Telomerase promoter reprogramming and interaction with general transcription factors in the human mesenchymal stem cell

Nedime Serakinci¹,
Stacey F Hoare²,
Moustapha Kassem³,
Stuart P Atkinson² &
W Nicol Keith^{2†}

[†]Author for correspondence

¹University of Southern
Denmark, Institute of
Medical Biology, Department
of Anatomy and
Neurobiology, DK-5000

Odense C, Denmark
²University of Glasgow,
Centre for Oncology and
Applied Pharmacology,
Cancer Research UK Beatson
Laboratories, Garscube
Estate, Switchback Road,
Bearsden,

Glasgow G61 1BD, UK
Tel.: +44 141 330 4811;
Fax: +44 141 330 4127;
E-mail: n.keith@
beatson.gla.ac.uk

³University Hospital of
Odense, Department of
Endocrinology and
Metabolism, DK-5000
Odense C, Denmark

The human adult mesenchymal stem cell (hMSC) does not express telomerase and has been shown to be the target for neoplastic transformation after transduction with hTERT. These findings lend support to the stem cell hypothesis of cancer development but by supplying hTERT, the molecular events required to upregulate hTERT expression in cancer development are missed. Therefore, the hMSC is ideal for the identification of molecular mechanisms regulating telomerase gene expression in stem cells. This study shows that the repression of hTERT expression in hMSC is chromatin based and that modifications of the chromatin environment lead to reactivation of telomerase gene expression. It is shown that repression of hTERT expression in hMSCs is due to promoter-specific histone hypoacetylation coupled with low Pol II and TFIIB trafficking. This repression is overcome by treatment with Trichostatin A (TSA), an HDAC inhibitor, concomitant with increases in promoter-specific histone acetylation and increases in Pol II and TFIIB tracking. hTR expression is also increased in TSA-treated hMSCs, concomitant with changes in Pol II and TFIIB dynamics.

Stem cells are unique in their ability to both self renew and to give rise to differentiated tissues. Mesenchymal stem cells (hMSC) are multipotential cells. Thus, hMSC have the ability both *in vivo* and *in vitro*, to differentiate into a variety of adult mesenchymal tissues, such as bone, cartilage, adipose and muscle [1,2] and therefore may be of use in the treatment of a diverse variety of clinical conditions. In addition, there are now strong links between normal stem cells and cancer stem cells [3], suggesting that stem cells are targets for neoplastic transformation. Interestingly, despite having stem cell properties, when passaged in culture the hMSC gradually reduce their proliferative rate and enter replicative senescence [1,2]. hMSCs also lack activity for the immortalizing enzyme telomerase which can counteract senescence by maintaining telomere sequence [1,2]. Further, when hTERT is ectopically expressed in the hMSC, neoplastic changes are accumulated suggesting that the adult hMSCs can be targets for neoplastic transformation [4].

The expression of telomerase is regulated in normal human cells. During development, telomerase expression is extinguished after embryonic differentiation in most cells. Notable exceptions are male germ cells, lymphocytes and some stem cell populations, which maintain their proliferative capacity in adult life, are telomerase competent and can express low levels of telomerase. By contrast,

telomerase is highly active in the vast majority of human tumors [5–7]. Thus, a key issue is how this complex pattern of expression is maintained in adult tissues and in particular the adult stem cell and how this is deregulated during neoplastic progression [8].

Telomerase activity is achieved through the expression of two genes, the *hTR* gene, which codes for the essential RNA component of telomerase, and the *hTERT* gene, which codes for the telomerase protein component that has reverse transcriptase activity. A major mechanism to differentially regulate telomerase is transcriptional control of the *hTERT* and *hTR* genes. Although much remains to be clarified, the cloning of the *hTR* and *hTERT* promoter region has enabled the identification of several positive and negative regulators of transcription [9].

