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Immediate and gradual gene expression changes in telomerase over-expressing fibroblasts

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ABSTRACT

Most human somatic cells contain no or very low levels of telomerase. The over-expression of the catalytic subunit (hTERT) of human telomerase is a common method to generate cells with a greatly prolonged lifespan. These cells serve as models for cells that are either difficult to cultivate or have a limited lifespan *in vitro*. In addition, hTERT over-expressing cells are thought to be a useful resource for tissue engineering and regenerative medicine.

While tumour suppressors and cell cycle checkpoints are maintained for an extended period in most hTERT over-expressing cells we found that there is a gradual change in gene expression over a range of 130 population doublings (PD) for the majority of genes analysed. Seven genes were significantly down-regulated with increasing population doublings (PDs), while only two were up-regulated. One gene, stanniocalcin 2, was highly expressed in parental fibroblasts but completely diminished as a consequence of hTERT transgene expression.

These data demonstrate that in hTERT over-expressing cells two different types of expression changes occur: one can be directly associated with hTERT transgene expression itself, while others might occur more gradual and with varying kinetics. These changes should be taken into account when these cells are used as functional models or for regenerative purposes.

1. Introduction

hTERT over-expression greatly extends the lifespan of various human cell types such as fibroblasts, skin keratinocytes, vascular endothelial, retinal, urothelial and breast epithelial cells. The main cause for the extended proliferation capacity is telomere maintenance thereby overcoming cellular senescence and the Hayflick limit [1]. These cells are being widely used as models for the functional characterisation of the different primary cell types that they are derived from, since these cells are thought to retain many properties of the original cell type such as physiological function and differentiation capacity [2–5].

Telomerase over-expressing cells are also regarded as a source for cell transplantation and to create bioengineered tissues for regenerative medicine in order to treat various diseases as well as age-related conditions [6,7].

At the same time it has been shown that telomerase expression can change the gene expression pattern of cell cycle regulated genes, signalling pathways and confer resistance to apoptosis. Although it seems that the most important cell cycle and tumour suppressors are unperturbed during the initial 50 population doublings after hTERT over-expression [8,9] changes have been found in expression of tumour suppressors such as p53 and p16 at later time points [10,11].

The aim of the study was to analyse whether changes in gene expression between parental MRC-5 fibroblasts and their hTERT over-expressing derivatives occur as an immediate effect of hTERT over-expression or rather continuously with time of culture. Here we analysed gene expression changes of 11 genes in non-clonally derived progeny from a retroviral mass transfection of hTERT into MRC-5 fibroblasts over a range of 130 population doublings [12]. These genes had been identified previously as being differentially regulated in young, senescent and telomerase over-expressing fibroblasts and might be part of a retrograde response signature in these cells [12,13].

We found that expression of most genes changed gradually with increasing population doublings while the disappearance of STC2 expression occurred immediately after transfection and seemed to be directly correlated to the presence of the hTERT transgene.

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2. Materials and methods

2.1. Cells and culture

MRC-5 cells were purchased from ECACC (London). Senescent cells were generated by serial passaging of MRC-5 fibroblasts reaching senescence at around 50 PD±5. hTERT-over-expressing MRC-5 was generated using retroviral transfection using pLCphTERT (Clontech) into MRC-5 at PD 30 [12]. After initial selection, the transfected mass culture was aliquoted at a PD < 41 and MRC-5/hTERT cells were independently grown to the final PDs (between 41 and 173). In all cultures, main tumour suppressors, p53 and p16, remained stress-inducible until final PD (data not shown).

All cells were cultured in DMEM (PAA) with 10% FCS (Sigma), 1% penicillin/streptomycin (GIBCO) and 2 mM L-glutamine (PAA), and grown in 5% CO_2 atmosphere.

