Stem Cell Research 25 (2017) 6–17

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Chromosome copy number variation in telomerized human bone marrow stromal cells; insights for monitoring safe *ex-vivo* expansion of adult stem cells

Jorge S. Burns [a,b,*], Linda Harkness [a,c], Abdullah Aldahmash [a,d], Laurent Gautier [e], Moustapha Kassem [a,f,g,h]

[a] Department of Endocrinology and Metabolism, University Hospital of Odense, DK-5000 Odense C, Denmark.
[b] Laboratory of Cell Biology and Advanced Cancer Therapies, Department of Oncology Hematology and Respiratory Disease, University Hospital of Modena and Reggio Emilia, Modena, Italy
[c] Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Brisbane, QLD, Australia
[d] College of Medicine, Department of Anatomy, Stem Cell Unit, King Saud University, Riyadh 11461, Saudi Arabia
[e] Center for Biological Sequence Analysis/DTU Multi Assay Core, Department for Systems Biology, Technical University of Denmark, DK-2800 Lyngby, Denmark
[f] Institute of Cellular and Molecular Medicine & DanStem, Panum Institute, University of Copenhagen, Copenhagen, Denmark
[g] Department of Cellular and Molecular Medicine, The NovoNordisk Center for Stem Cell Biology (DanStem), University of Copenhagen, Copenhagen, Denmark
[h] Stem Cell Unit, Department of Anatomy, College of Medicine, King Saud University, Saudi Arabia

ARTICLE INFO

Article history:
Received 24 April 2017
Received in revised form 14 July 2017
Accepted 20 September 2017
Available online 25 September 2017

Keywords:
Karyotype
Bone marrow stromal cell
Sky
Array CGH

ABSTRACT

Adult human bone marrow stromal cells (hBMSC) cultured for cell therapy require evaluation of potency and stability for safe use. Chromosomal aberrations upsetting genomic integrity in such cells have been contrastingly described as "Limited" or "Significant". Previously reported stepwise acquisition of a spontaneous neoplastic phenotype during three-year continuous culture of telomerized cells (hBMSC-TERT20) didn't alter a diploid karyotype measured by spectral karyotype analysis (SKY). Such screening may not adequately monitor abnormal and potentially tumorigenic hBMSC in clinical scenarios. We here used array comparative genomic hybridization (aCGH) to more stringently compare non-tumorigenic parental hBMSC-TERT strains with their tumorigenic subcloned populations. Confirmation of a known chromosome 9p21 microdeletion at locus *CDKN2A/B*, showed it also impinged upon the adjacent *MTAP* gene. Compared to reference diploid human fibroblast genomic DNA, the non-tumorigenic hBMSC-TERT4 cells had a copy number variation (CNV) in at least 14 independent loci. The pre-tumorigenic hBMSC-TERT20 cell strain had further CNV including 1q44 gain enhancing *SMYD3* expression and 11q13.1 loss downregulating *MUS81* expression. Bioinformatic analysis of gene products reflecting 11p15.5 CNV gain in tumorigenic hBMSC-TERT20 cells highlighted networks implicated in tumorigenic progression involving cell cycle control and mis-match repair. We provide novel biomarkers for prospective risk assessment of expanded stem cell cultures.

1. Introduction

Successful trials showing autologous stem cell subpopulations isolated from human adult bone marrow tissue can be grown *ex vivo* and subsequently reintroduced into patients to improve regenerative repair of large bone defects (Quarto et al., 2001) have prompted need for standardized protocols to broaden the therapeutic scope (Panchalingam et al., 2015). Associated with optimal cell sourcing approaches and cell dosage requirements, the monitoring of procedural risk is of fundamental concern. Cultured cells are susceptible to stochastic acquisition of hereditable changes that may subvert function or introduce a detrimental outcome. Oncogenic changes can be monitored with very sensitive specific techniques, yet the wide diversity of potential aberrations can confound analysis and suitable biomarkers in the context of expanded "hBMSC" populations have yet to be defined. Significant oncogenic changes can range from discrete single codon mutations, gene amplification, loss of tumor suppressor gene function and epigenetic modifications. Since such changes usually reflect or evoke chromosome abnormalities, it was reasonable to propose that karyotypic analysis might suffice to provide an overview of whether the expanded stem cells are fit for therapeutic use (Saito et al., 2011), although this view has raised controversy (Ferreira et al., 2012).

