nevertheless, upregulation of OCT-4 and Nanog was observed during the formation of embryonic body (Darr et al., 2006; Zeineddine et al., 2006)- the early differentiation of embryonic stem cells, and overexpression of Oct-4 was also proved to driving primitive endoderm (Niwa et al., 2000), primitive mesoderm (Niwa et al., 2000) and early cardiac (Zeineddine et al., 2006) differentiation of embryonic stem cells. Since 3A6 was developed on the purpose to have a BMSC cell line with the potential of ESCs, we then investigated whether 3A6 has a differential expression pattern of Oct-4 and Nanog similar to that of ESCs during the process of early differentiation. To illustrate the relationship between expression profile of Oct-4 and Nanog and early differentiation state of 3A6, we exploited osteogenic and neuronal differentiation as models in search of transient expression alteration of Oct-4 and Nanog during early differentiation. With induction of osteogenic differentiation, quantitative real-time PCR revealed continuous upregulation of Oct-4 and Nanog until day 7 which were followed by the downregulation of both genes thereafter (Fig. 2Ba). Similarly, during neuronal differentiation, the upregulation of Oct-4 and Nanog expression in early differentiation state were noeted (Fig. 2Bb). These results

indicated that 3A6 has a differential gene expression of embryonic markers similar to the early differentiation of ESCs, suggesting the most primitive state of hTERT-expressed BMSCs

Downregulation of developmental markers and lineage-specific genes in hTERT-expressed hBMSCs

To further clarify the reversion of stemness, that is, the recovery to a more primitive state after ectopic expression of hTERT at 3A6, we compared the relative expression level of developmental markers and lineage-specific genes between 3A6 and KP, which would be another indicator of the primitive state of stem cells besides embryonic markers, Oct-4 and Nanog. Among these genes, we choose trophoectoderm specific markers, Cdx2 and CGß; germ-line specific markers, Dazl, Vasa and Scp3; osteogenic specific genes, Bsp and Ocn; neuronal specific genes, Pax6 and Nestin as targets of comparison. The RT-PCR results (Fig. 3A) disclosed generalized downregulation of these developmental markers and lineage-specific genes at 3A6 than that at KP.

Improvement of differentiation potential by introducing hTERT to hMSCs

After characterization of 3A6 and unraveling its relative quiescent state, it is of great interest if the differentiation potential of 3A6 would be sustained, enhanced and reversed to a considerably primitive state, as with ESCs. We first examined if 3A6 sustained the normal capabilities of hMSCs, such as mesenchymal (osteogenic, adipogenic and chondrogenic) and non-mesenchymal (neural) differentiation and hematopoietic supporting potential (cobblestone forming). 3A6 had normal or elevated osteogenic and chondrogenic differentiation potential compared with one KP-derived single cell clone, whereas 3A6 had decreased adipogenic differentiation potential (Fig. 3A). These data are consistent with previous studies that overexpression of hTERT increased osteogenic potential and the inverse relationship between osteogenic and adipogenic differentiation. For neural differentiation, 3A6 adopted the typical morphology of neural progenitor cells, including bipolar elongated cell processes and retracted cell bodies, and expressed neural lineage specific markers, such as Nestin and Pax6 on stimulation with noggin in serum free conditions for 14 days (Fig. 3B). For co-cultured CD34+ hematopoietic stem cells with 3A6 cells, we noted the formation of cobblestone areas from hematopoietic cells that transmigrated beneath the layer of 3A6 cells (Fig. 3C).

Previously, only ESCs has proven to be able to successfully differentiate toward trophoectoderm (Xu et al., 2002) and germline (Clark et al., 2004) in vitro, but Johnson

and others (Johnson et al., 2005) detected the expression of germline markers in bone marrow and peripheral blood, and Nayernia and others (Nayernia et al., 2006) further implied the germline differentiation potential of mouse MSCs. No literature so far, however, has revealed the differentiation potential of MSCs toward trophoectoderm. To test the most versatile differentiation potential of hMSCs after ectopic expression of hTERT, we directed 3A6 towards trophoectoderm and germline differentiation upon stimulation with BMP4 (Xu et al., 2002) and retinoic acid (RA) (Geijsen et al., 2004), respectively. This has been used to initiate trophoblast and germline differentiation in human ESCs. As demonstrated by RT-PCR, 3A6 started to express the trophoectoderm specific markers, such as CDX2 and CGß (Fig. 3D), and germline specific markers (Clark et al., 2004), such as Stella, Dazl, Vasa, and Scp3 (Fig. 3E) after differentiation. These results together suggest 3A6 not only sustained normal potential as hMSCs, but also adopted the potential that was previously exclusive to ESCs.

Enhanced differentiation efficiency of hTERT-transfected hMSCs

Besides the differentiation potential, another significant issue would be the differentiation efficiency of 3A6. Spontaneous differentiation, noted during expansion of primary hMSCs and KP, might hamper differentiation efficiency because less uncommitted cells could be directed toward specific lineage. Thus, we expected 3A6 to have better

differentiation efficiency because of its less committed state. To clarify this hypothesis, we directed KP and 3A6 toward osteogenic or neural lineage and compared their differentiation efficiency by histochemical staining and lineage-specific gene expression. We observed 3A6 had higher alkaline phosphatase and Alizarin Red S staining compared with KP no matter at day 3, day 7 or day 14 of osteogenic differentiation (Fig. 4A). The expression levels of osteogenic markers- BSP and OCN were also elevated in 3A6 compared with KP during osteogenic differentiation. The expression levels of neural markers- Nestin and Pax6 were also elevated in 3A6 during neural differentiation (Fig. 4B).