2.2. RNA isolation and PCR

Total RNA was isolated using the RNAeasy kit from Qiagen. Purity and quality of the RNA was monitored using Nanodrop at 260 and 280 nm. All RNAs had at least a ratio of 1.6 for 260/280 nm. RNA was then denatured and reverse transcribed using random primers (Promega) and Superscript III™ (Invitrogen). PCR was performed using 50 ng cDNA in a Hybaid thermocycler at 94 °C for 4 min, 94 °C for 30 s, annealing temperature in Table 1 for 30 s, elongation 72 °C for 30 s using primers and cycle numbers specified in Table 1 using primers and conditions listed in Table 1. Equal RNA loading was ensured by optimising cDNA input for GAPDH at unsaturated cycle number. Products were visualised by separation on 1.5% TBE agarose gels and ethidium bromide staining. Each PCR was repeated at least once. Images were generated using a gel doc (BioRad).

2.3. Quantification

For semi-quantitative analysis of PCR products we used AIDA image software (Raytest) for 2D densitometry using quantum level modus on the gel images. Intensity of the amplification product band was measured as percentage of all signals on the image.

Regression analysis was performed using Sigma Plot software 7.0 (SYSDAT, USA) for the genes of interest.

2.4. Microarray analysis

Affymetrix HGU133plus2 arrays were normalised and analysed in GeneSpring GX11 (Agilent). Using the MAS5 algorithm a list of probe sets which had a present or marginal flag in 66% of samples was generated. Affymetrix control probe sets were removed, and this list was used for downstream analysis. Normalised intensity values for the remaining probesets were derived using GCRMA (algorithm for summarisation and normalisation of probe sets from affymetrix arrays). Comparisons of differentially expressed probe sets between experimental groups were made using 2-fold changes as cut-offs. Early and late passage hTERT over-expressing cells were independently compared to young MRC-5 for that purpose. Three early passage hTERT over-expressing cells (PD 43, 58 and 62) were used along with three late passage cells (PD 113, 130 and132). Six young MRC-5 fibroblasts were used at PDs between 21 and 36.

3. Results

The aim of our study was to characterise the long term effects of hTERT over-expression on gene expression in fibroblasts over a large range of population doublings. Comparing parental MRC-5 fibroblasts with hTERT over-expressing MRC-5 at high passage (PD 125 ± 10), we recently found significant changes in the expression of various genes related to mitochondrial (dys-)function and retrograde response [12]. To determine whether these changes were due to hTERT over-expression itself or rather gradual changes due to prolonged time in culture, we analysed expression levels for a number of these genes over a wide range of population doublings. In addition to genes identified as potential retrograde response signature genes before [12,13] we included in our analysis PPARGC1 (involved in mitochondrial biogeneses), DNER (Delta and Notch-like epidermal growth factor-related receptor) and STC2. The latter two genes were found to be prominently changed in a previous microarray analysis [12]. GAPDH was used as a housekeeping gene. Its variation over all the 11 analysed samples was less than 8%.

To avoid random drift effects, we used frozen aliquots of independent subcultures (generated over a time period of 4 years) for each of the indicated population doublings that were analysed. In addition, we included young parental (PD 29) as well as replicatively senescent MRC-5 fibroblasts (PD 49). Analysis of the transgene expression confirmed that hTERT was only expressed in hTERT over-expressing MRC-5 and that there was no change in the expression levels of hTERT gene expression over time (Fig 1B). hTERT protein content did also not change with time (data not shown) and telomeres remained at a stable length of around 10 kb [12].

Representative analyses of gene expression of 11 selected genes in young, senescent and nine different passages of hTERT over-expressing cells are shown in Fig. 1A and quantitative evaluations of all experiments are given in Fig. 1B. Fig. 2 shows a linear regres-

Table 1
Primer sequences and PCR conditions.

