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Genetic and epigenetic instability of human bone marrow mesenchymal stem cells expanded in autologous serum or fetal bovine serum

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ABSTRACT Culture of mesenchymal stem cells (MSCs) under conditions promoting proliferation and differentiation, while supporting genomic and epigenetic stability, is essential for therapeutic use. We report here the extent of genome-wide DNA gains and losses and of DNA methylation instability on 170 cancer-related promoters in bone marrow (BM) MSCs during culture to late passage in medium containing fetal bovine serum (FBS) or autologous serum (AS). Comparative genomic hybridization indicates that expansion of BMMSCs elicits primarily telomeric deletions in a subpopulation of cells, the extent of which varies between donors. However, late passage cultures in AS consistently display normal DNA copy numbers. Combined bisulfite restriction analysis and bisulfite sequencing show that although DNA methylation states are overall stable in culture, AS exhibits stronger propensity than FBS to maintain unmethylated states. Comparison of DNA methylation in BMMSCs with freshly isolated and cultured adipose stem cells (ASCs) also reveals that most genes unmethylated in both BMMSCs and ASCs in early passage are also unmethylated in uncultured ASCs. We conclude that (i) BMMSCs expanded in AS or FBS may display localized genetic alterations, (ii) AS tends to generate more consistent genomic backgrounds and DNA methylation patterns, and (iii) the unmethylated state of uncultured MSCs is more likely to be maintained in culture than the methylated state.

KEY WORDS: *bisulfite sequencing, COBRA, genomic stability, comparative genomic hybridization*

Introduction

The potential for *in vitro* expansion, differentiation and oncogenic transformation of mesenchymal stem cells (MSCs) has been extensively investigated primarily because of their potential use in cell therapy for tissue repair (Brooke *et al.*, 2007) and as immunosuppressive vehicle (Le Blanc K. and Ringden, 2007). Bone marrow MSCs (BMMSCs) can differentiate into mesodermal lineages (Pittenger *et al.*, 1999) and a small subpopulation seems to exhibit more extensive differentiation ability (Jiang *et al.*, 2002). MSCs with mesodermal differentiation potential can also be obtained in large numbers from adipose tissue (Zuk *et al.*, 2001; Boquest *et al.*, 2005; Katz *et al.*, 2005).

Using MSCs in a therapeutic context necessitates large-scale *in vitro* expansion, increasing the probability of genetic and epigenetic instabilities. Spontaneous oncogenic transformation commonly affects mouse MSCs (e.g., Miura *et al.*, 2006; Tolar *et al.*, 2007). However, reports of transformation of human MSCs are scarce. Most human MSC types cultured to late passages display normal karyotypes (Rubio *et al.*, 2005; Miura *et al.*, 2006; Zhang *et al.*, 2007), genomic stability (Bernardo *et al.*, 2007b), and absence of telomerase expression and activity (Bernardo *et*

Abbreviations used in this paper: AS, autologous serum; ASC, adipose stem cell; BM, bone marrow; CGH, comparative genomic hybridization; FBS, fetal bovine serum; MSC, mesenchymal stem cell.

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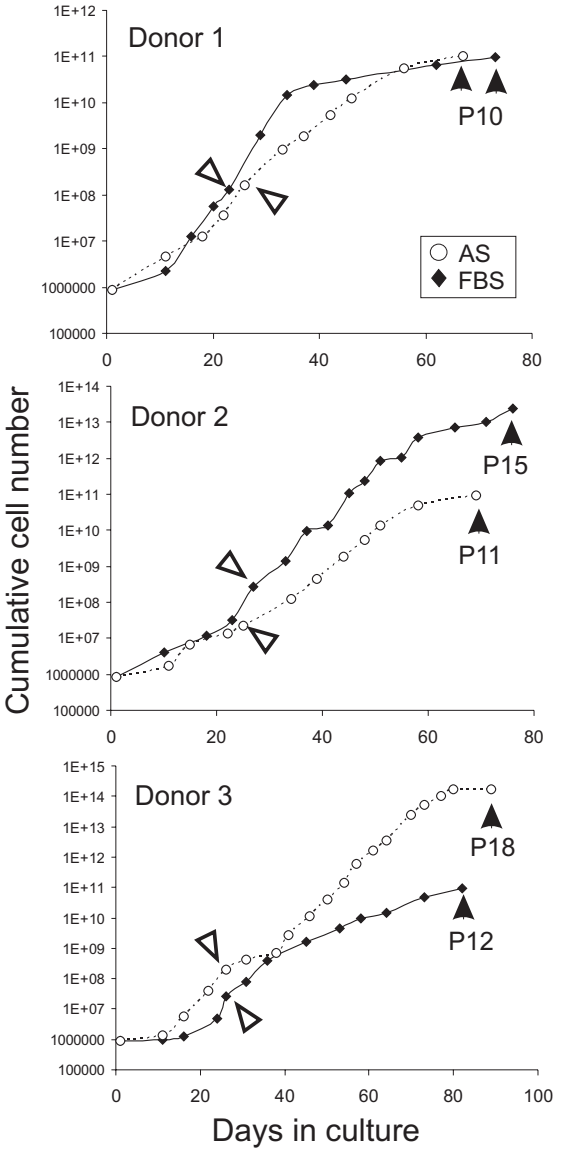

Fig. 1. Proliferation of human BMMSCs isolated from three donors and expanded in AS or FBS. *White arrows point to P4, while black arrows point to the passage number at which "late passage" was defined in this study. Cells were collected and analyzed at P4 and in late passage.*

al., 2007b). Nevertheless, some human BMMSC cultures can bypass senescence and give rise to spontaneously transformed clones (Rubio *et al.*, 2005; Rubio *et al.*, 2008b) with characteristics of poorly differentiated carcinomas (Rubio *et al.*, 2008a). Interestingly, these cells express embryonic antigens and can integrate into blastocysts without forming tumors in chimeric mice, suggesting that some de-differentiation has taken place (Rubio *et al.*, 2008a). Telomerase-immortalized BMMSCs can also display transformation and tumorigenicity (Burns *et al.*, 2005). Moreover, human neuronal stem cells derived from glioma tissue can also transform into tumorigenic cells and undergo genomic instability driven by a high number of DNA double strand breaks and a constitutively overactivated DNA damage response (Shiras *et al.*, 2007). We recently reported that one out of six long-term cultures of adipose stem cells (ASCs) display minor telomeric deletions, primarily in early passage and in a subpopulation that is subsequently and spontaneously eliminated from culture (Meza-Zepeda *et al.*, 2008). Thus, chromosomal aberrations may occur in a fraction BM- and adipose-derived MSC cultures, but their incidence appears to be negligible in long-term human MSC cultures (Bernardo *et al.*, 2007b).