Global hypomethylation of development and differentiation associated genes in hTERT-transfected hMSCs

To prove the recovery of pluripotency after hTERT transfection might be attributed to epigenetic remodeling, we conducted a genome-wide analysis of DNA methylation between 3A6 and KP cells, which contained about 240000 probes for 24000 CpG islands. The average methylation level of 3A6 (1.630 ± 9.456) was significantly lower than KP (1.762 ± 17.187) (Supplementary Fig. 2). The number (percentages) of annotated genes detected as hypermethylated by the probes were 6703 (16.2 %) and 7239 (17.6 %) for 3A6 and KP, respectively. These results are consistent with the finding CpG islands are more frequently associated with housekeeping genes in an active state with

hypomethylated DNA(Saxonov et al., 2006) and reveal KP has greater DNA methylation level than 3A6. Since global DNA demethylation occurs immediately following fertilization and ESCs are nearly devoid of methylation markers(Li, 2002; Meshorer and Misteli, 2006), the decrease in global CpG island methylation level in 3A6 further demonstrates its primitive state.

Due to the decrease in numbers of hypermethylated genes in 3A6, we then analyzed genes demethylated after hTERT overexpression according to different gene categories using Gene Ontology (Fig. 5). Notably, the demethylated genes were highly associated with development (p value= 1.09E-16) and cellular differentiation (p value= 0.0208). However, we didn't find a relatively higher expression level of the demethylated genes in 3A6 than in MSCs and differentiated ESCs by comparing their transcriptome microarrays (data not shown), suggesting the hypomethylated state didn't actually assure the gene expression, but rather, kept these genes in a state poised for activation.

Ectopic expression of hTERT downregulated three major DNMT genes transcription in hMSCs

Attempting to discover factors that might induce DNA demethylation in 3A6, we used real-time RT-PCR to quantify the expression level of three major DNMTs between 3A6

and KP. Surprisingly, the levels of DNMT1, DNMT3A and DNMT3B were markedly suppressed in 3A6 compared with KP (Supplementary Fig. 3A). Since DNA methylation could also be controlled by the polycomb group protein, EZH2(Vire et al., 2006), we checked the expression of EZH2 by real-time RT-PCR. The expression levels of EZH2 were not different between 3A6 and KP (Supplementary Fig. 3B). From these results, the decrease in CpG island methylation in 3A6 is associated with the decrease in DNMT gene expression.

The hTERT-transfected hMSC expression profile converges toward ESCs

To gain insight into the convergence of 3A6 toward ESCs, we compared the expression profile of 3A6 with various normal human tissues, MSCs and ESCs. This data set therefore contained different tissues from embryo, endoderm, epithelial, or mesenchymal origins. The expression profiles of each chip were compared using principal component analysis (PCA) to discover the similarity of the expression profiles within and across the cells or tissues. PCA using all probe sets showed ESC and MSC each formed a distinct group and were quite different from all the normal human tissues. Interestingly, the 3A6 expression profile located very close to ESCs rather near MSCs, signaling the expression profile of 3A6 converged toward ESCs (Figure 6).

D. Discussions

To circumvent the problems associated with expanded hMSCs, we found that ectopic expression of hTERT enhanced proliferation and pluripotency, and blocked spontaneous differentiation in a previously developed hMSC line. Surprisingly, the hTERT-transfected hMSCs had differentiation potential far beyond the normal hMSCs. They expressed trophoectoderm and germline specific markers at day 7 of induced differentiation with BMP4 and RA, respectively. These findings didn't guarantee these induced cells would behave like normal trophoblasts or germ cells, and their identity should be elucidated in detail. Besides pluripotency and unlimited differentiation potential, we further showed hTERT-transfected hMSCs displayed higher osteogenic and neural differentiation efficiency than their parental cells. The increased differentiation efficiency was attributable to the decrease in committed cells that have spontaneously undergone differentiation and might be limited in directed differentiation potential.

DNA methylation and chromatin structure are major epigenetic factors that regulate gene expression (Meshorer, 2007). Increase in CpG island methylation was noticed during ESC differentiation (Kremenskoy et al., 2003; Shen et al., 2006) and deleting the three major DNMTs would cause hypomethylation and thorough blockage of differentiation of ESCs (Carlone et al., 2005; Jackson et al., 2004). These findings plus the fact global methylation marks are erased after fertilization and formation of embryo,

and increase during in vitro expansion (Maitra et al., 2005) suggest the CpG island methylation profile may serve as an indicator of "primitiveness" of stem cells. Therefore, the decrease in CpG island methylation in 3A6 indicates the induction of primitiveness by ectopic expression of hTERT. More importantly, DNA demethylation occurred mainly in the CpG islands of development and differentiation associated genes, and ensured these genes the accessibility for activation upon cues of stimulation and further explained the unlimited differentiation potential.

In the current study, CpG island hypomethylation did not induce an increase in the average gene expression level in 3A6. Weber (Weber et al., 2007) clarified most of the unmethylated promoters with high CpG frequency (HCPs) remain inactive. Mikkelsen and others (Mikkelsen et al., 2007) further explored the chromatin state of HCPs in ESCs and revealed monovalent promoters (H3K4me3) generally regulate genes with "housekeeping" functions, and otherwise, bivalent promoters (H3K4me3 and H3K27 me3) are associated with genes related to key developmental transcription factors. Most importantly, they found low activity of bivalent HCPs, compatible with the findings that most of the development associated genes are quiescent in pluripotent cells. Therefore, the low activity of demethylated development-associated genes in 3A6 might be due to transient repression by chromatin modifications, and indeed the hypomethylated state of

these genes enable them to recapitulate expression upon later development or cellular differentiation.