Primer	AT[a]	Cy[b]	Forward	Reverse
BAMBI	50	27	ACA GCT TGA AGG CCT TTT GA	TGC AAA TAA CCC TGG TGA CA
DNER	55	30	TTG GGA AGT GGT CAG AGG TC	GTT AAG CGC AAA GCC ACA AT
DSG2	50	30	GTG GAT CGA ATC CTC TGG AA	GTG GTG TTC CTA GCC GTC AT
GAPDH	55	19	CTC AGA CAC CAT GGG GAA GGT GA	ATG ATC TTG AGG CTG TTG TCA TA
hTERT	55	30	GCC TGA GCT GTA CTT TGT CAA	CGC AAA CAG CTT GTT CTC CAT GTC
IGFBP3	55	30	TTG CAC AAA AGA CTG CCA AG	CAA CAT GTG GTG AGC ATT CC
MAOA	50	27	CAC ACC TTT TGG GAA ATG CT	TGG ACT GGC ATT CAT TTT GA
PPARGC1	55	30	CCT GCA TGA GTG TGT GCT CT	AAG AGG CTG GTC TTC ACC AA
PTGDS2	55	27	AAC CAG TGT GAG ACC CGA AC	TGG TAT CCT CTG TGA AGC CC
STC2	55	25	GGA TTC CAG GAC AGG AGT GA	CCC AGC CCA GAC AGT ACA AT
SULF1	55	30	AAG GTT AAT CAG CCC CGT CT	ACC AAC CCG TCA CTT TG

[a] AT: annealing temperature.
[b] Cy: cycle number.

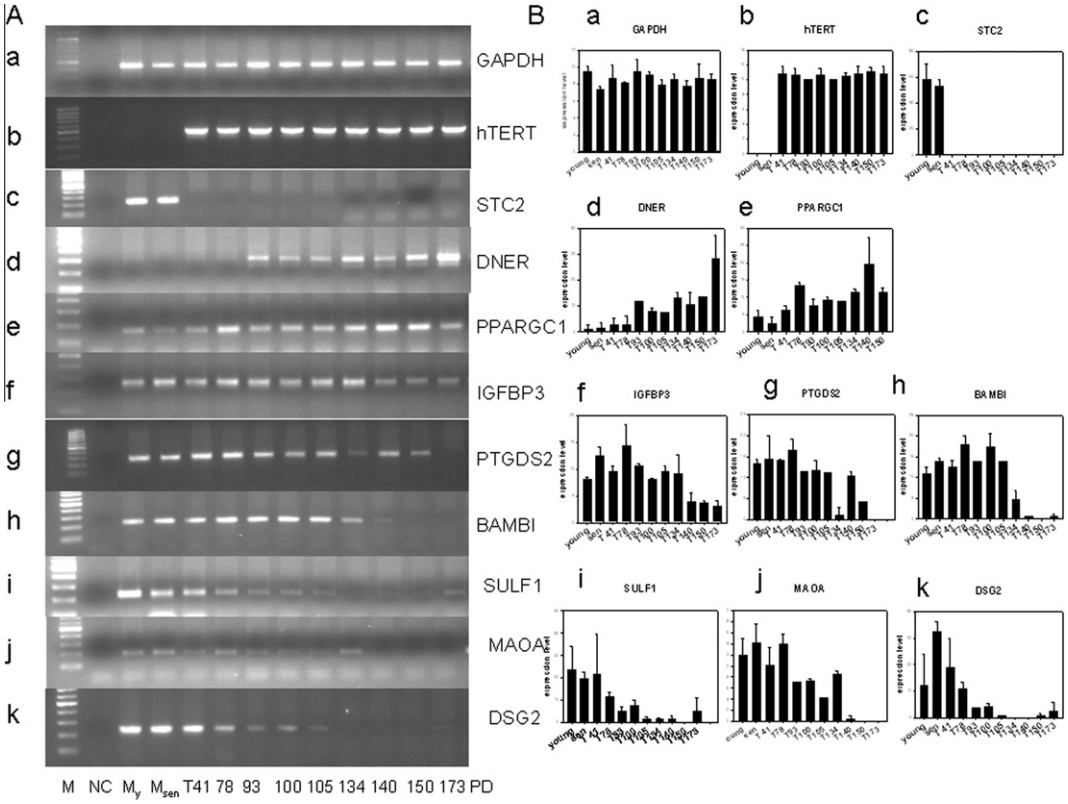

Fig. 1. Gene expression analysis in young (M_y), senescent (M_{sen}) MRC-5 fibroblasts and 130 PDL of TERT over-expressing, non-clonally derived cells. (A) Representative gel images after RT-PCR for all genes analysed on young, senescent and hTERT over-expressing MRC-5 fibroblasts at the indicated PDs. (B) Quantification of expression changes from two to three independent experiments. Bars represent mean and SD.

sion analysis of the data in hTERT over-expressing cells. Among all examined genes, stanniocalcin 2 was the only one that changed expression immediately in response to over-expression of hTERT. All young and senescent MRC-5 fibroblasts analysed to date expressed constantly high levels of *STC2*, while all TERT over-expressing MRC-5 cells independent of their PD lost its expression completely (Fig 1C).