Fetal bovine serum (FBS) remains to date the primary source of growth supplements and low molecular weight bioactive compounds for long-term *in vitro* expansion of MSCs (Kume *et al.*, 2006). FBS may however have undesirable effects in therapeutic applications due to risks of contamination by pathogens or transmission of xenogeneic proteins (reviewed in Mannello and Tonti, 2007). Yet, there are to date no documented significant effects of FBS in published clinical trials using human MSCs (Sotiropoulou *et al.*, 2006; Berger *et al.*, 2006; Mannello and Tonti, 2007; Le Blanc K. *et al.*, 2008). Nevertheless, alternative sources of growth supplements are being investigated. Replacement of FBS with pooled allogeneic AB serum (Kocaoemer *et al.*, 2007; Kunisaki *et al.*, 2007), thrombin-activated platelet-rich plasma (KocaoemerÄ*et al.*, 2007), human platelet lysate (Lange *et al.*, 2007; Schallmoser *et al.*, 2007), or bovine fibroblast growth factor (Battula *et al.*, 2007) supports equal or greater proliferation and/or multilineage differentiation of human BM-, adipose- or amniotic fluid-derived MSCs (Mannello and Tonti, 2007). BMMSCs expanded in medium containing autologous serum (AS) proliferate faster and differentiate less rapidly than cells cultured with FBS (Shahdadfar *et al.*, 2005). Gene expression profiling also shows that BMMSCs in AS display enhanced stability in gene expression, suggesting that they may be expanded more stably in AS than in FBS (Shahdadfar *et al.*, 2005). On the other hand, BMMSCs expanded to late passage in FBS-supplemented medium have shown no signs of genetic instability or transformation in a study involving 10 donors (Bernardo *et al.*, 2007b). There is however to date no side-by-side study on how FBS and AS affect genomic and epigenetic stability of MSCs during extended culture.

Epigenetic processes are heritable modifications of DNA and chromatin that affect gene expression without altering genomic sequence. A primary component of epigenetics is methylation of cytosines in cytosine-phosphate-guanine (CpG) dinucleotides. DNA methylation favors genomic integrity and ensures proper regulation of gene expression. Corruption of DNA methylation in long-term culture of primary cells, including ASCs (Noer *et al.*, 2006; Boquest *et al.*, 2007), are predominantly caused by stochastic CpG methylation events reflecting errors in the maintenance methylation machinery (Graff *et al.*, 2000; Bird, 2002). DNA methylation is associated with long-term gene silencing (Antequera, 2003), thus methylation of tumor suppressor genes may lead to cellular transformation (Laird, 2005). Similarly, hypermethylation of specific promoters may affect the fate of cultured MSCs.

Using array comparative genomic hybridization (CGH), we examine here to what extent human BMMSCs cultured in FBS or AS are prone to localized DNA gains and losses in a range of passages at which cells may be used clinically. We also analyzed by combined bisulfite restriction analysis (COBRA)

and bisulfite sequencing the CpG methylation pattern in the promoter of cancer-related genes in BMMSCs expanded in FBS- or AS-containing medium.

Results

Expansion of BMMSCs in AS and FBS

There was variability in the proliferative capacity of BMMSCs between donors, in either FBS or AS, and no serum supported proliferation better than the other (Fig. 1). Consistent with our previous observations (Shahdadfar *et al.*, 2005), MSCs from donor 3 proliferated faster in AS than in FBS (population doubling time in log phase of growth was 62 h vs. 93 h, respectively). However, for the other two donors population doubling time was shorter in FBS than AS (donor 1: 65 h vs. 82 h; donor 2: 58 h vs. 70 h). Late passage characteristics were marked by increased population doubling time, variation in cell size and shape, and a flattened appearance consistent with features of senescent human fibroblasts (data not shown) (Hayflick, 2003).

CGH analysis of changes in DNA copy number in BMMSCs expanded in AS and FBS

We investigated at high resolution DNA gains or losses in BMMSCs expanded in AS or FBS to early passage (P4) and late passage (P10-P18 depending on the culture; Fig. 1), by CGH using 385,000 oligonucleotide arrays. Genomic gains and losses detected on each chromosome in all cultures are summarized in Figure 2A, with whole-genome profiles‘of DNA copy numbers shown in Figure 2B (donor 1) and Supplementary Figure 1 (see: http://dx.doi.org/10.1387/ijdb.082663jd; donors 2 and 3). The cultures showed primarily telomeric deletions on a variable number of chromosomes. For donor 1, telomeric deletions on chromosomes 4, 5, 7, 8, 10-13, 21 and X were detected, along with a deletion of chromosome 22, but only in FBS and at P4 (Fig. 2A,B). The deleted regions contained variable numbers of genes (Table 1; listed in Supplementary Table 1) and on the basis of the $\log_2$ ratios (Table 1) affected only a subpopulation of cells. Notably, none of the deletions were significant in late passage cells (Fig. 2A-C), suggesting that most cells in the subpopulation harboring these deletions were eliminated from the culture beyond P4. Cells from donor 1 expanded in AS did not show significant DNA copy number alterations at any passage (Fig. 2A-C).

Cells from donor 2 remained stable in early and late passage regardless of serum origin, with only two minor telomeric deletions on 5p15.33 and 12q24.33 in FBS at P15 (Fig. 2A). Each of these deletions encompassed a small number of genes (Table 1; Supplementary Table 1). Cells expanded in AS showed a normal DNA content. In cells from donor 3, the alterations detected were independent of passage number (Fig. 2A). In FBS, telomeric deletions occurred‘at P4 on 5p15.55 and 8p23.3 and abnormalities increased at P12. In contrast in AS, telomeric deletions‘were detected on chromosomes 4, 5, 13 and 18 together with a chromosome 22 deletion at P4, all in a subpopulation of cells (Table 1). These however were no longer significant at P18 (Fig. 2A), arguing again for the elimination of the majority of the affected cells in late passage in AS.

