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Tumourigenicity and radiation resistance of mesenchymal stem cells

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Abstract

Background. Cancer stem cells are believed to be more radiation resistant than differentiated tumour cells of the same origin. It is not known, however, whether normal nontransformed adult stem cells share the same radioresistance as their cancerous counterpart. *Material and methods*. Nontumourigenic (TERT4) and tumourigenic (TRET20) cell lines, from an immortalised mesenchymal stem cell line, were grown in culture prior to irradiation and gene expression analysis. Radiation resistance was measured using a clonogenic assay. Differences in gene expression between the two cell lines, both under nontreated and irradiated conditions, were assessed with microarrays (Affymetrix Human Exon 1.0 ST array). The cellular functions affected by the altered gene expressions were assessed through gene pathway mapping (Ingenuity Pathway Analysis). *Results*. Based on the clonogenic assay the nontumourigenic cell line was found to be more sensitive to radiation than the tumourigenic cell line. Using the exon chips, 297 genes were found altered between untreated samples of the cell lines whereas only 16 genes responded to radiation treatment. Among the genes with altered expression between the untreated samples were PLAU, PLAUR, TIMP3, MMP1 and LOX. The pathway analysis based on the alteration between the untreated samples indicated cancer and connective tissue disorders. *Conclusion*. This study has shown possible common genetic events linking tumourigenicity and radiation response. The PLAU and PLAUR genes are involved in apoptosis evasion while the genes TIMP3, MMP1 and LOX are involved in regulation of the surrounding matrix. The first group may contribute to the difference in radiation resistance observed and the latter could be a major contributor to the tumourigenic capabilities by degrading the intercellular matrix. These results also indicate that cancer stem cells are more radiation resistant than stem cells of the same origin.

The cancer stem cell (CSC) theory assumes that cancer arises from the malignant transformation of either adult stem cells or their immediate progenies. Recent studies have also shown that cells isolated from human tumours that were positive for stem cell markers were more radiation resistant than tumour cells without the markers [1–3]. In addition, increased resistance to chemotherapy has been reported for CSC marker positive tumour cells [4,5]. Therefore, the presence of CSC and their response to anticancer treatment, especially radiotherapy, may be detrimental for the final clinical outcome.

It is not known, however, whether normal nontransformed adult stem cells share the same radioresistance as their cancerous counterparts. The currently believed theory is that CSCs retain several characteristics from their stem cell origin, such as enhanced damage repair and slow division rate, and that these stem cell characteristics are the main cause for the observed radiation resistance phenotype.

To study this issue one needs a model in which both the normal stem cells and CSC are derived from the same source. Such a model is the human mesenchymal stem cell, which after immortalisation with retroviral inserted telomerase and passaged over a long time period in cell culture, obtained carcinogenic properties [6,7]. The only external factor that differed between the tumourigenic and nontumourigenic cell lines was the different split ratios of this prolonged passage. The model is well characterised and multiple samples have been frozen at different stages of development, thereby making it possible to pinpoint some of the events that occur on the path away from normality.

In the current study, cells from late passages of both the tumourigenic and nontumourigenic cell

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lines were compared for their growth characteristics and radiation sensitivity. In addition, they were subjected to gene expression analysis before and after irradiation, to investigate whether there are possible common genetic events that link tumourigenicity with radiation response.

Material and methods

The human mesenchymal cell lines hMSC-TERT4 (TERT4) and hMSC-TERT20 (TERT20) were used and are described in detail elsewhere [6,7]. All cell lines were passaged in MEM media containing 10% foetal calf serum, 1% nonessential amino acid, 1% penicillin-streptomycin and 1% sodium pyruvate, and incubated in a 5% CO_2 atmosphere at 37°C. The population doubling levels of the cell lines used in the experiments were 301 for TERT4 and 317 for TERT20. The growth rates were assessed by seeding 10*5 cells into T25 flasks and counting three flasks daily in a Bûrker-Tûrk counting chamber, from which the doubling time was calculated.