None of the remaining analysed genes showed a different expression early after transfection, but all altered expression levels gradually with increasing PD. While *MAOA, DSG2, SULF1 BAMBI, IGFBP3 and PTGDS* were all gradually down-regulated (Figs 1F–K and 2C–H) *DNER* was significantly up-regulated with time in culture (Figs. 1D and 2D), while changes in *PPARGC1* expression were not significant (Fig. 1E and 2B).

Expression levels changed with different kinetics for the different genes (Fig 2). *DNER* expression increased steadily from early to late PDs. For five out of six down-regulated genes expression ceased before the end of the observation period. *DSG2* and *SULF1* showed the earliest down regulation. Both genes were expressed only at a very low level by PD 105. *BAMBI* and *MAOA* still showed significant expression at PD 134 but remained at background levels from PD 140 onwards. Finally, *PTGDS2* and *IGFBP3* showed a more gradual decline from early to late population doubling levels with *PTGDS2* reaching negligible expression at PD 173 and only *IGFBP3* still being detectable at low expression levels at the highest PD.

Microarray analysis comparing normalised expression levels in six MRC-5 fibroblast cultures, (29.5±7.4), three early PDs (PD 55±10,) and three higher PDs (125±10) of hTERT over-expressing MRC-5 confirmed the described differences in all nine analysed genes while the transgene hTERT and GAPDH did not change (Fig. 3).

4. Discussion

The present study analyses gene expression changes in hTERT over-expressing MRC-5 fibroblasts, starting at 11 PDs after retroviral transfection and spanning 130 PDs in total.

We specifically choose independent subcultures for all time points in order to avoid any amplification of randomly appearing changes in subsequent culturing of the same subculture.

Our results demonstrate that expression of six of the analysed genes changed gradually and significantly with increasing culture age: *DSG2, IGFBP3, BAMBI, DNER, SULF1*, and *PTGDS*, while changes in PPARGC1 were not significantly different in early and late passage hTERT cells and expression of *GAPDH* and the transgene *hTERT* remained unchanged. Thus, the observed changes in gene expression cannot be attributed to a change in the expression level of *hTERT* itself. All cells analysed in this study had functional p53 and p16 checkpoints (data not shown) corresponding to the original findings [8,9] and have no obvious chromosomal aberrations [12] similar to what others have described [14]. Therefore we are confident that inactivation of the main tumour suppressor genes are not involved in these slow changes in expression levels. Most expression levels early after hTERT transfection were similar to those of the parental young MRC-5 at PD 30 when the culture was transfected but changed more or less continuously afterwards. Neither telomerase activity nor TERT levels were changed during the time of cultivation (data not shown) as expected for a constitutively over-expressing system. We found a different kinetics for the regulation of most genes. Some expression changes occured as early as PD 90 (*SULF1, DSG2, DNER*) while other genes were relatively stably expressed for more than 100 PD (*BAMBI, PTGDS2, IGFBP3*) but changed thereafter within a short range of PDs and disappeared in some cases altogether (Fig. 2).