These results indicate that BMMSC culture in either FBS or AS may cause donor-dependent occasional aberrations in

TABLE 1

ARRAY CGH ANALYSIS OF BMMSCS EXPANDED TO LATE PASSAGE IN FETAL BOVINE SERUM OR AUTOLOGOUS SERUM

Sample	NimbleGen ID	Chromosome	Position start of gain or loss (Kb)	Position end of gain or loss (Kb)	Fragment size (Kb)	$\log_2$ ratio[a]	No. genes[b]
Donor 1							
FBS, P4	1178902	4p16.3-16.1	30	9810	9780	-0.302	136
		5p15.33	90	3930	3840	-0.384	36
		7p22.3-22.2	150	3270	3120	-0.392	44
		8q24.23-24.3	139170	146421	7251	-0.324	122
		10q26.3	133350	135328	1978	-0.458	39
		11p15.5-15.4	210	3810	3600	-0.387	112
		12q24.33	129090	132264	3174	-0.403	42
		13q34	109110	114122	5012	-0.322	41
		21q22.3	41430	46923	5493	-0.428	96
		22q11.21-q13.33	14670	49570	34900	-0.277	595
		Xq28	152190	153510	1320	-0.486	55
Donor 2							
FBS, P15	1207802	5p15.33	90	3810	3720	-0.345	36
		12q24.33	129090	131910	182820	-0.338	42
Donor 3							
FBS, P4	1193602	5p15.33	90	3810	3720	-0.303	36
		8p23.3	150	2190	2040	-0.388	16
FBS, P12	1182202	2p25.3-25.2	30	4050	4020	-0.384	21
		2q14.2	120810	121710	900	-0.437	4
		3q29	194910	199323	4413	-0.254	54
		4p16.3-16.1	30	9750	9720	-0.302	136
		5p15.33	90	2850	2760	-0.441	35
		8p23.3	270	2190	1920	-0.526	13
		8q24.23-24.3	139710	146241	6531	-0.351	122
AS, P4	1183902	4p16.3-16.1	30	9950	9920	-0.325	136
		5p15.33	90	2790	2700	-0.428	33
		13q34	109590	114122	4532	-0.307	40
		18q23	74370	76096	1726	-0.393	14
		22q11.21-q13.33	14670	49570	34900	-0.277	595

[a] A positive $\log_2$ value indicates a DNA gain, a negative value indicates a DNA loss.

[b] Genes are listed in Supplementary Table 1.