However, gene expression is not only determined by the availability of combinations of transcription factors but also by chromatin context and in particular by covalent modifications of the core histones including acetylation, phosphorylation and methylation [10]. Distinct modification patterns at promoter sequences have been linked to expression and repression, and the dynamics of histone modification in transcriptional control is ideally suited to the task of organizing complex programs of gene activity necessary for many cellular processes including cell lineage commitment, cellular senescence and tumorigenesis [11–13]. Formation

Keywords: chromatin,
epigenetics, hTERT, hTR,
mesenchymal, telomerase,
stem cell

future
medicine

of the basal transcriptional machinery into transcriptional complexes on promoter sequences is also a major step in gene transcription. It is known that telomerase has a critical role in tumor progression and cancer cell survival; therefore it is crucial that the mechanisms regulating telomerase gene expression are well understood. Studies have shown that distinct patterns of histone modifications are linked to *hTR* and *hTERT* gene regulation [14] and alterations in expression levels are concomitant with dynamic changes in basal transcription machinery binding [15].

Thus, the lack of *hTERT* expression in the hMSC provides an ideal model to study telomerase gene regulation in the normal adult stem cell. This allows us to study all stages of promoter function including the key steps of transcription preinitiation complex formation, promoter clearance and elongation in the context of chromatin remodeling. The histone deacetylase inhibitor Trichostatin A (TSA) has been shown previously to cause an increase in histone acetylation at the *hTERT* promoter and cause re-expression of the *hTERT* gene [16–18]. To this end, we have studied *hTERT* and *hTR* expression, the acetylation status of the *hTR* and *hTERT* promoters and RNA Pol II and TFIIB dynamics in the hMSC by chromatin immunoprecipitation (ChIP), before and after treatment with TSA.

Material & methods

Isolation of hMSC & cell culture

Primary hMSC isolated from bone marrow aspirates by centrifugation (700 g for 15 min at 4°C) over a Ficoll–Hypaque gradient (Sigma) as previously described by Pittenger and colleagues [19]. The resulting cells were cultivated in high-glucose (4.5 g/l) Dulbecco's modified Eagles medium (DMEM, Gibco, Life Technology, USA) supplemented with 10% fetal bovine serum (Gibco, Life technology), 100 U/ml of penicillin and streptomycin (Gibco, Life technology) and 2 mM of L-glutamine. Cells were plated out with a confluence of 75% and the next day TSA was added at a final concentration of 350 µM for 17 h. This was followed by fixing in neutral buffered formalin (at a final concentration of 3.7%) for ChIP assay.

Chromatin immunoprecipitation assays

ChIP assays for histone acetylation were performed following the instructions recommended by the kit supplier (Upstate

Biotechnology, UK). Antibodies used were anti-acetylated histone H3 (AcH3), which recognizes acetylated lysines 9 and 14 and anti-acetylated histone H4 (AcH4), which recognizes acetylated lysines 5, 8, 12 and 16 (Upstate Biotechnology, Cat. No. 06-599 and 06-866). Sonication was optimized to give chromatin fragments of around 500 bp in length (8 × 10 second pulses at 5 µ with 20 s rest between each pulse on ice using an MSE Soniprep150 sonifier) and the resultant DNA from each immunoprecipitation (IP) was purified using the QIAquick PCR Purification Kit™ (Qiagen, UK). ChIP assays using anti-Pol II and anti-TFIIB (both Autogen Bioclear, UK) were undertaken as in Gomez-Ramon and colleagues [20]. In both ChIP techniques and in each individual experiment, a no-antibody control is used to detect any background, which if present is subtracted from the IPs to normalize.