The decrease in CpG island methylation of 3A6 might be attributable to mechanisms involving DNA demethylation. Passive demethylation would take place during DNA synthesis by blocking DNMT1, enzyme for preserving methylation in successively replicating cells (Razin and Riggs, 1980). Though we noted substantial downregulation of DNMT1 and a decrease in global CpG island methylation level in 3A6, we discovered the genes demethylated after hTERT transfection were mainly associated with development and cellular differentiation. This high specificity excludes the possibility passive demethylation alone would be responsible for DNA demethylation in 3A6. Gius and others (Gius et al., 2004) revealed both DNA demethylation and increase in gene expression were observed at 24 hr of treatment with DNA methylation inhibitor, 5-Aza-2'-deoxycytidine (5-Aza-CdR) (after 1-2 cell division at most). The effects of 5-Aza-CdR were the same after one or five days, suggesting the existence of active demethylation (Gius et al., 2004). Indeed, active demethylation could be justified by the model DNA methylation patterns constitute a steady-state balance of methylation and demethylation reactions and inhibition of DNMTs would leave demethylation reactions unopposed, leading DNA demethylases to induce a new unmethylated state (D'Alessio and Szyf, 2006). Therefore, demethylation highly specified to development- and cellular

differentiation-related genes caused by the downregulation of three major DNMTs after hTERT transfection might be attributed to both passive and active DNA demethylation.

Despite further investigations needed to elucidate exact demethylation mechanism, the effect of global DNA demethylation on pluripotency and behavior of stem cells is still of great significance. Taylor and others first described that treatment with 5-AzaC increased the differentiation potential of C3H/10T1/2 cells (Taylor and Jones, 1979). Similarly, 5-AzaC also induced dedifferentiation in partially differentiated ESCs (Tsuji-Takayama et al., 2004) or trophoblast stem cells (Hattori et al., 2004). These findings support our findings that 3A6 with a significant global decrease in CpG island methylation level behaved like ESCs and such alteration in pluripotency might be achieved by DNA demethylation of development and differentiation associated genes after hTERT transfection.

Therefore, besides bypass of senescence, transfection of hMSCs with hTERT might elicit change of epigenetic marks to reverse pluripotency, which finally contributes to unlimited differentiation potential and increased differentiation efficiency. Although the hidden ulterior connection between hTERT and epigenetic remodeling in stem cell biology is needed, the current results are a great step forward in establishing the feasibility and applicability of adult stem cells in future clinical applications.

E. Conclusions

In the current study, we found that ectopic expression of hTERT not only blocked spontaneous differentiation and enhanced differentiation efficiency of hMSCs, but, what's most fascinating, we successfully induced the extraordinary differentiation potential of hMSCs to germline and trophoectoderm lineages. We further demonstrated that hTERT might elicit phenomenon of dedifferentiation of hMSCs through epigenetic remodeling, especially DNA demethylation. A global CpG island methylation profile analysis revealed decreased methylation level of hTERT-transfected hMSCs and notably the demethylated genes were highly associated with development and differentiation genes which ensure the recapitulation of expression of these genes upon proper stimulation. Principal component analyses further indicated that the expression profile of the hTERT-transfected hMSCs converged toward ESCs. These data implicated great importance of the dedifferentiation effect of hTERT, which reversed pluripotency and blocked spontaneous differentiation of hMSCs through epigenetic mechanism and the current results take a great step forward in establishing the feasibility and applicability of adult stem cells in future clinical applications.