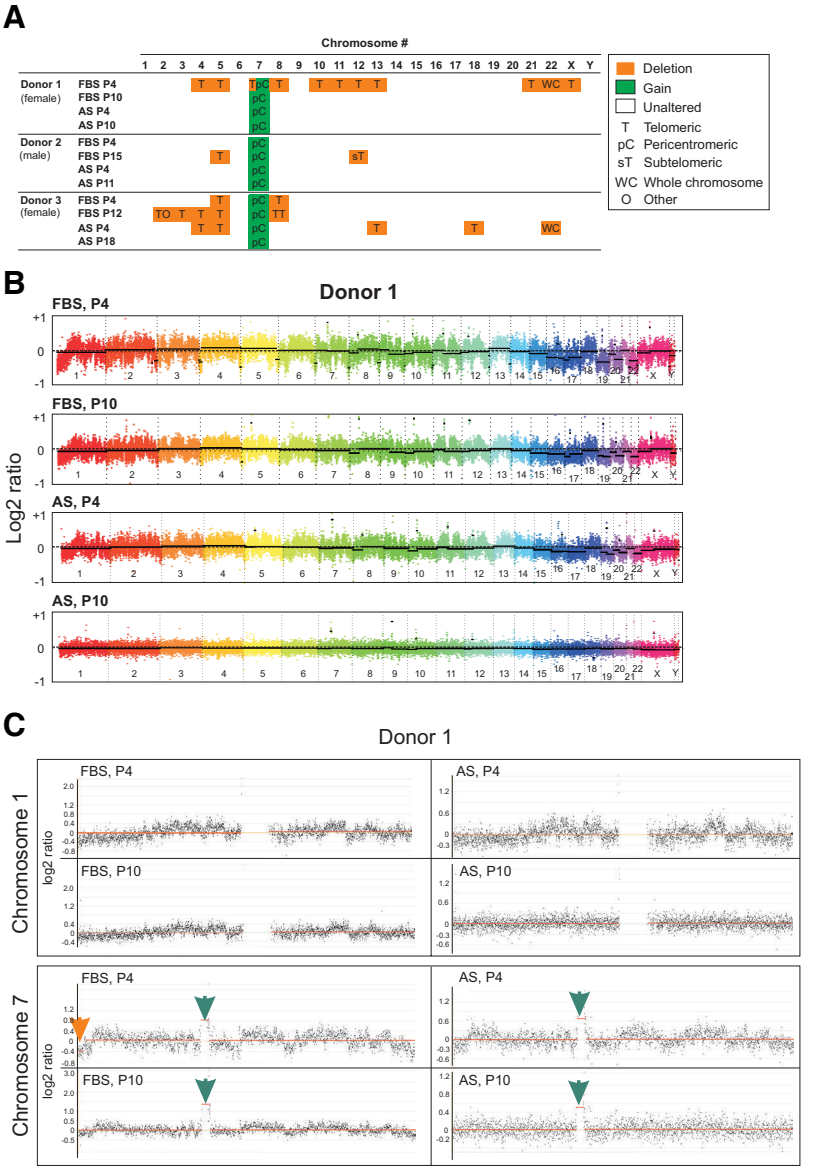

Fig. 2. Array comparative genomic hybridization (CGH) profile of bone marrow mesenchymal stem cells (BMMSCs) expanded in autologous serum (AS) or fetal bovine serum (FBS) to P4 and to late passage. (A) *CGH summary, shown as DNA gains (*green boxes*) and deletions (*orange boxes*) in indicated chromosome regions. Genomic regions were defined by nucleotide number according to the Ensembl database in Table 1, and the list of genes included in each region is given in Supplementary Table 1 (see: http://dx.doi.org/10.1387/ijdb.082663jd).* **(B)** *Whole-genome array CGH analysis of BMMSCs cultured to P4 and P10 (late passage) in FBS- and AS-supplemented medium for donor 1. DNA gains and losses are shown as* $\log_2$ *values relative to DNA from uncultured cells from the same donor (reference diploid DNA), with a window size of 300 Kb. Profiles of each chromosome (numbered) are shown. Data for donors 2 and 3 are shown in Supplementary Figure 1.* **(C)** *DNA copy number changes throughout chromosomes 1* (top panels) *and 7* (bottom panels) *in BMMSCs cultured in FBS* (left panels) *or AS* (right panels) *to P4 and P10* (donor 1). *Chromosome 1 shows no significant changes in DNA copy number. Chromosome 7 displays a telomeric deletion* (orange arrow) *in FBS at P4, while the deletion is absent at P10. All cultures show apparent pericentromeric gains on chromosome 7* (green arrows). *Normalized* $\log_2$ *ratios (*y*-axis) using a window size of 60 Kb are plotted in black and their segmentation drawn in red.*

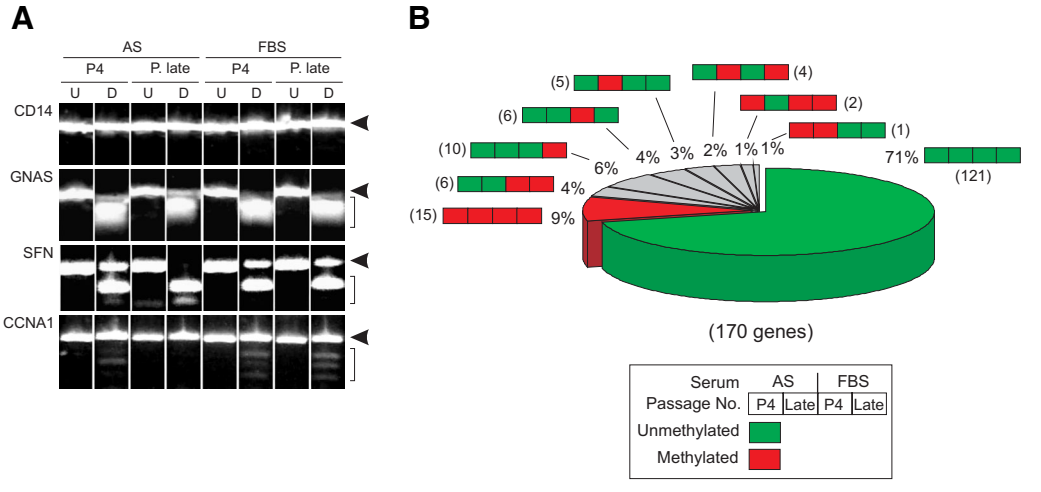

Fig. 3. COBRA analysis of DNA methylation in the promoter of 170 genes in BMMSCs expanded to P4 and late passage in AS or FBS. (A) *COBRA methylation profiles of* CD14, GNAS, SFN *and* CCNA1 *detected by agarose gel electrophoresis. Arrowheads point to uncut PCR products (umethylated DNA) while brackets delineate PCR products digested by the enzymes (methylated DNA). U, undigested sample; D, sample digested with enzymes.* **(B)** *Percentage of genes showing unmethylated (green) or methylated (red) DNA patterns under each culture condition; number of genes is indicated in parenthesis. P. late, late passage (pool of P10-P18 cells; see Fig. 1).*

gene copy number primarily in the form of telomeric deletions, in a subpopulation of cells. However, for the three donors, cells in AS generated late passage cultures without significant imbalanced chromosomes rearrangements. In addition, all cultures showed a pericentromeric DNA gain in chromosome 7 (Fig. 2A), consistently at the same location (Fig. 2C, green arrows) and in a segment containing no annotated genes. Similar pericentromeric gains were recently reported in ASCs (Meza-Zepeda *et al.*, 2008). The possibility remains at present that these alterations represent hybridization artifacts caused by their proximity to areas with satellite repeats or reflect instability of microsatellite repeats in culture.

AS shows a higher propensity than FBS to maintain DNA methylation patterns

To assess the degree of epigenetic stability of BMMSCs during long-term culture in FBS or AS, we examined the state of DNA methylation in the regulatory region of a panel of 170 cancer-related genes by COBRA. An NCBI (www.ncbi.nlm.nih.gov) and HUGO (www.genenames.org) database search revealed that these genes encompass oncogenic, tumor suppressor, cell cycle regulation, cell adhesion/migration, DNA metabolism and cell metabolism functions (Supplementary Table 2). To ensure enough material for both COBRA and bisulfite sequencing (see below), and at the same time average out putative between-donor variation in methylation patterns, BMMSC DNA from the three donors was pooled. This was motivated by our earlier observations that ASCs show the same variation in DNA methylation patterns between donors as between cultured or uncultured cells from one donor (Noer *et al.*, 2006; Boquest *et al.*, 2007).

We found that 71% of the genes examined were unmethylated at P4 and at late passage both in AS and FBS (see e.g., Fig. 3A, *CD14*), while 9% were methylated in both sera (Fig. 3A, *GNAS*, *SFN*; Fig. 3B; Supplementary Fig. 2). Thus, 80% of the genes maintained their methylation state between P4 and late passage in both sera. Thirty-four genes (20%) displayed a different methylation pattern in AS and FBS (Fig. 3A, *CCNA1*; Fig. 3B; Supplementary Fig. 2). Among these, 22 were stably unmethylated in AS while being stably methylated or changing methylation state in FBS, whereas significantly fewer genes (6; $P<0.01$; Fischer's test) were stably unmethylated in FBS while undergoing methylation or being stably methylated in AS. In addition, 4 genes underwent methylation between P4 and late passage in both sera (Fig. 3B; Supplementary Fig. 2), reflecting for these genes a serum-independent effect of culture on methylation. Therefore, ≥90% of the genes examined maintain their DNA methylation state between P4 and late passage in either serum. Among these, nearly 90% display a similar methylation state in both sera, with most genes being unmethylated. There is also a higher propensity of AS than FBS to maintain the unmethylated state in long-term culture, and overall, all functional groups were represented in genes exhibiting specific methylation states.