Quantitative polymerase chain reaction

Products from the ChIP assay were quantified by quantitative polymerase chain reaction (Q-PCR) Opticon2 DNA Engine (MJ Research, Massachusetts) using primers to *hTR* and *hTERT* core promoter sequences and exon 12 of *hTERT* using SYBR Green Q-PCR Buffer (Finnzymes, Espoo, Finland) [14,15]. *hTR* Primers, 29SF 5'CCC GCC CGA GAG AGT GAC 3' and 5ALTR 5' AAG TCA GCG AGA AAA ACA GC 3', *hTERT* primers, TERTSF 5' TCC CCT TCA CGT CCG GCA TT 3' and TERTSR 5' AGC GGA GAG AGG TCG AAT CG 3' and Exon 12 primers, Ex12Fa 5'CAG GAC AAG GAA GCG GGA GGA 3' Ex 12Rab 5'CAG CCG CAA GAC CCC AAA GA 3' were used.

Expression analysis

hTR expression was analyzed by Q-PCR using the following primers (TRC3F – 5' CTAAC-CCTAACTGAGAAGGGCGTA 3' and TRC3R – 5' GGCGAACGGGCCAGCAGCTGACAT T 3' and adjusted to GAPDH expression (primers – GAPDH0.45F – 5' ACCACAGTCCATGCCATCAC 3' and GAPDH0.45R – 5' CCA CCA CCC TGT TGC TGT A 3') [14,15]. *hTERT* expression was analyzed by methods outlined in Keith and colleagues [21] using primers which detect all splice variants (primers HT2026F 5' GCCTGAGCTGTACTTTGTCAA 3' AND HT2482R 5' GCCAAACAGCTTGTTC-CATGTC 3'). *hTERT* expression is displayed as total amounts of all four main splice variants.

Results

hTERT gene expression in hMSC & chromatin remodeling at the hTERT promoter

hTERT expression was studied before and after treatment with TSA and levels of histone acetylation at the promoter were also studied by ChIP assays in order to link changes in the chromatin environment with any changes in gene expression. In control (untreated) hMSCs, there was a lack of expression of any *hTERT* transcripts (Figure 1A). Acetylation of histone H3 was not detected and acetylation of histone H4 was low (Figure 1B), linking the lack of expression of *hTERT* with low levels of promoter proximal histone acetylation. Upon treatment with TSA, hMSCs showed re-expression of full-length *hTERT* transcripts (Figure 1A) and this was associated with increased levels of AcH3 and AcH4, (Figure 1B) linking remodeling of the chromatin environment of the *hTERT* promoter with re-expression of *hTERT*.

Basal transcription machinery dynamics at the hTERT gene in hMSCs.

As well as monitoring acetylation, Pol II and TFIIB occupancy at the *hTERT* promoter and exon 12 of *hTERT* were also studied, to understand the trafficking of the basal transcription machinery along the *hTERT* gene. Exon 12 is located some 31 kb downstream from the promoter and therefore can give excellent data on the extent of basal transcription machinery tracking along the gene-coding sequence. Pol II levels were low at the *hTERT* promoter before treatment with TSA and decreased slightly after treatment (Figure 1C) perhaps signifying that any Pol II that was present at the promoter but was not engaged in transcription. Levels of Pol II at exon 12 were increased after treatment with TSA (Figure 1C), indicating that increasing amounts of Pol II was involved in the process of transcription of the *hTERT* gene.

TFIIB levels in hMSCs were increased at the promoter upon TSA treatment, perhaps due to the relaxing of the chromatin environment allowing the stabilization of the preinitiation complex and increased transcription of *hTERT* by Pol II. Low levels of TFIIB at exon 12 before and after TSA treatment suggests that the role of TFIIB is primarily at the promoter in stabilizing the preinitiation complex (Figure 1D).

hTR gene expression in hMSC & chromatin remodeling at the hTR promoter

hTR gene expression and promoter chromatin environment was also studied in hMSC before

and after treatment with TSA. Figure 2A shows that hMSCs express *hTR* and levels increase upon treatment with TSA. However, ChIP analysis did not detect any change in acetylation of histones H3 and H4 before and after treatment with TSA (Figure 2B).