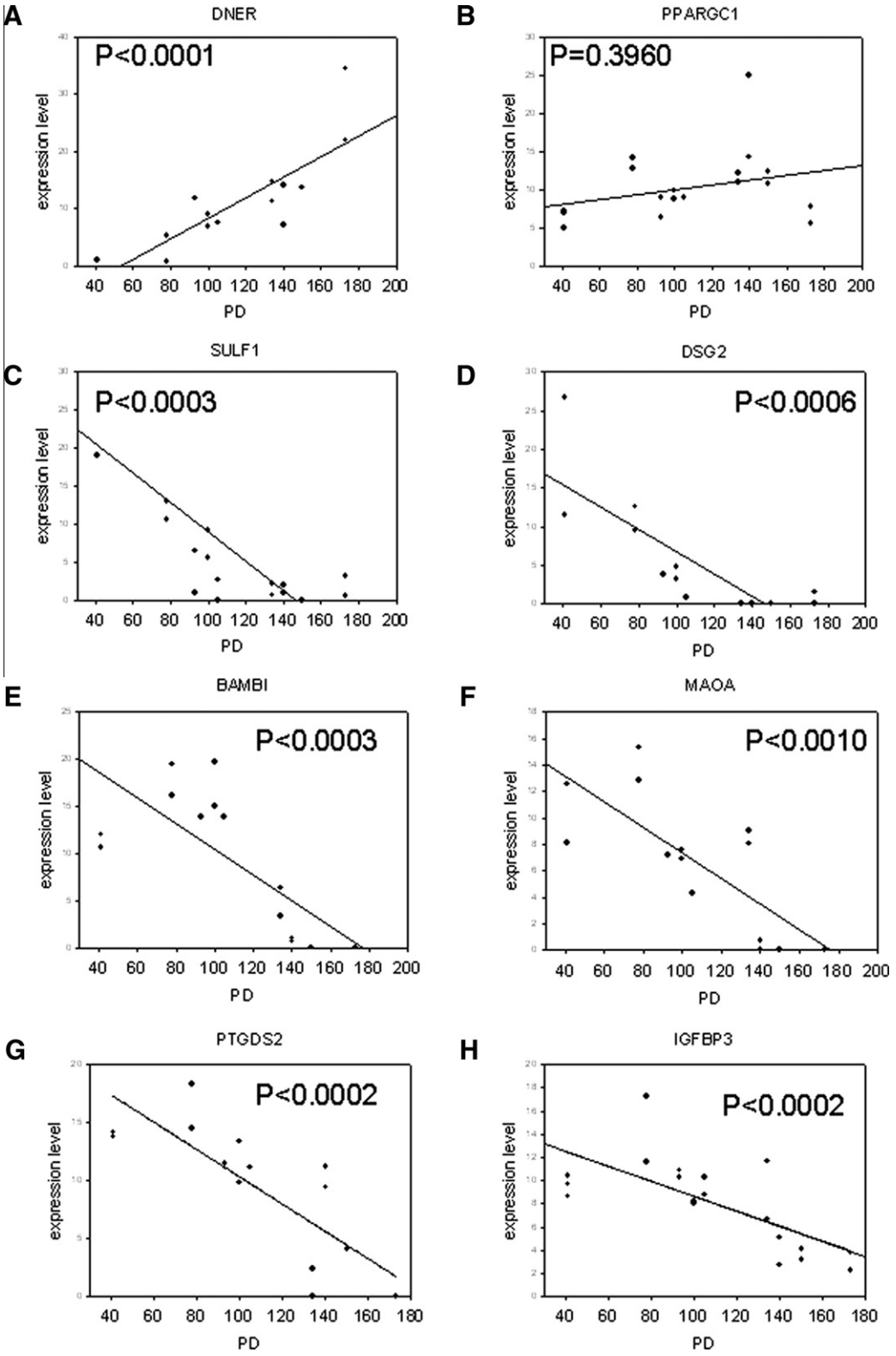

Fig. 2. Regression analysis for changes in gene expression in hTERT over-expressing MRC-5 fibroblasts over the range of PDs indicated. *P*-values are for the slope (A) of the regression line.

Strikingly, the gene *STC2* seemed the only one to be directly down-regulated by the presence of hTERT. Mammalian stanniocalcin-2 (*STC2*) is a secreted glycoprotein hormone with a putative role in calcium- and phosphate homeostasis, unfolded protein response and apoptosis. *STC2* has been described to be over-expressed in mammary carcinoma and correlated to metastasis [15,16]. However, it has also been shown that the STC2 promoter is frequently hypermethylated in various cancer cell lines leading to a diminished expression [17].