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Figures

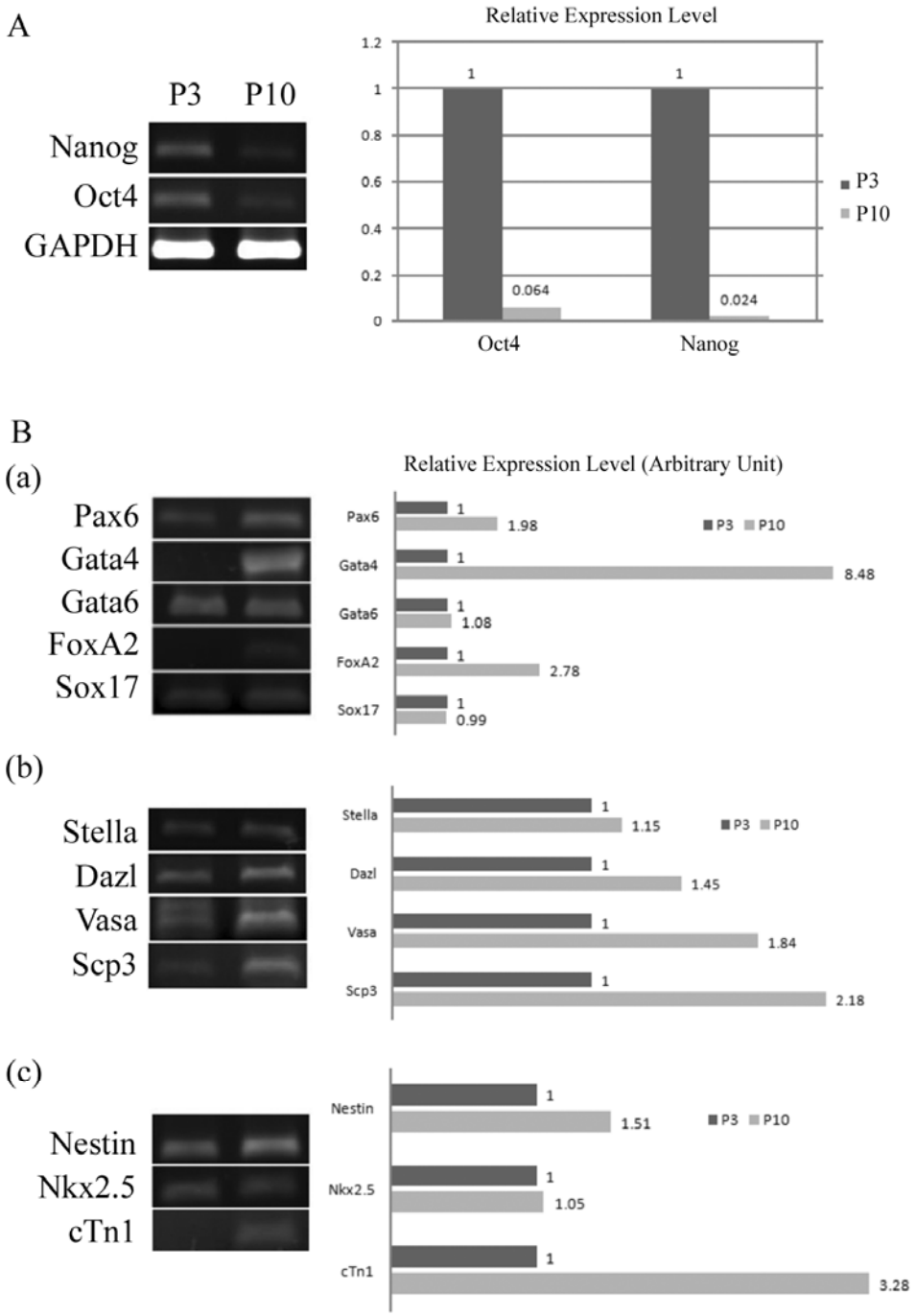


Fig. 1

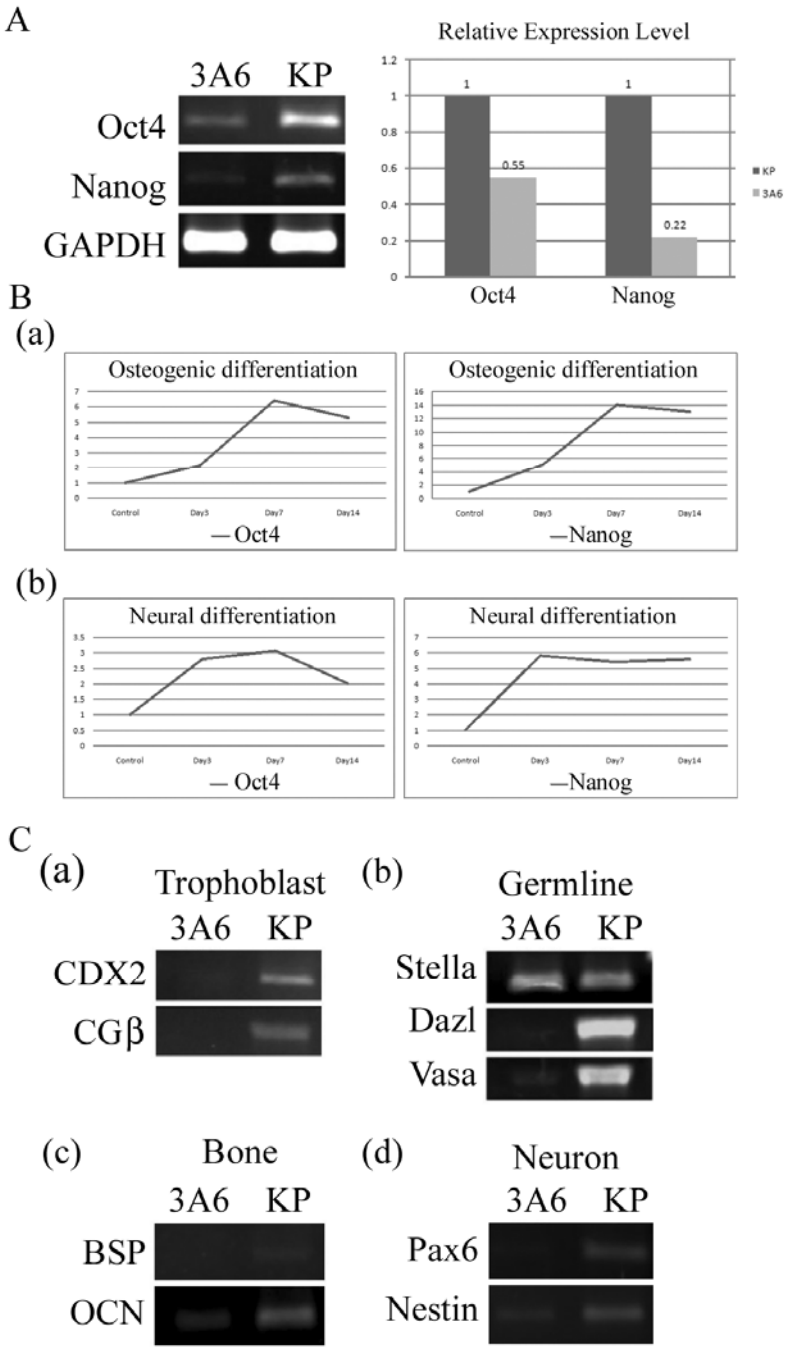

A
3A6
KP
Oct4
Nanog
GAPDH
Relative Expression Level
1
0.55
1
0.22
Oct4
Nanog
KP
3A6
B
(a)
Osteogenic differentiation
— Oct4
Osteogenic differentiation
—Nanog
(b)
Neural differentiation
— Oct4
Neural differentiation
—Nanog
C
(a)
Trophoblast
3A6
KP
CDX2
CGβ
(b)
Germline
3A6
KP
Stella
Dazl
Vasa
(c)
Bone
3A6
KP
BSP
OCN
(d)
Neuron
3A6
KP
Pax6
Nestin