Radiation sensitivity was determined with an in vitro clonogenic surviving fraction (SF) assay, which has been previously described in detail [8]. Briefly, varying concentrations of cells were seeded in culture flasks and exponentially growing cells were irradiated with single doses of 1–8 Gy at a dose rate of 0.58 Gy/min using a 230 kV x-ray machine (Phillips, Holland) at 23°C. Two weeks later the colonies in the flasks were stained with methylene blue and counted under a stereo microscope. Experiments were performed in triplicate and the SF at each radiation dose was calculated using the formula (SF = colonies counted/(cells seeded*PE), where PE is the counted colonies divided by the number of cells seeded for the 0 Gy flasks. Alpha and beta values for the SF curves were estimated by nonlinear regression and subsequently plotted (SF = exp(Dose*alpha-Dose*Dose*beta)). Statistical analysis was done in Stata 10 (StataCorp) and visualised by Sigmaplot (Systat Software Inc.). A two sided Student's t-test was used to test for significant differences.

Samples for the Affymetrix Human Exon 1.0 ST array chip analysis (Aros Applied Biosystem A/S, Denmark) were purified (miRNeasy Mini kit, Qiagen) from 10^6 cells four hours after irradiation with either 0 Gy or 2 Gy. Normalisation was performed using Expression Console (Affymetrix, USA) with the RMA gene core algorithm. Genes with linearised expression values above a background threshold of 40 and with at least a two-fold or more difference between control and treated samples were further analysed with the Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, www.ingenuity.com), which displayed the gene networks and biological function. Clustering analysis of gene expression levels was done with Gene Cluster 2.11 (Michael Eisen, rana.lbl.gov/EisenSoftware.htm) using Pearson's correlation and complete linkage and Java Treeview (ver. 1.1.3) for visualisation of the heat map.

Validation of gene expression was done with quantitative Real-Time PCR (q-RT-PCR) on a 7900HT Fast RealTime PCR machine (Applied Biosystem, Europe) using TaqMAN probes as indicated in Table III (Applied Biosystem, Europe). The CHCHD1 probe (Hs00415054_m1) was included as a control. A Student's t-test with a significance level of 0.05 between the different treated samples was used to estimate the likelihood of a true difference.

Results

The growth rates (Figure 1A) showed that TERT4 was slower growing that TER20 and this was supported by the cell line's rate of cell population doubling (0.8 days for TERT20 and 1.3 days for TERT4). The plating efficiencies for the two lines were found to be 0.292 (TERT4) and 0.395 (TERT20), and although the TERT20 had a higher value the difference was not significant.

The response of both cell lines to radiation is shown in Figure 1B. TERT20 had a consistently higher SF value than TERT4 at all dose levels, with this difference becoming significant at 2 Gy and above. The regression estimation showed nearly similar beta values (0.038 for TERT4 and 0.034 for TERT20), while the estimated alpha values showed a larger difference (0.59 for TERT4 and 0.42 for TERT20). The alpha/beta ratio for the cell lines was 15.5 and 12.4 for TERT4 and TERT20, respectively.

In total we found 313 genes with altered expression between TERT4 and TERT20 (Table I and Figure 2). To obtain an overview of these genes, we sorted them into untreated cell line specific and radiation responsive genes. For 297 genes, the gene expression for control and irradiated cells in both cell lines did not differ, but there was a difference between results for the TERT4 and TERT20 cell lines. With 13 genes, no difference was found between the controls for TERT4 and TERT20, nor between the irradiated samples for both cell lines, but for each cell line the irradiated samples were different from the control values. Finally, three genes (GDF15, DYNLL1 and SLC14A1) displayed a difference between treated and nontreated for both cell lines and for values between the cell lines.

Using an Ingenuity Pathway Analysis of biological function we found a high possibility for cancer to be the main disorder induced by the altered gene expressions. Ordering the genes into pathway networks, as