We used genomic bisulfite sequencing to verify the CpG methylation profiles at the single nucleotide level of 34 randomly chosen genes previously analyzed by COBRA (Supplementary Fig. 3). We found robust consistency between COBRA and sequencing results for genes that were stably unmethylated or stably methylated, as well as for genes with variable methylation patterns (Fig. 4). For a handful of genes, we detected an apparent

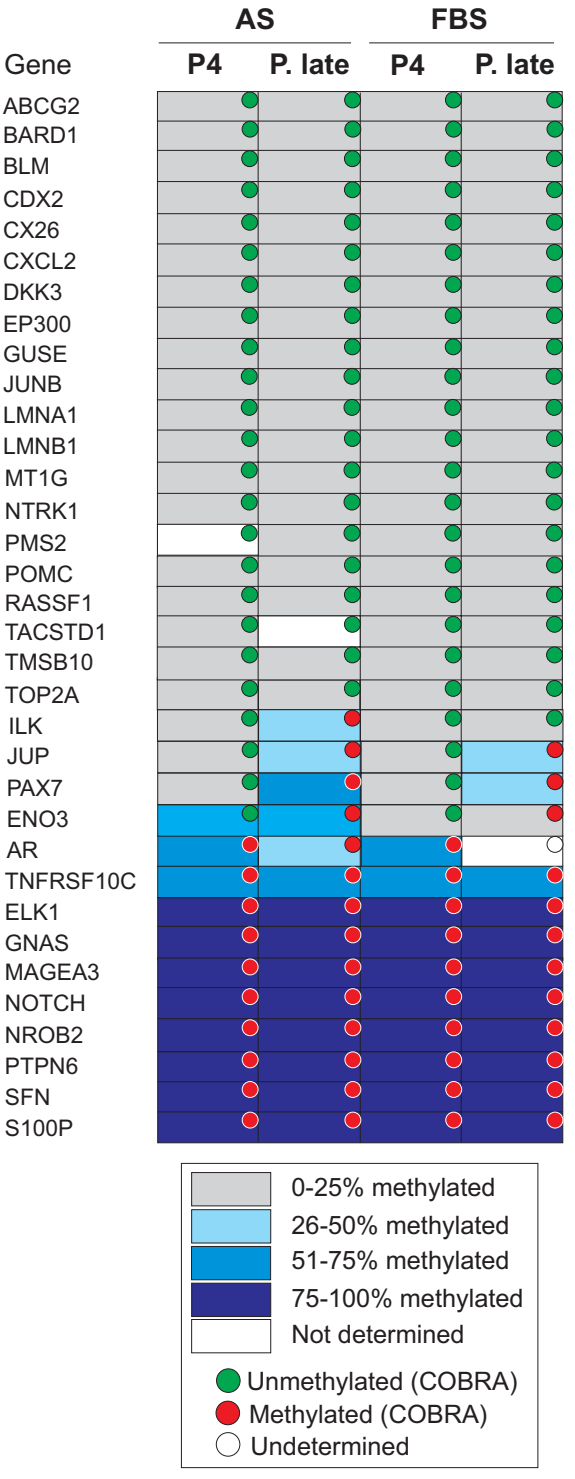

Fig. 4. Bisulfite genomic sequencing analysis of CpG methylation. *CpG methylation was determined by direct sequencing of PCR products for 34 genes out of the 170 examined by COBRA in Figure 3. Each window is color-coded to represent the average percentage of methylation calculated from bisulfite sequencing data. CpG methylation profiles for each locus are shown in Supplementary Figure 3. The red and green dots symbolize the DNA methylation state identified by COBRA. Blank boxes and dot indicate that no data were obtained. P. late, late passage (pool of P10-P18 cells; see Fig.1).*