Fig. 2

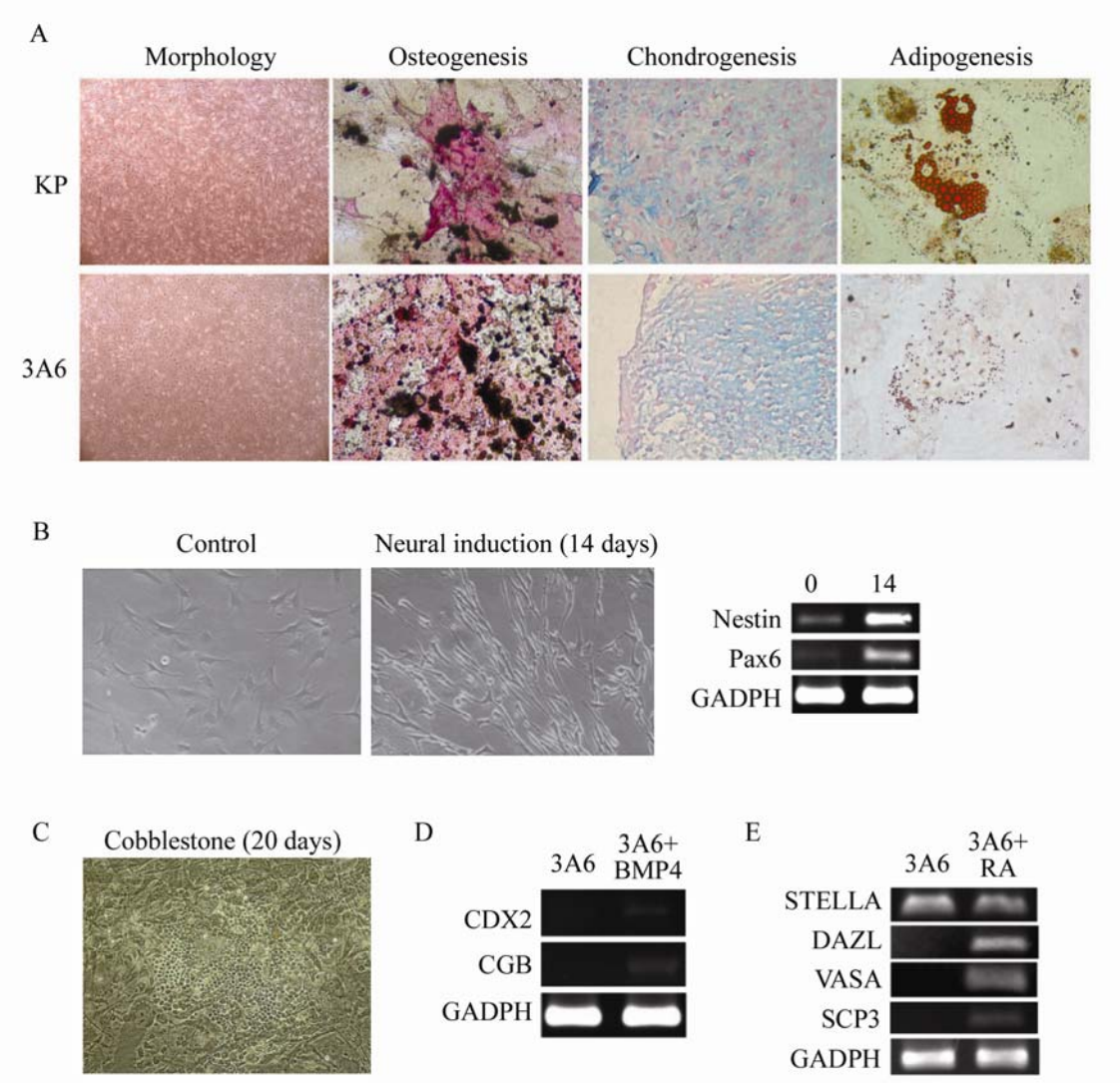

A
Morphology
Osteogenesis
Chondrogenesis
Adipogenesis
KP
3A6
B
Control
Neural induction (14 days)
0
14
Nestin
Pax6
GADPH
C
Cobblestone (20 days)
D
3A6
3A6+ BMP4
CDX2
CGB
GADPH
E
3A6
3A6+ RA
STELLA
DAZL
VASA
SCP3
GADPH