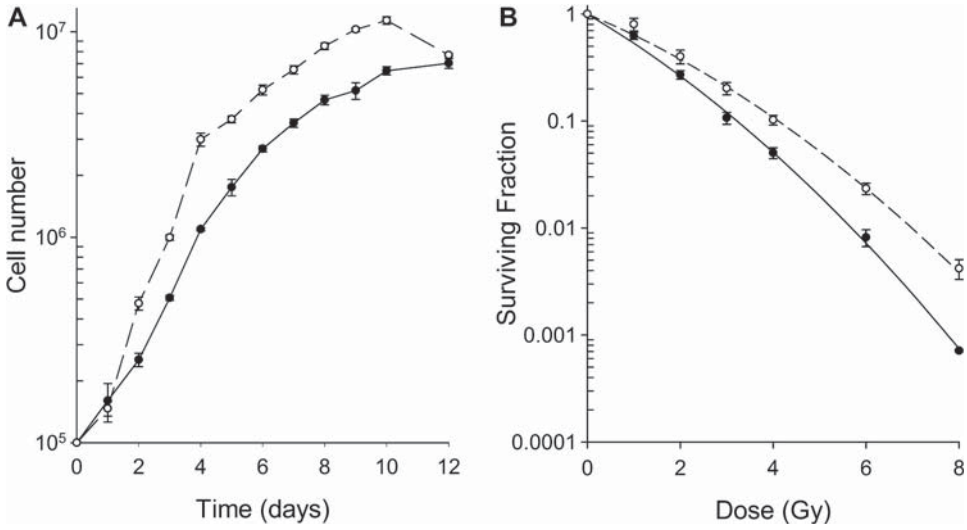

Figure 1. Growth and SF curves for the TERT4 (•) and TERT20 (○) cell lines. A. Curves show the growth of the cell lines in vitro as a function of time after seeding the cells in T25 flasks. The lines were fitted by eye. B. SF for the cell lines measured 14 days after irradiating the cells in culture. The lines through the data are the linear-quadratic curves based on the alpha and beta values. For both the growth and SF data the points show means (± 1 S.E.) for a minimum of three values.

seen in Table II, we found that the top five networks implicated genes in tumour morphology and cell death; cellular and connective tissue development, organisation and assembly; dermatological, immunological, and inflammatory disease; cellular and tissue development and function; and cancer.

To strengthen the findings from the array and gene interaction network assays, gene level verification was done with q-RT-PCR (Table III and Figure 3). A total of 16 RNA samples from two separate experiments were used; eight from each cell line (four controls and four irradiated). The difference in mRNA levels of the selected central genes involved in the two top networks in Table II was verified by q-RT-PCR. We also verified the altered expression of CD24 and CD44, and the radiation responsive genes MDM2, CDKN1A and GDF15. TERT was included as a positive control and similar levels were found in both cell lines. CDKN2A was used as a negative control since the cell lines share a deletion in the gene CDKN2A, which results in no transcription. The q-RT-PCR assay failed to detect any CDKN2A mRNA in any of the 16 samples.

Discussion

In this pre-clinical study we did a comparison between a nontumourigenic (TERT4) and tumourigenic (TERT20) cell lines that were originally derived from the same immortalised mesenchymal stem cell line. We found that the TERT4 grew significantly slower than the TERT20, but was more sensitive to the cell killing effect of radiation. The two cell lines also displayed differences in the expression of a variety of genes involved in a number of biological functions.

Our finding that TERT20 was more resistant to radiation than TERT4 implies that when applying radiation therapy the normal stem cells would be more susceptible to radiation damage than the tumour stem cells. The significant difference in radiation sensitivity between the two cell lines was seen at a radiation dose as low as 2 Gy, which is typically the standard daily dose given in a conventional fractionation schedule. Thus, our findings are totally applicable to the clinical situation. The best cellular markers for CSC are generally considered to be CD133 that was particularly positive in gliomas [1,9] and the combination of $CD44^+/CD24^-$ for breast cancer [10]. Our gene expression analysis in TERT20 indicates a $CD44^+/CD24^-$ status, whereas TERT4 expressed both CD44 and CD24 mRNA, confirming CSC phenotype for TERT20, but not TERT4. Both clones showed a lack of PROM1 gene expression, which is the gene encoding the CD133 surface marker. This was expected since the model is based on mesenchymal stem cells and CD133 is associated with endothelial, gliomas and haematopoietic stem cells. Even if the founder cell line did express CD133, then this would likely be lost due to the long time period that the cells were kept in culture, since this is known to promote the loss of the CD133 marker [11].

Analysis of the microarrays data showed that the genes listed in the first Ingenuity network (Table II) centre on PAWR, PLAU and PLAUR, all of which influence the cellular apoptotic pathways. The RNA levels of these three genes were verified by q-RT-PCR

Table I. Gene expression levels in TERT4 and TERT20.