The polyclonal transfection method used to generate MRC-5 hTERT expressing fibroblasts makes it highly unlikely that *STC2* inactivation is a result of a specific genomic integration event. In

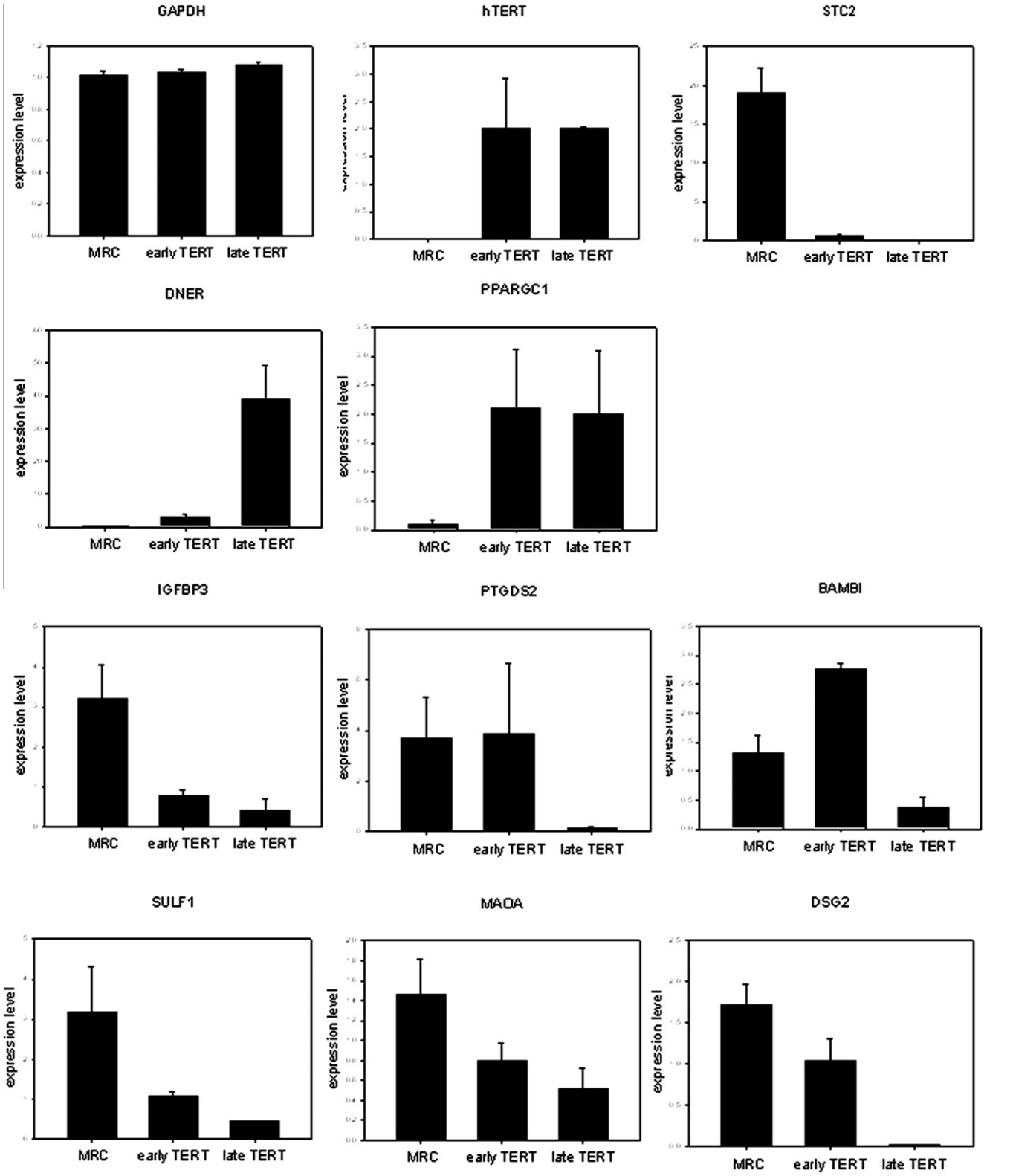

Fig. 3. Results of microarray analysis of the analysed genes in parental MRC-5 ($n = 6$), early ($n = 3$) and late ($n = 3$) PDs of hTERT over-expressing MRC-5 fibroblasts. Values are mean from normalised expression levels and SE.

addition, we have also analysed *STC2* expression in another hTERT over-expressing cell system: BJ/BJ5ta [1] and confirmed that *STC2* is also substantially down-regulated in these cells (data not shown). This result suggests that down regulation of STC2 could be a direct effect of hTERT over-expression.