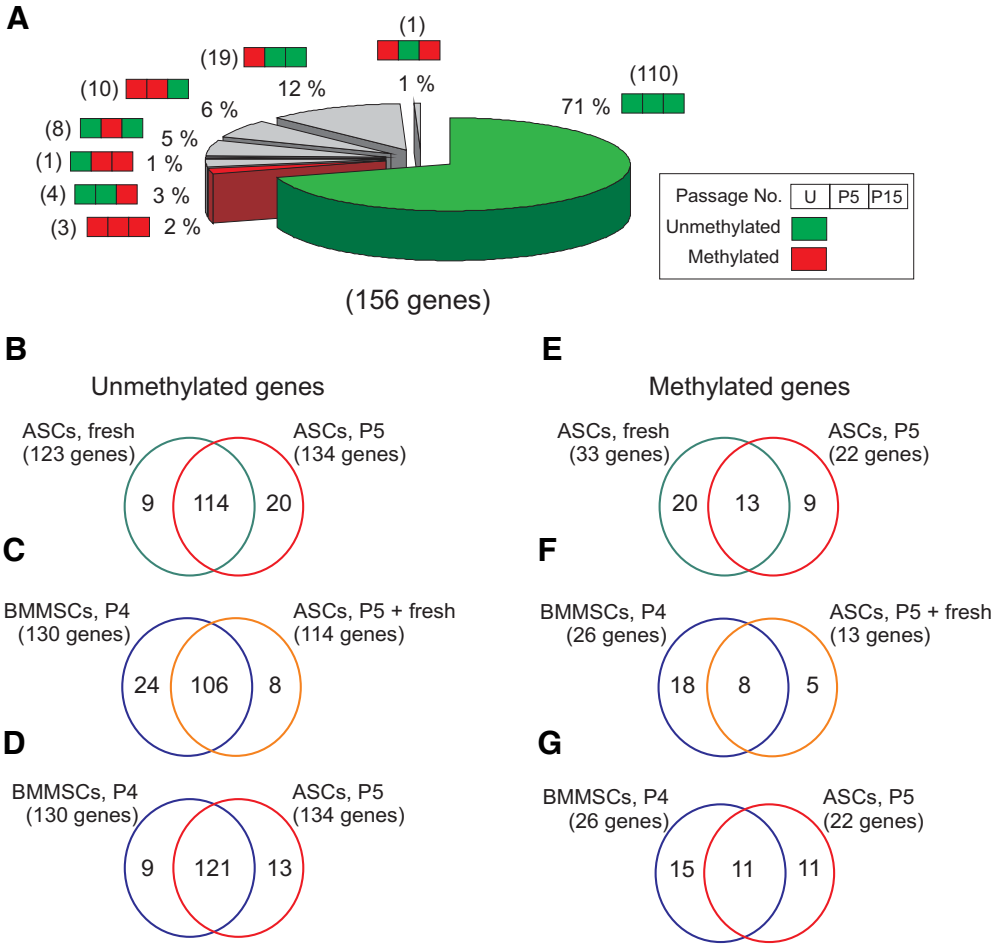

Fig. 5. Relationship between DNA methylation patterns in ASCs and BMMSCs. (A) *COBRA analysis of DNA methylation in ASCs that were uncultured (freshly isolated), cultured to P5 and cultured to P15 (late passage) in FBS-supplemented medium. Numbers of genes in each category are indicated in parentheses.* **(B-G)** *Venn diagrams of the relationship between methylation states in ASCs (freshly isolated and/or cultured to P5, as indicated) and BMMSCs cultured to P4. Venn diagrams for unmethylated genes* **(B-D)** *and methylated genes* **(E-G)** *are shown.*

discrepancy between COBRA (unmethylated pattern) and bisulfite sequencing (methylated pattern), which was due to the location of these CpGs outside the cutting sites for *BstU*I and *Taq*1 enzymes used in the COBRA assay (Supplementary Fig. 3).

Relationship between DNA methylation and DNA copy number

We next examined whether DNA methylation changes detected correlated with an alteration in DNA copy number. Only 9 (5%) genes examined by COBRA were affected by changes in copy number in one or more donors. Among these, four (*CDKN1C, CHFR, S100P, BDH1*) were affected by deletions in donors 1 (FBS, P4) and 3 (FBS, P12; Supplementary Table 1), but showed stable methylation (Supplementary Fig. 2, genes marked with an asterisk). Five genes included in the COBRA assay localized on chromosome 22, which CGH analysis revealed to be deleted in a proportion of cells in donor 1 (FBS, P4) and 3 (AS, P4) (Fig. 2A). These genes (*JUNB, LIM2, GIPC1, STK11, VAV1*) were stably unmethylated in both AS and FBS. These observations confirm that deletions detected by CGH concern a subpopulation of cells and do not affect the methylation level detected by COBRA.

The unmethylated state of DNA in freshly isolated MSCs is preserved more efficiently than the methylated state during culture

To examine how culture *per se* would affect the DNA methylation state of freshly isolated MSCs, we turned to ASCs which, after purification from the stromal vascular fraction of adipose tissue, can be analyzed in the freshly isolated, uncultured state (Boquest *et al.*, 2005). Isolation of BMMSCs, in contrast, involves—a culture step, and there is currently no published way of determining the methylation state of the putative mesenchymal population of progenitor cells in BM. In addition, although both BMMSCs and ASCs are heterogeneous in nature (Boquest *et al.*, 2005; Kucia *et al.*, 2005), ASCs have a similar immunophenotype and gene expression profile to BMMSCs (Freitas and Dalmau, 2006: Kern *et al.*, 2006), display a similar *in vitro* differentiation potential (Kern *et al.*, 2006) and similar genome-wide DNA methylation profiles on promoters (A.L. Sørensen and P.C., unpublished data). We have therefore analyzed by COBRA the methylation state of the 170 cancer-related genes examined above, in ASCs that were either freshly isolated, cultured to P5, or cultured to P15 (late passage) in FBS, under the same conditions as BMMSCs. Out of the 170 genes we obtained methylation information on 156 in ASCs (Fig. 5A; Supplementary Fig. 4). Of these, 71% were unmethylated in freshly isolated ASCs and remained unmethylated throughout culture to P15. In addition, 2% of genes methylated in uncultured ASCs remained methylated in culture while 30% of methylated genes underwent demethylation by P5 (19 genes) or P15 (10 genes). A minor proportion of genes unmethylated in uncultured cells were methylated upon culture (Fig. 5A). Thus, DNA methylation in ASCs behaves overall as in BMMSCs in FBS-supplemented culture, with most genes (72%) maintaining their methylation pattern, and the vast majority of which are unmethylated.

We next focused our analysis on methylation states in freshly isolated (uncultured) and early passage ASCs relative to that of early passage BMMSCs. Among the 156 genes examined, 134 were unmethylated at P5 in ASCs; 123 genes were also found to be unmethylated in freshly isolated, uncultured cells, of which 114

(93%) remained unmethylated at P5 (Fig. 5B). Thus in ASCs, most genes unmethylated in early passage culture are also found to be unmethylated in freshly isolated cells. Interestingly, among the 114 unmethylated genes, 106 (93%) were shared with the 130 genes unmethylated in BMMSCs at P4 (Fig. 5C). Of the 134 unmethylated ASC genes at P5, we found 121 (90%) also unmethylated at BMMSCs at P4 (Fig. 5D). This indicates that most genes unmethylated in early passage culture of BMMSCs are also unmethylated in early passage culture of ASCs, and importantly, nearly all genes unmethylated commonly in BMMSCs and ASCs in early passage culture are unmethylated in freshly isolated ASCs.

There was less consistency among methylated genes. Of the 156 genes with methylation information, 22 were methylated at P5 in ASCs. Thirty-three genes were also methylated in freshly isolated cells, of which 13 (39%) remained methylated at P5 (Fig. 5E). Among these 13 genes, 8 (61%) were shared with the 26 genes found to be methylated in BMMSCs at P4 (Fig. 5F). Of the 22 genes methylated in ASCs at P5, 11 (50%) were also found to be methylated in BMMSCs at P4 (Fig. 5G). Therefore, approximately half of the genes found to be methylated in early cultures of both ASCs and BMMSCs are also methylated in freshly isolated ASCs. These results collectively argue that the methylated state of promoters in uncultured ASCs is less likely to be maintained upon culture than the unmethylated state ($P<0.004$; Fisher's exact test).