Gene Symbol	TERT4 control	TERT4 irradiation	TERT20 control	TERT20 irradiation	Gene Description	Cytoband	Fold difference	p-value
Genes with known similar expression								
TERT	1466	1407	1271	1154	telomerase reverse transcriptase	5p15.33	0.8	0.11
CD44	225	227	293	266	CD44 molecule (Indian blood group)	11p13	1.2	0.15
CDKN2A	10	12	13	12	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	9p21	no expression	n.a.
Genes with altered expression after radiation treatment								
CDKN1A	130	541	166	431	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	6p21.2	3.4	0.08
TP53INP1	42	124	30	95	tumour protein p53 inducible nuclear protein 1	8q22	3.1	0.08
FAS	107	202	56	185	Fas (TNF receptor superfamily, member 6)	10q24.1	2.6	0.11
MDM2	214	521	187	417	Mdm2 p53 binding protein homolog (mouse)	12q14.3-q15	2.3	0.10
EDA2R	67	181	130	244	ectodysplasin A2 receptor	Xq12	2.3	0.12
FST	143	271	154	359	follistatin	5q11.2	2.1	0.16
TNFRSF10B	91	189	122	233	tumor necrosis factor receptor superfamily, member 10b	8p22-p21	2.0	0.07
XPOT	438	291	455	223	exportin, tRNA (nuclear export receptor for tRNAs)	12q14.2	0.6	0.10
C18orf32	156	71	108	56	chromosome 18 open reading frame 32	18q21.1	0.5	0.19
Genes with altered expression after radiation treatment in TERT20 only								
SLC5A3	383	427	411	127	solute carrier family 5 (sodium/myo-inositol cotransporter), member 3	21q22.12	0.3	n.a.
Genes with altered expression after radiation treatment in TERT4 only								
CTSL1	252	118	171	154	cathepsin L1	9q21-q22	0.5	n.a.
ANP32B	151	69	105	77	acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	9q22.32	0.5	n.a.
HMGB2	174	68	50	68	high-mobility group box 2	4q31	0.4	n.a.
Genes with altered expression after radiation treatment and between cell lines								
SLC14A1	549	496	207	80	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	18q11-q12	0.6	n.a.
DYNLL1	437	258	193	87	dynein, light chain, LC8-type 1	12q24.23	0.5	n.a.
GDF15	29	124	65	265	growth differentiation factor 15	19p13.11	4.2	n.a.
Genes with 5 fold or more regulation between the cell lines								
MMP1	62	51	2215	1782	matrix metallopeptidase 1 (interstitial collagenase)	11q22.3	35.5	0.07
HLA-DRB5	4	7	184	109	major histocompatibility complex, class II, DR beta 5	6p21.3	28.5	0.17
EREG	9	8	215	246	epiregulin	4q13.3	27.2	0.04
PAMR1	12	9	233	198	peptidase domain containing associated with muscle regeneration 1	11p13	20.7	0.05
HLA-DRA	9	9	229	159	major histocompatibility complex, class II, DR alpha	6p21.3	20.7	0.12
HLA-DRB1	56	47	936	524	major histocompatibility complex, class II, DR beta 1	6p21.3	14.0	0.19
HLA-DPA1	35	22	462	307	major histocompatibility complex, class II, DP alpha 1	6p21.3	13.8	0.13
CTSK	26	19	257	235	cathepsin K	1q21	11.0	0.02
CD74	51	41	454	435	CD74 molecule, major histocompatibility complex, class II invariant chain	5q32	9.8	0.00
AREG	19	21	135	178	amphiregulin	4q13-q21	7.8	0.10
NTNG1	26	22	190	156	netrin G1	1p13.3	7.1	0.07
TGM2	35	37	265	195	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	20q12	6.4	0.11

(Continued)

Table I. *(Continued)*.