Basal transcription machinery dynamics at the hTR gene in hMSCs

Although there did not seem to be an increase in histone acetylation, hMSCs treated with TSA showed an increase in Pol II and TFIIB levels at the promoter sequence (Figure 2C). An increase in Pol II and TFIIB at the promoter could indicate an increase in stability of the transcriptional complex and therefore an increase in transcription levels of *hTR* that would account for the increase in expression. As the *hTR* gene is relatively short, extended analysis of Pol II and TFIIB trafficking was not possible.

Discussion

A common set of features distinguish cancer cells from their normal counterparts including loss of cell cycle regulation, loss of control over invasion and metastasis, failure of apoptotic mechanisms and bypass of senescence [7,22]. Cellular models designed to recapitulate these features of multi-stage neoplastic progression have recently been developed by a number of groups and epitomized by the work from the Weinberg lab [21]. A common element of these models is to use ectopic expression of the *hTERT* gene to overcome senescence and thus provide the immortalization step of cancer progression. However, whilst this model for neoplastic transformation may be of considerable use in defining a number of the key events in progression, by supplying telomerase, the molecular events required to upregulate *hTERT* in cancer development are inevitably missed. Given the critical role that telomerase has in tumor progression and with the cancer cell reliant on telomerase for its survival, it is essential that the mechanisms regulating telomerase gene expression are investigated [8]. Our findings give essential information about the regulation of endogenous telomerase gene expression in normal hMSCs. Understanding telomerase gene regulation in the hMSC, and similar cell types, is of importance. Any differences in regulation may reflect distinct regulatory pathways for telomerase gene expression in these cell types, which need not be the same as in normal and cancer cell lines, which has been published previously [14,15].

Figure 1. hTERT expression, chromatin environment and basal transcriptional machinery dynamics.

(A) hTERT splice variant expression analysis of hMSC before and after treatment with TSA, corrected to GAPDH expression and visualized using the Agilent Bioanalyser (Agilent Technologies). Lane 1 shows size markers, Lane 2, the negative control for the PCR reaction, Lane 3, hTERT expression for the hMSC, Lane 4, hTERT expression for the hMSC treated with TSA, and Lane 5, hTERT expression in the MCF-7 cell line as comparison. ChIP assays were performed for (B) histone acetylation (histones H3 and H4) status of the hTERT promoter (C) Pol II and (D) TFIIB occupancy of the promoter and exon 12 before and after treatment of hMSCs with TSA. Each IP is expressed as a percentage of an input sample to normalize and detected by Q-PCR in triplicate to generate error bars. No antibody controls were analyzed within each experiment and Q-PCR allowed the subtraction of any background from each IP.

hMSC: Human mesenchymal stem cell; IP: Immunoprecipitation; Q-PCR: Quantitative polymerase chain reaction; TSA: Trichostatin A.

The data presented shows that *hTERT* expression is repressed in the hMSC and that the mode of repression is chromatin based. Upon TSA treatment of hMSCs, *hTERT* becomes re-expressed concomitant with an increase in promoter histone acetylation. Beside *hTERT* expression, *hTR* expression is also increased upon TSA treatment although large changes in histone acetylation are not apparent. This suggests that chromatin and epigenetic mechanisms are involved in telomerase gene regulation in the hMSC. The chromatin-based repression of *hTERT* transcription and hence repression of telomerase activity may act as a tumor suppressor mechanism, whereby hMSCs would have a limited lifespan and can-

not divide long enough to accumulate neoplastic changes and become tumorigenic. Basal transcription machinery dynamics were also changed upon TSA treatment. Association of Pol II with gene-coding regions has been previously shown to coincide with active gene transcription [23,24], and this agrees well with the data for *hTERT* for which we see an increased level of Pol II at exon 12 after treatment with TSA. Also apparent is a significant level of Pol II at the promoter sequence before treatment with TSA when *hTERT* is repressed. This may be due to a paused Pol II molecule being present at the promoter, perhaps indicating that the *hTERT* gene may be permissive for transcription, only needing changes in the

Figure 2. hTR expression, chromatin environment and basal transcriptional machinery dynamics.