As a model system exemplifying accumulated genetic instability in long-term continuously expanded cell cultures, we previously reported that telomerized human bone marrow stromal cell strains hBMSC-TERT (aka "hMSC-TERT"), ordinarily forming heterotopic bone when transplanted with osteoconductive scaffold into immune deficient mice

* Corresponding author at: Laboratory of Cell Biology and Advanced Cancer Therapies, Department of Oncology Hematology and Respiratory Disease, University Hospital of Modena and Reggio Emilia, Modena, Italy.
E-mail address: Jorge.Burns@Unimore.it (J.S. Burns).

https://doi.org/10.1016/j.scr.2017.09.006

(Simonsen et al., 2002), could eventually spontaneously evolve a neoplastic phenotype (Serakinci et al., 2004). Notably, however, the tumorigenic cells retained a normal diploid karyotype (Burns et al., 2008). To improve detection of cytogenetic changes in cultured cells, we hereby describe use of array comparative genomic hybridization (aCGH) to identify segments of the genome existing as different copy number variants (CNV). A reference human diploid fibroblast genomic DNA was compared to bone-forming hBMSC-TERT4 and hBMSC-TERT20 cell strains, versus six sub-cloned tumorigenic cell lines derived from the extended culture neoplastic hBMSC-TERT20 strain of high population doubling level (PDL) (Burns et al., 2005). Inclusion of single-cell derived clones in our comparative analysis was advantageous, since aCGH may fail to detect low-level mosaicism (≤10%) among a heterogeneous cell strain (Elliott et al., 2010). Use of a progressive cell model with comprehensively measured growth kinetics in closely matched cell strains allowed verification of CNV reproducibility. The capacity to correlate genetic aberrations with population doubling level and acquisition of a tumorigenic phenotype, helped identify specific CNV more likely to harbour potentially causative genetic changes. Furthermore, prior cytohistological data for cancer and differentiation pathway proteins in hBMSC-TERT20 populations provided a verified context for exploring the potential relevance of CNV-associated genes. A biological database search tool for interacting genes/proteins confirmed that many CNV-specific genes were highly germane for tumorigenic progression, sharing relevance with events prevalent in human cancers and sarcomas.

2. Materials and methods

2.1. Cell culture

From concern that broad casual use of the term MSC can introduce confusion (Robey, 2017; Caplan, 2017) cells called “hMSC-TERT” in our prior publications are here renamed hBMSC-TERT. The hBMSC strain were cultured from a healthy male donor, 46, XY (age 33) transduced at population doubling level (PDL) 12 with a retroviral vector overexpressing the human telomerase reverse transcriptase gene (hBMSC-TERT cells) was used to derive the hBMSC-TERT2, hBMSC-TERT4 and hBMSC-TERT20 populations. Notably, the hTERT vector wasn't tagged with an independent co-selectable marker, thus selection relied purely on hTERT function driving growth beyond the senescence observed in untransduced control primary hBMSC. As previously described (Abdallah et al., 2005), eight passages after retroviral transduction (PDL 23), cells were partitioned into two passage regimes, with split ratios of 1:2 or 1:4 to generate hBMSC-TERT2 or hBMSC-TERT4 populations. By PDL 47, hBMSC-TERT4 cells were further partitioned into a 1:20 split ratio hBMSC-TERT20 population. Cell number was calculated for each weekly passage. Prior analysis of neoplasia detected loss of the $p16^{INK4A}$/ARF locus in both hBMSC-TERT4 and hBMSC-TERT20 cells, plus *Kras* Q61H mutation specific to hBMSC-TERT4 cells and oncogene methylation of the *DBCCR1*/*DBC1*/*BRINP1* promoter specific to hBMSC-TERT20 cells (Serakinci et al., 2004). At late passage (PDL 440), hBMSC-TERT20 was used to derive single-cell sub-clones designated -BB3, -BC8, -BD6, -BD11, CE8 and DB9 as described (Burns et al., 2005). Subsequent histological analysis of hBMSC-TERT20 strains and subclones examined a panel of cancer pathway prognostic biomarkers (Burns et al., 2008). All cells were grown in phenol red-free minimal essential medium (MEM) supplemented with 10% batch-tested fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco Invitrogen) and maintained in a 5% CO2 humidified incubator at 37 °C.