Fig. 3

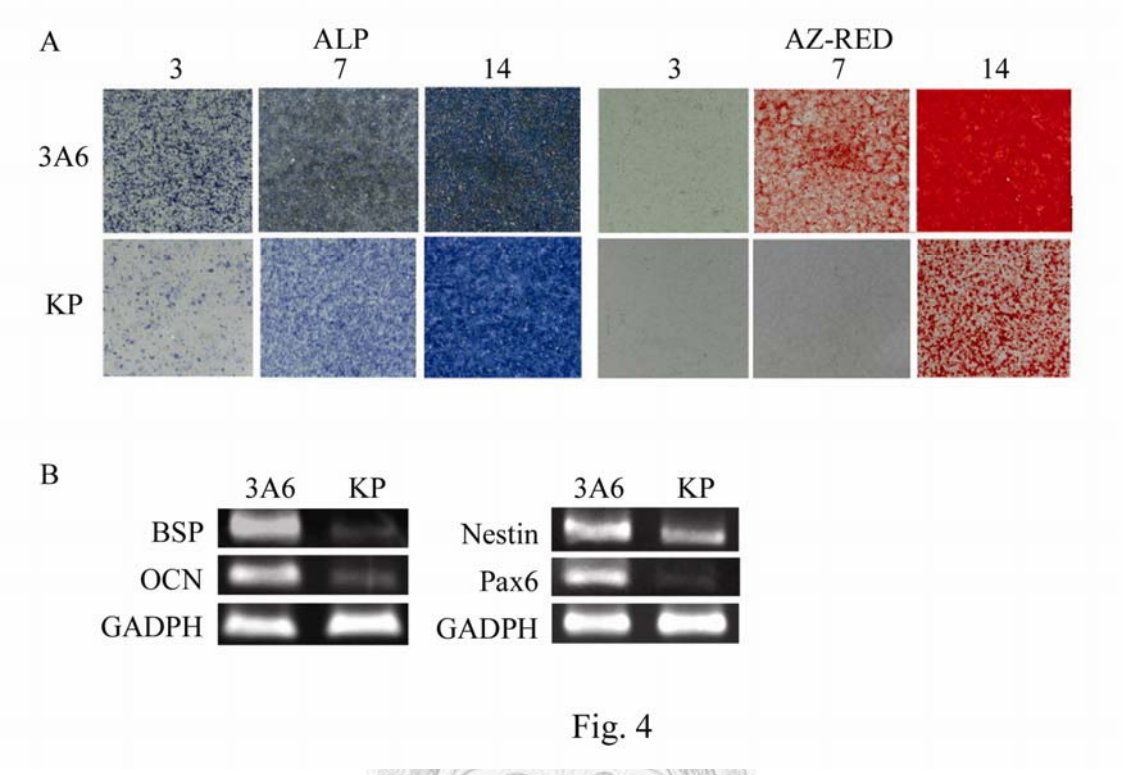

A
ALP
AZ-RED
3
7
14
3
7
14
3A6
KP
B
3A6
KP
BSP
OCN
GADPH
3A6
KP
Nestin
Pax6
GADPH

Fig. 4

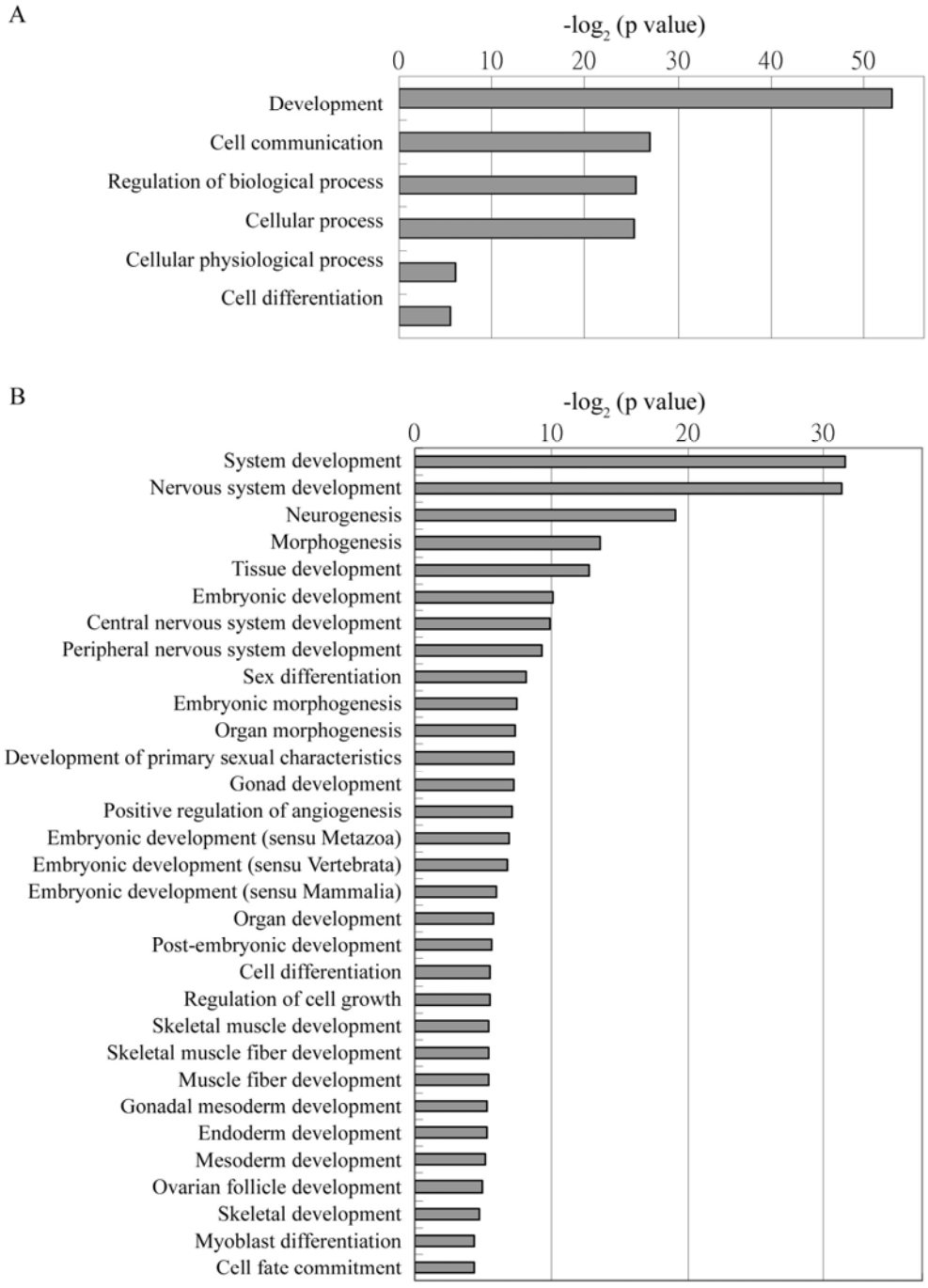

A
-log2 (p value)
0
10
20
30
40
50
Development
Cell communication
Regulation of biological process
Cellular process
Cellular physiological process
Cell differentiation
B
-log2 (p value)
0
10
20
30
System development
Nervous system development
Neurogenesis
Morphogenesis
Tissue development
Embryonic development
Central nervous system development
Peripheral nervous system development
Sex differentiation
Embryonic morphogenesis
Organ morphogenesis
Development of primary sexual characteristics
Gonad development
Positive regulation of angiogenesis