(A) hTR expression analysis for hMSCs before and after treatment with TSA, detected by Q-PCR and corrected to GAPDH expression. ChIP assays were performed for (B) histone acetylation status (histones H3 and H4) of the hTR promoter and (C) Pol II and TFIIB occupancy of the hTR promoter. Q-PCR: Quantitative polymerase chain reaction; TSA: Trichostatin A.

chromatin environment of the promoter to allow transcription (Figure 3). The apparent decrease in Pol II binding at the promoter and the large increase at exon 12 can be accounted for by increased Pol II tracking along the gene, being no longer paused at the promoter.

Apparent differences in Pol II dynamics between *hTR* and *hTERT* may reflect the different regulation of each of these genes. Changes in the chromatin environment of promoter sequences may therefore drive changes in the dynamics of the basal transcription machinery and complex interactions between transcriptional complexes and chromatin may therefore be important in mediating transcriptional activity.

While TSA treatment does lead to an increase in *hTERT* and *hTR* expression when compared with the telomerase-positive MCF7 breast carcinoma cell line (Figure 1A) *hTERT* expression in the hMSC treated with TSA is very low. It has also been observed that these cells display no detectable telomerase activity as measured by telomerase repeat amplification protocol (TRAP) [DATA NOT INCLUDED]. This observation indicates that while modulation of the chromatin environment by TSA is important in the control of *hTERT* and *hTR* expression, this action alone is not enough to increase levels of *hTERT* and *hTR* expression to a level high enough to reconstitute telomerase activity. This may be due to the lack of other factors that can influence gene expression.

It is known that hyperacetylation of promoter-specific histones at promoter sequences will cause relaxation of chromatin environment relative to a compacted state when histones are hypoacetylated. This relaxation of the chromatin environment will allow the binding of transcription factors, basal transcription machinery and other chromatin modifying enzymes to the promoter DNA. However, if such factors are not freely available within the cell, the relaxation of the chromatin environment alone will not be sufficient to mediate high levels of transcription. Analysis of the binding of regulatory factors influencing *hTERT* and *hTR* expression in the hMSC will therefore be an important next step. Further analysis of histone modifications involved in telomerase gene repression in the hMSC may allow the identification of enzymes and co-regulators involved, which can then be targeted to allow a more specific method of modulating telomerase gene expression other than TSA.

Although TSA treatment has been shown to cause chromatin changes at the *hTERT* and *hTR* promoter, it is not known if this is a direct action. However, the use of TSA does allow us to see that changes in the chromatin environment, be they direct or indirect, can lead to changes in telomerase gene expression. These data provide

an impetus for further, more detailed, analysis of the interplay between the chromatin environment, general transcription machinery and other

important regulatory factors which may affect telomerase gene expression.

Overall, this study shows that the lack of *hTERT* expression in hMSCs is due to a repressive chromatin environment surrounding the *hTERT* gene promoter and that modulation of the chromatin environment has an important role in the re-expression of *hTERT* in these cells. This provides an excellent platform for the further investigation of the control of telomerase gene expression in the hMSC and its implications for tissue regeneration and cancer stem cells.

Acknowledgements

This work was supported by Cancer Research UK, European Community grants FIGH-CT-2002-00217, LSHC-CT-2004-502943, Glasgow University and Danish Cancer Society.

Executive summary
<ul style="list-style-type: none">• Human mesenchymal stem cells (hMSC) have limited replicative capacity and lack expression of the immortalizing enzyme telomerase.• Lack of telomerase may act as a tumour suppressor mechanism as ectopic expression of telomerase in hMSC results in neoplastic transformation and reveals a cancer stem cell potential.• Lack of telomerase gene expression is due to the repressive chromatin environment surrounding the telomerase gene promoters.• Activation of telomerase gene expression is accompanied by chromatin remodelling and dynamic shifts in the basal transcriptional complex.• hMSC represent an excellent model for studying telomerase gene expression with direct implications for cell replacement therapies and tissue engineering.