Discussion

Genomic stability and DNA methylation patterns in long-term BMMSC culture

Despite advancements in cell culture techniques, clinical applications of MSCs require the elaboration of better suited culture media with human-derived and/or defined factors (Moore, 2006; Mannello and Tonti, 2007). We have earlier reported that AS supports BMMSC proliferation and mesodermal multilineage differentiation and maintains a more stable gene expression profile than FBS (Shahdadfar *et al.*, 2005). We now show that AS maintains long-term genomic stability of BMMSCs at least as well as FBS and tends to preserve DNA methylation states better than FBS. Thus at present, there is no cellular-based counter-indication for supplementing culture media with AS for expanding human MSCs. Alterations in DNA copy number on several chromosomes can occur during culture in both AS and FBS, however all AS cultures yielded normal gene copy numbers for all donors in late passage. Unlike in our study, no significant chromosomal aberrations were found in a CGH analysis of BMMSCs cultured to P3 and P11-15 with FBS (Bernardo *et al.*, 2007a; Bernardo *et al.*, 2007b). However, the limited scale of the changes in DNA copy numbers detected in our study (as judged by the $\log_2$ ratios of DNA copy number relative to normal diploid cells), thresholds for the identification of significantly affected regions, and differences in array platforms and analysis algorithms may account for the apparent discrepancy between these and our studies. The results nonetheless indicate that genomic instability in BMMSC cultures reflects donor variability and/or variability between bovine serum lots.

Among genes displaying changes in promoter DNA methylation in BMMSCs during culture, 90% were located in genomically stable regions, indicating that methylation changes observed are unrelated to aberrant gene copy number. Additionally, we detected more genes undergoing methylation over time (regardless of serum source), than genes undergoing demethylation. Bisulfite sequencing analysis illustrates that methylation occurs over the entire regions examined (Supplementary Fig. 3), yet a stochastic component cannot be excluded (Noer *et al.*, 2007). We have notably identified four genes showing methylation upon prolonged culture in either FBS or AS, suggesting that these may be programmedÄto be transcriptionally repressed (Jones and Takai, 2001). Two of these genes are involved in cell adhesion, genomic stability and oncogenesis. *CD248/TEM1* (endosialin) encodes an embryonic fibroblast antigen also expressed in cancers (Rettig *et al.*, 1992) and involved in cell adhesion, migration and tumor invasion (Tomkowicz *et al.*, 2007). *CD248/TEM1* methylation in long-term culture may permanently inactivate the gene, eliminating the possibility of endosialin-dependent migration of BMMSCs. Furthermore, *JUP* encodes junction plakoglobin (γ-catenin), a desmosome component implicated in cancer progression (Barker and Clevers, 2000; Chidgey and Dawson, 2007). γ-catenin overexpression also increases *MYC* expression, which leads to genomic instability (Pan *et al.*, 2007). Thus as for *CD248/TEM1*, *JUP* promoter methylation may indirectly contribute to inhibition of cell migration and maintenance of genomic stability. Methylation driven changes in cancer cell invasive properties as a function of microenvironment have been shown for the E-cadherin promoter in breast cancer cells (Graff *et al.*, 2000).

Dynamics of DNA methylation in BMMSCs expanded in AS and FBS

DNA methylation reflects the establishment of a long-term transcriptional program, thus in a context where the genes examined in our study were found to be not significantly up- or downregulated in long-term culture (Shahdadfar *et al.*, 2005), we did not expect to see dramatic methylation changes. Indeed, despite the methylation change reported for 4 genes (see above), the majority of the genes examined in this study showed unaltered methylation between P4 and late passage (P10-P18) both in AS and FBS. Thus, both sera overall preserve CpG methylation patterns, and only focused methylation changes occur. Interestingly however, among genes with a different methylation state in AS and FBS, we identified significantly more genes that are stably unmethylated in AS than in FBS. This suggests that AS has a greater propensity than FBS to maintain an unmethylated state upon long-term culture. We previously found that BMMSCs cultured in FBS differentiate more readily than in AS (Shahdadfar *et al.*, 2005), suggesting the initiation of a differentiation program in FBS. A possibility is that components in FBS, more so than AS, elicit a non-random DNA methylation drift during MSC culture, on top of seemingly stochastic alterations (Noer *et al.*, 2007), such as that suggested to take place in embryonic stem cell cultures (Maitra *et al.*, 2005; Bibikova *et al.*, 2006; Allegrucci *et al.*, 2007). These methylation changes may pre-program BMMSCs toward mesodermal differentiation by, notably, affecting expression of cell cycle inhibitors (Shahdadfar *et al.*, 2005). It is conceivable, therefore, that AS contributes to perpetuating a less differentiated state than FBS by maintaining methylation patterns.

In light of these observations, what is the DNA methylation pattern of MSCs *in vivo*? Current protocols for isolation of BMMSCs

require a culture step, thus there is no data on DNA methylation profiles in the putative MSC subpopulation in bone marrow. To get one step closer to resolving this issue, we analyzed DNA methylation in freshly isolated and in cultured ASCs, and compared methylation states with that of BMMSCs, This approach was motivated by reports that ASCs resemble (but are not identical to) BMMSCs at the morphological, transcriptional and surface marker expression levels (Boquest *et al.*, 2005; Fraser *et al.*, 2006), as well as at the genome-wide DNA methylation level (A.L. Sørensen and P.C., unpublished data). We conclude from these observations that ~80% of genes examined in ASCs retain their methylation state between isolation and culture, and most of these genes are unmethylated in the cultured state. Moreover, the vast majority of genes that are unmethylated commonly in BMMSCs and in ASCs in early passage culture are also unmethylated in freshly isolated ASCs. Thus, we tentatively speculate that most genes unmethylated in BMMSCs cultured to P4, regardless of serum origin, are also unmethylated *in vivo*.