2.2. Array-based Comparative Genomic Hybridization (aCGH)

Genomic DNA was prepared using the Puregene DNA Purification Kit (Qiagen). aCGH was performed following the standard Agilent protocol (V6.1). Briefly, 700 ng of hBMSC-TERT lineage cell genomic DNA and 700 ng of reference diploid human fibroblast genomic DNA 46,XX was digested with *Alu*I and *Rsa*I and labeled with Cy5- or Cy3-dUTP (Agilent). Following purification with Microcon YM-30 filters (Millipore), the labeled DNA yield and quality was checked on a NanoDrop ND- 1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer using Bioanalyzer DNA High Sensitivity (Agilent Technologies). The labeled samples were hybridized at 65 °C on Agilent dual colour arrays (Sureprint G3, 2x400k cat.#G4825A; Agilent Technologies) for 24 h in a rotator oven at 20 rpm. The annotation files corresponded to NCBI build 36.1 of the human genome hg18 (March 2006) containing 411,056 60-mer probes. The comprehensive probe coverage allowed genome-wide DNA CNV profiling focused on known genes, promoters, miRNAs, pseudoautosomal and telomeric regions. Slides were washed according to the protocol and scanned immediately at 2 μM resolution on an Agilent G2565CA high-resolution scanner. Data was extracted using Agilent Feature Extraction software and the resulting files were processed with the Bioconductor limma package version 2.18.2, with the raw data cleaned for background noise using the normexp convolution model. The resulting signal was processed using a quantiles-based normalization procedure. Figures were generated with help of R software ggplot2 implementation of the Grammar of Graphics.

2.3. RT-PCR Analysis of gene expression

Cellular RNA from hBMSC-TERT4 PDL-175 and hBMSC-TERT20 PDL-337 was isolated using a single-step method with TRIzol (Invitrogen, Taastrup, Denmark) according to manufacturer's instructions. First-strand complementary cDNA was synthesized from 4 μg of total RNA in accordance to a Revertaid H minus first-strand cDNA synthesis kit (Fermentas). Real-time polymerase chain reaction (RT-PCR) utilized the StepOne PlusTM RT-PCR system (Applied Biosystems) with double-strand DNA-specific SYBR → Green I luminescent dye. In a total reaction volume of 10 μL, 20 pmol/mL of each primer (DNA Technology A/S or Eurofins, Ebersberg, Germany) and 10 pmol/mL for each reference gene was used with Fast SYBR® Green master mix (ABI). The cycle conditions included an initial denaturation step at 95 °C for 20 s and 40 cycles of 95 °C for 3 s, and 60 °C for 30 s. Normalization was achieved via the reference gene β2-microglobulin (Lupberger et al., 2002) (primer sequences in Supplementary Table 1).

In vivo xenograft of hBMSC-TERT cells in immuno-deficient mice hBMSC-TERT4 PDL 192 cells (5×10^5) combined with hydroxyl- apatite/tricalcium phosphate 1–2 mm diameter granules (HA/β-TCP, 40 mg, Zimmer Scandinavia, Denmark) were transplanted subcutaneously into the dorsum of 8-week- old female NOD/SCID mice (NOD/LtSz-Prkdcscid) as described (Burns et al., 2010). The transplants were recovered eight weeks after transplantation, transferred to 4% neutral buffered formalin for about 45 min, and then formic acid was added for 2 days. Adopting standard histopathologic methods, the HA/β-TCP implants were embedded in paraffin and 4-μm tissue sections were stained with hematoxylin and eosin Y (H&E) (Bie & Berntsens Reagenslaboratorium). Similarly, tumorigenic hBMSC-TERT20 PDL 480 cells (5×10^5) combined with Matrigel™ were transplanted subcutaneously into the murine dorsal region and recovered two weeks after transplantation, fixed in 4% neutral buffered formalin as above and embedded in paraffin for subsequent H&E staining of 4-μm tissue sections.

2.4. Bioinformatic characterization of copy number variable genes

To explore CNV relevance to known hBMSC-TERT cell biology, we referenced prior molecular studies of tumorigenic events, including genetic abnormalities and the expression of proteins involved in pathways to cancer, the cell cycle, DNA mismatch repair and ossification. Associations between such hBMSC-TERT qualified molecules and CNVs were made using the Search Tool for Retrieval of Interacting Genes/Proteins database (STRING, version 10.0), a database comprised known and predicted interactions, including direct (physical) and indirect (functional)