Bibliography

1. Zimmermann S, Voss M, Kaiser S *et al.*: Lack of telomerase activity in human mesenchymal stem cells. *Leukemia* 17, 1146–1149 (2003).
2. Simonsen JL, Rosada C, Serakinci N *et al.*: Telomerase expression extends the proliferative lifespan and maintains the osteogenic potential of human bone marrow stromal cells. *Nat. Biotechnol.* 20, 592–596 (2002).
3. Dick JE: Breast cancer stem cells revealed. *Proc. Natl Acad. Sci. USA* 100, 3547–3549 (2003).
4. Serakinci N, Guldberg P, Burns JS *et al.*: Adult human mesenchymal stem cell as a target for neoplastic transformation. *Oncogene* 23, 5095–5098 (2004).
5. Shay JW, Bacchetti S: A survey of telomerase activity in human cancer. *Eur. J. Cancer* 33, 787–791 (1997).
6. Kim NW, Piatyszek MA, Prowse KR *et al.*: Specific association of human telomerase activity with immortal cells and cancer. *Science* 266, 2011–2015 (1994).
7. Holt SE, Shay JW: Role of telomerase in cellular proliferation and cancer. *J. Cell. Physiol.* 180, 10–18 (1999).
8. Keith WN, Bilsland A, Evans TRJ, Rosalind M: Telomerase-directed molecular therapeutics. *Expert Rev. Mol. Med.* www.expertreviews.org/02004507h.htm (2002).
9. Horikawa I, Barrett JC: Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanisms. *Carcinogenesis* 24, 1167–1176 (2003).
10. Strahl BD, Allis CD: The language of covalent histone modifications. *Nature* 403, 41–45 (2000).
11. Narita M, Nunez S, Heard E *et al.*: Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113, 703–716 (2003).
12. Bachman KE, Park BH, Rhee I *et al.*: Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell* 3, 89–95 (2003).
13. Fisher AG: Cellular identity and lineage choice. *Nat. Rev. Immunol.* 2, 977–982 (2002).
14. Atkinson SP, Hoare SF, Glasspool RM *et al.*: Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the hTR and hTERT gene promoters. *Cancer Res.* 65, 7585–7590 (2005).
15. Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE, Keith WN: Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. *Oncogene* doi:10.1038/sj.onc.1209011. Epub ahead of print (2005).
16. Cong YS, Bacchetti S: Histone deacetylation is involved in the transcriptional repression of *hTERT* in normal human cells. *J. Biol. Chem.* 275, 35665–35668 (2000).
17. Hou M, Wang X, Popov N *et al.*: The histone deacetylase inhibitor trichostatin A derepresses the telomerase reverse transcriptase (*hTERT*) gene in human cells. *Exp. Cell Res.* 274, 25–34 (2002).
18. Takakura M, Kyo S, Sowa Y *et al.*: Telomerase activation by histone deacetylase inhibitor in normal cells. *Nucleic Acids Res.* 29, 3006–3011 (2001).
19. Pittenger MF, Mackay AM, Beck SC *et al.*: Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147 (1999).
20. Gomez-Roman N, Grandori C, Eisenman RN *et al.*: Direct activation of RNA polymerase III transcription by c-Myc. *Nature* 421, 290–294 (2003).
21. Keith WN, Hoare SF: Detection of telomerase *hTERT* gene expression and its splice variants by RT-PCR. *Methods Mol. Med.* 97, 297–309 (2004).
22. Hahn WC, Weinberg RA: Modelling the molecular circuitry of cancer. *Nat. Rev. Cancer* 2, 331–341 (2002).
23. Sandoval J, Rodriguez JL, Tur G *et al.*: RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic Acids Res.* 32, e88 (2004).
24. Zhang L, Johnson M, Le KH *et al.*: Interrogating androgen receptor function in recurrent prostate cancer. *Cancer Res.* 63, 4552–4560 (2003).