We demonstrate here localized genomic and DNA methylation instabilities during *in vitro* expansion of human BMMSCs in a range of passages where cells may be used clinically. Although other studies report the absence of genomic alterations in BMMSCs cultured in FBS (Bernardo *et al.*, 2007a; Bernardo *et al.*, 2007b), the risk of alterations in gene copy number and spontaneous oncogenic transformation exists for human MSCs (Meza-Zepeda *et al.*, 2008; Rubio *et al.*, 2008a), irrespective of serum source (this study). Epigenetic drifting may also occur at the DNA methylation level, although this seems to affect a limited number of genes. Thus, in a clinical setting, caution should be exerted prior to transplanting MSCs by applying appropriate tests to ensure integrity of the genome and epigenome.

Materials and Methods

Autologous serum

From each BM donor, ~500 ml of whole blood was allowed to clot for 4 h at 4-8ºC and centrifuged at 1,800 g at 4°C for 15 min. Serum was collected, filtered through a 0.2 μm membrane and aliquots (AS) were stored at -20°C (Shahdadfar *et al.*, 2005).

Isolation and culture of BMMSCs

MSCs were isolated from bone marrow from three healthy donors (one male, two females) as described (Shahdadfar *et al.*, 2005). Cells were plated overnight in DMEM/F12 containing 20% AS or FBS, and antibiotics. On day 1, the medium was replaced with a fresh portion containing 20% AS or FBS. Cells were subcultured by trypsinization at 50% confluency and seeded at 5,000 cells/cm^2. After the first passage, amphotericin B was removed and 10% of either AS or FBS was used throughout the culture. Viable cells were counted at each passage and medium replaced every 2-3 days. Cells were harvested at passage 4 (P4) and at late passage (P10-P18 depending on the culture; see Fig. 1) and frozen as dry pellets or in DMSO as viable cells.

Isolation and culture of ASCs

ASCs were purified from the stromal vascular fraction of human liposuction material from three donors as described previously (Boquest *et al.*, 2005). Briefly, stromal cells were isolated by collagenase and DNase digestion, sedimentation and straining. CD45$^+$ and CD31$^+$ cells were removed from the stromal cells by double negative selection, resulting in CD45$^-$CD31$^-$ cells (ASCs) (Boquest *et al.*, 2006). ASCs were plated overnight in DMEM/F12 with 50% FBS and further cultured in DMEM/F12/10% FBS. Cells were passaged 1:3 by trypsinization. Cultures from three donors were pooled to eliminate any donor variation. Purified uncultured ASCs were directly processed for DNA isolation and COBRA.

DNA isolation and amplification

For array CGH, DNA was purified by double phenol-chloroform-isoamylalcohol extraction and one chloroform-isoamylalcohol extraction after cell lysis in 10 mM Tris-HCl, pH 8.0, 100 mM EDTA and 0.5% SDS, and digestion with 0.1 mg/ml Proteinase K overnight. DNA was diluted to 250 ng/μl in nuclease-free H_2O. DNA concentration was measured by Picogreen fluorometry (Invitrogen). DNA was amplified using the Qiagen REPLI-g Mini kit (www.qiagen.com), cleaned up using the QIAmp DNA micro kit (Qiagen) and concentration determined with Picogreen. For bisulfite conversion, DNA was purified as above.

Combined bisulfite restriction analysis

COBRA of DNA methylation relies on the existence of one or more restriction sites for an enzyme in the amplicon of interest, which after bisulfite conversion will still contain cytosine residues indicating methylation of the region prior to bisulfite treatment (COBRA however excludes assessment of CpGs outside such restriction sites). COBRA was performed (Xiong and Laird, 1997) on a panel of 170 cancer-related genes (Human Genetic Signatures; HGS; www.geneticsignatures.com). Genomic DNA was isolated from BMMSC cultures, bisulfite-converted using the MethylEasy™ *Xceed* (HGS) and fully nested PCRs were performed on the converted DNA using commercially available primers (HGS). PCR conditions were for each gene 95ºC for 3 min and 30 cycles of 95ºC 1 min, 50ºC 2 min and 72ºC 2 min, followed by 10 min at 72ºC. The same PCR product was used for COBRA and direct sequencing. Products were double digested with *BstU*I (recognition sequence CGCG [TGTG after conversion if Cs are not methylated]) and *Taq*1 (TCGA [TTGA after conversion if Cs are not methylated]) at 60ºC for 1 h. Amplicons were screened to ensure they contained at least one and in most cases multiple recognition sites for either enzyme. Undigested control PCR products were resolved next to digested products in 2% agarose gels. Products from methylated DNA templates were digested by the enzymes while those from unmethylated DNA were not.

Bisulfite genomic sequencing

PCR products generated for COBRA were directly sequenced (SUPAMAC; www.supamac.com) and sequences analyzed with the ABI Sequencing Analysis Software v5.2 using the 3-base genome option to determine relative peaks heights for adenine (reflecting a converted unmethylated cytosine) and guanine (reflecting a non-converted methylated cytosine) (Clark *et al.*, 2006). Extent of methylation for each CpG is shown as a color- and number-coded box in the region examined.

Comparative genomic hybridization

CGH was performed on BMMSC cultures as described (Meza-Zepeda *et al.*, 2008). Samples were hybridized onto NimbleGen high-density oligonucleotide microarrays containing 385,000 probes spanning non-repetitive genic and intergenic regions of the human genome at a median spacing of ~6,000 bp (HG18_WG_CGH; www.nimblegen.com). DNA from peripheral blood lymphocytes from the same BMMSC donors was used as reference diploid sample. Test and reference samples were co-hybridized onto the arrays and scanned (NimbleGen). After linking signal intensity to genome coordinates, signals were normalized using qspline normalization (Workman *et al.*, 2002). After normalization, data were prepared for segmentation using an averaging step, with probes within a defined base-pair window size averaged using a Tukey's biweight mean (Lu, 2004). Windows of 60, 120 and 300 kb were used. Data segmentation was performed using a binary segmentation algorithm (Olshen *et al.*, 2004), which breaks DNA segments into sub-segments by determining the *t* statistics of the means. We have used 1,000 permutations and a *P*-

value of 0.01 to call breakpoints. DNA copy number changes were scored as aberrant when they contained a segmentation $\log_2$ value of more than 0.25 (gains) or less than -0.25 (losses), segments contained at least 10 consecutive oligonucleotides using raw normalized data, and aberrations were seen in at least 2 segmentation windows.

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