Embryonic development (sensu Metazoa)
Embryonic development (sensu Vertebrata)
Embryonic development (sensu Mammalia)
Organ development
Post-embryonic development
Cell differentiation
Regulation of cell growth
Skeletal muscle development
Skeletal muscle fiber development
Muscle fiber development
Gonadal mesoderm development
Endoderm development
Mesoderm development
Ovarian follicle development
Skeletal development
Myoblast differentiation
Cell fate commitment

Fig. 5

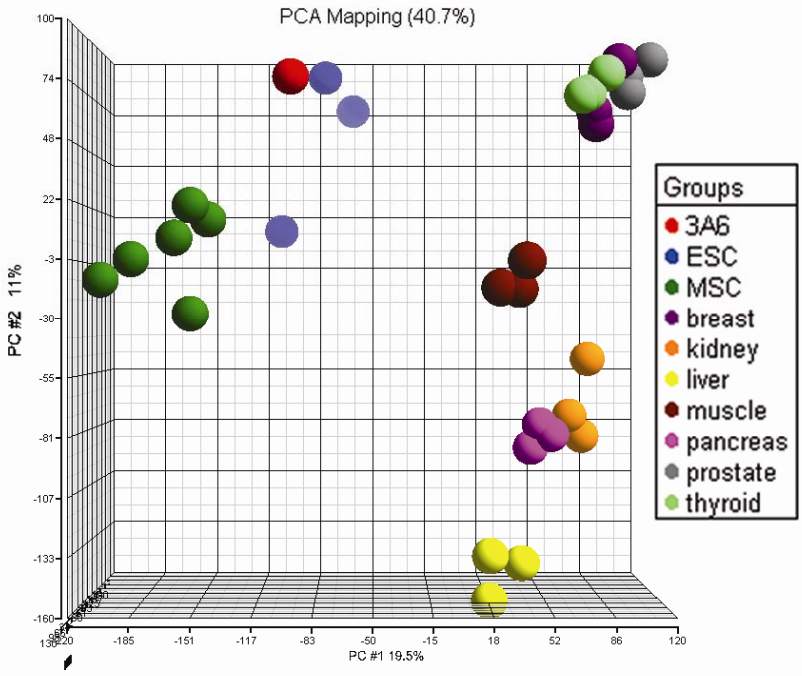
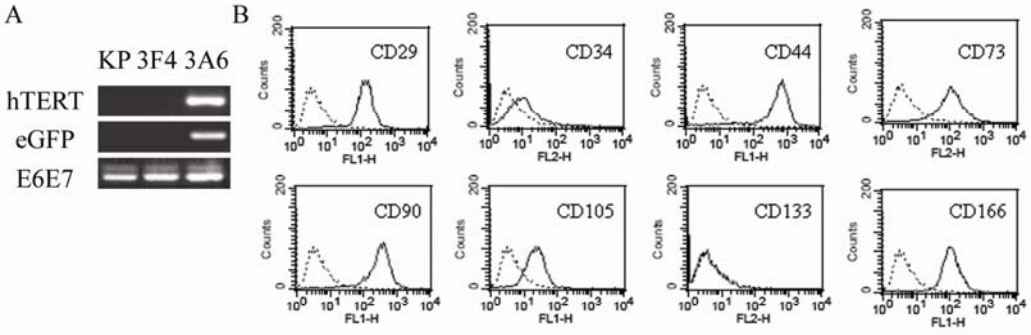
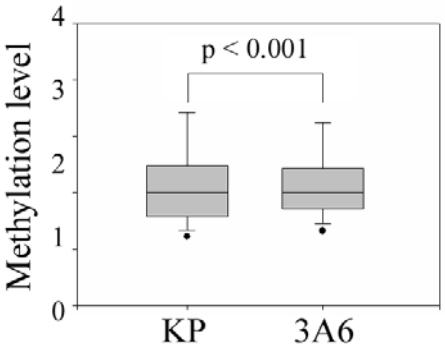

PCA Mapping (40.7%)
PC #2 11%
PC #1 19.5%
Groups
3A6
ESC
MSC
breast
kidney
liver
muscle
pancreas
prostate
thyroid