Choi and colleagues using an inducible system for TERT expression in mice have demonstrated that gene expression changes occur as early as 24 h after transgene expression and that hTERT regulated genes were chromosomally clustered [18]. Some changes were similar to ours, including for *STC2* and *IGFBP3* and upregula-

tion of *PPARGC1* while other genes such as *BAMBI* and *PTGDS2* were regulated differently to our results. That could reflect differences in time of analysis and the fact that these authors analysed mouse epithelial cells.

Kumazaki and coworkers analysed the expression level of 12 genes at three different time points in two cell types over a range of more than 100 PDs starting only at PD 100 [19]. Similar to our results they found expression differences at different PDs in some genes [19]). However, they rarely found a consistent unidirectional change. More often gene expression went up and down at various population doubling levels analysed. The reason for that could be that the authors specifically analysed genes involved in proliferation and apoptosis that might vary more randomly during culturing.

Interestingly, in general, more genes were down-regulated than up-regulated in our PCR analysis-a trend that we found as well in the microarray results, similar to other related studies [14,19]. This would suggest that epigenetic events such as promoter silencing could be the underlying mechanism for the large amount of down-regulated genes during higher population doublings of hTERT over-expressing cells. However, the fact that some genes are also up-regulated over time would mean that both directions of epigenetic modifications are possible.

Additional events such as damage induced down regulation of gene expression with specifically sensitive promoters have been described during in vivo brain ageing [20]. It is possible that such a mechanism could also play a role for cells with a greatly increased lifespan *in vitro* despite stable telomeres and no obvious mitochondrial dysfunction.

We conclude that some changes occur immediately in response to transgene expression while most expression changes occur later and gradually, apparently independent of the transgene hTERT. These slow changes in gene expression pattern occur in cells without obvious genomic instability [12]. The fact that we found a continuous pattern of changes in cultures grown independently to similar PDs strongly suggest that (i) they are not the result of a single, low-probability chance event and (ii) that it is not simply random drift causing them. We have no evidence that these changes are selected for during prolonged growth, however one could speculate that a gradual down-regulation of genes taking part in the retrograde response to mitochondrial dysfunction as observed here could well be caused by a selection for improved mitochondrial function during long-term culture. This could be either due to telomere stabilisation by telomerase or to a newly described role of hTERT in improving mitochondrial function [12]. We did not observe faster growth rates or changed sensitivity to DNA-damaging stress at late PD levels as have been found following inactivation of the tumour suppressor genes p53 or p16 at high passages of hTERT over-expressing fibroblasts [10,11].

It is not known yet whether gradual changes in gene expression can increase the probability of neoplastic transformation of hTERT over-expressing cells. Chapman et al. (2008) [14] described similarities in gene expression of unstressed hTERT over-expressing urothelial cells at higher PDs (around 150) to urothelial cancer cells. If they additionally stress these cells the authors observe additional changes in gene expression such as down regulation of p16 expression and greatly increased chromosomal aberrations [14]. Similar observations have been reported for other hTERT immortalised epithelial cells [21,22]. This data suggests that there is a certain continuum of changes occurring over time which are further exacerbated under stressful culture conditions that impose an additional selective pressure. It is difficult to say how much the gene expression changes found in fibroblasts are resembling changes in cancer cells since fibroblasts hardly form any tumours.

5. Conclusion

Telomerase over-expression is used to generate material for functional analysis of various cell types as well as a source for regenerative medicine. Although major tumour suppressor genes are functionally maintained for extended periods of culturing [7,8] the current study demonstrates that that expression of selected genes is not stable and can change substantially during extended culture periods. These changes could be either immediately associated with hTERT transgene expression or occur gradually during ongoing cultivation. Further studies are necessary in order to determine what functional consequences these expression changes have for the various telomerase over-expressing cell types.

Conflict of interest

None of the authors has any conflict of interest.

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