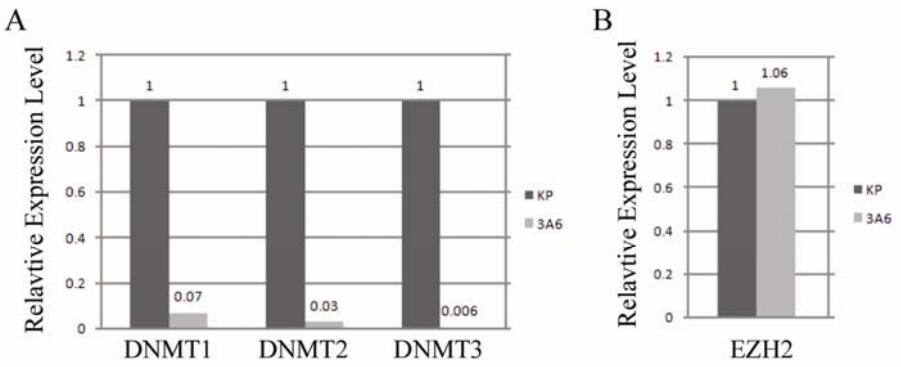
Fig. 6



Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3

Figure Legends

Figure 1. Comparisons of Differential gene expression between primary cultured hBMSCs passage 3 (P3) and passage 10 (P10). **A.** Semiquantitative RT-PCR (left panel) and real-time RT-PCR (right panel) analysis reveal higher mRNA levels of Oct4 and Nanog at P3 than those at P10. **B.** The expression levels of ectoderm (Pax6), primitive endoderm (Gata4 and Gata6) and definitive endoderm (Sox17 and FoxA2) markers are explored by RT-PCR. (a) The expression levels of Pax6, Gata4 and FoxA2 are higher at P10 than those at P3; (b) the expression levels of Stella, Dazl, Vasa and Scp3 are similarly elevated at P10; and (c) Cells at P10 show an increased expression of Nestin and cTn-1.

Figure 2. Representative differential gene expressions in 3A6 and KP cells, and alteration of pluripotency related markers during 3A6 differentiation. **A.** RT-PCR and real-time RT-PCR analysis unravel downregulation of pluripotency related genes (Oct4 and Nanog) in 3A6 cells when compared with KP cells. **B.** Differential expression of Oct4 and Nanog during differentiation of 3A6 cells in culture. (a) In osteogenic differentiation, a continuous upregulation of Oct4 and Nanog are noticed until day 7 and then followed by a downregulation of both genes at day 14; (b) Similarly, in neural differentiation, the upregulation of Oct4 and Nanog is observed during early

differentiation. **C.** It exhibits a general downregulation for the expression of (a) trophoectoderm, (b) germline, (c) osteoblastic, and (d) neural lineage specific genes in 3A6 cells when compare with KP cells.

Figure 3. Versatile differentiation potential of 3A6 cells. **A.** 3A6 cells display normal or elevated osteogenic and chondrogenic differentiation potential when compare with one KP-derived single cell clone, whereas 3A6 cells show a decreased adipogenic differentiation potential. **B.** For neural differentiation, 3A6 adopted the typical morphology of neural progenitor cells, including bipolar, elongated cell processes and retracted cell bodies, and express neural lineage specific gene markers, such as Nestin and Pax6 on stimulation with noggin in serum free conditions for 14 days. **C**. In co-cultured of CD34+ hematopoietic stem cells and 3A6 cells, formation of cobblestone areas from hematopoietic cells that transmigrated beneath the layer of 3A6 cells is found. **D.** As demonstrated by RT-PCR, 3A6 cells express the trophoectoderm specific gene markers, such as CDX2 and CGβ. **E.** Other germline specific markers, such as Stella, Dazl, Vasa, and Scp3, appear after induction with BMP4 and RA, respectively.

Figure 4. Forced differentiation of osteogenic or neural lineage and elevation of their differentiation efficiency in KP and 3A6 cells are made by histochemical staining and

lineage-specific gene expression. **A.** 3A6 cells demonstrate a higher alkaline phosphatase and Alizarin Red S stainings than KP cells do at day 3, 7 and 14 during osteogenic differentiation. **B.** The expression levels of major osteogenic markers, BSP and OCN, are elevated in 3A6 when compare with KP cells during osteogenic differentiation. Similar observations are found concerning neural markers, Nestin and Pax6, in 3A6 during neural differentiation.

Figure 5. A. Methylation level of genomic DNA in 3A6 cells is investigated by labeling DNA with Cy5- or Cy3- conjugated dUTP and processes hybridization to human CpG island microarray later, and finally analyzes on microarray data. Due to the decrease in number of hypermethylated genes found in 3A6 cells, genes demethylated after hTERT overexpression are measured according to different gene categories using Gene Ontology. Notably, the demethylated genes examined are found to highly associate with those at the stage of development (p value= 1.09E-16) and cellular differentiation (p value= 0.0208). **B.** Sub-classification of relevant gene profiles at the stage of development.

Figure 6. Convergence of gene expression profile of the hTERT-transfected hMSC (3A6 cells) toward embryonic stem cells. Microarray expression and principal component analysis comparing the gene expression profiles among hTERT-transfected hMSCs

(3A6), Embryonic stem cells (ESC), mesenchymal stem cells (MSC), and various tissues is performed using all the transcriptome data. Each plotted data point represents a single profile. There is a marked intimate adherence of 3A6 cells to ESCs, which is far from the localization of BMSCs.

Supplementary Figure 1. A. Detection of hTERT, eGFP and E6E7 mRNA expression in KP, 3F4 and 3A6 cells. **B.** Flow cytochemical characterization of CD molecules in 3A6 cells. Cytofluorimetric profiles of 3A6 reacted first with (solid line) or without (broken line) mouse MAbs specific for each marker, and second with fluorescein-labeld antimouse Ig antibody. Flow cytometry demonstrated 3A6 has a normal surface protein profile like the normal hMSCs, which express CD29, CD44, CD73, CD90, CD105, SH2, and SH3, but otherwise, they lack expression of CD34 and CD166.

Supplementary Figure 2. Box plots show average methylation levels of genes contain CpG islands. P value was calculated using a *t*-test. The average methylation level of 3A6 (1.630 ± 9.456) was significantly ($p < 0.001$) lower than KP (1.762 ± 17.187).

Supplementary Figure 3. Real-time RT-PCR analysis of expression levels of DNA methylation related enzymes **A.** DNMT1, DNMT3A and DNMT3B**,** and **B.** EZH2 in 3A6

and KP. The levels of DNMT1, DNMT3A and DNMT3B were markedly suppressed in 3A6 compared with KP, but the expression levels of EZH2, however, were not different between 3A6 and KP.