Gene Symbol	TERT4 control	TERT4 irradiation	TERT20 control	TERT20 irradiation	Gene Description	Cytoband	Fold difference	p-value
DUSP6	29	31	212	157	dual specificity phosphatase 6	12q22-q23	6.1	0.11
CA12	104	99	602	477	carbonic anhydrase XII	15q22	5.3	0.09
RGS4	600	647	136	118	regulator of G-protein signaling 4	1q23.3	0.2	0.01
CRIP2	347	343	77	64	cysteine-rich protein 2	14q32.3	0.2	0.01
ACTG2	549	559	114	110	actin, gamma 2, smooth muscle, enteric	2p13.1	0.2	0.00
PSG9	188	165	42	27	pregnancy specific beta-1-glycoprotein 9	19q13.2	0.2	0.01
TXNIP	328	321	68	56	thioredoxin interacting protein	1q21.1	0.2	0.00
SEMA5A	417	410	82	76	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain ™ and short cytoplasmic domain, (semaphorin) 5A	5p15.2	0.2	0.00
PTPRJ	215	184	43	33	protein tyrosine phosphatase, receptor type, J	11p11.2	0.2	0.04
CDH6	147	120	27	22	cadherin 6, type 2, K-cadherin (fetal kidney)	5p15.1-p14	0.2	0.07
TGFB2	150	157	29	25	transforming growth factor, beta 2	1q41	0.2	0.00
DSP	177	169	30	28	desmoplakin	6p24	0.2	0.01
FAM69A	127	139	23	18	family with sequence similarity 69, member A	1p22.1	0.2	0.02
LITAF	168	143	24	24	lipopolysaccharide-induced TNF factor	16p13.13	0.2	0.06
EPHA5	536	555	103	63	EPH receptor A5	4q13.1	0.2	0.01
SLIT3	213	177	27	27	slit homolog 3 (Drosophila)	5q35	0.1	0.07
PCDHB14	225	235	43	18	protocadherin beta 14	5q31	0.1	0.02
PCDHB16	109	127	15	15	protocadherin beta 16	5q31	0.1	0.06
IFI6	822	769	89	109	interferon, alpha-inducible protein 6	1p35	0.1	0.01
MYPN	288	258	37	31	myopalladin	10q21.3	0.1	0.03
EFEMP1	781	680	81	95	EGF-containing fibulin-like extracellular matrix protein 1	2p16	0.1	0.05
C1R	218	179	26	20	complement component 1, r subcomponent	12p13	0.1	0.07
PCDHB2	121	96	11	13	protocadherin beta 2	5q31	0.1	0.08
TLR4	121	103	11	13	toll-like receptor 4	9q32-q33	0.1	0.05
CXCL12	463	362	51	37	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	10q11.1	0.1	0.08
BACE2	111	104	11	10	beta-site APP-cleaving enzyme 2	21q22.3	0.1	0.02
THBS2	944	947	104	83	thrombospondin 2	6q27	0.1	0.01
C14orf45	161	124	17	11	chromosome 14 open reading frame 45	14q24.3	0.1	0.08
CCL2	201	133	20	12	chemokine (C-C motif) ligand 2	17q11.2-q12	0.1	0.14
TNFRSF11B	277	247	25	24	tumour necrosis factor receptor superfamily, member 11b	8q24	0.1	0.04
MFAP5	134	110	10	13	microfibrillar associated protein 5	12p13.1-p12.3	0.1	0.07
CD24	106	86	10	8	CD24 molecule	6q21	0.1	0.07
SULF1	374	351	41	26	sulfatase 1	8q13.2-q13.3	0.1	0.00
ZC4H2	120	97	8	9	zinc finger, C4H2 domain containing	Xq11.2	0.1	0.07
ADAMTSL1	135	117	7	8	ADAMTS-like 1	9p22.2-p22.1	0.1	0.05
FDXACB1	346	311	17	19	ferredoxin-fold anticodon binding domain containing 1	11q23.1	0.1	0.04
SORT1	385	354	17	18	sortilin 1	1p21.3-p13.1	0.0	0.03
FLJ34690	156	135	6	5	hypothetical protein FLJ34690	17p12	0.0	0.05

(Continued)

Table I. *(Continued)*.

Gene Symbol	TERT4 control	TERT4 irradiation	TERT20 control	TERT20 irradiation	Gene Description	Cytoband	Fold difference	p-value
TFAP2D	321	278	6	6	transcription factor AP-2 delta (activating enhancer binding protein 2 delta)	6p12.1	0.0	0.05
MYOCD	685	628	8	8	myocardin	17p11.2	0.0	0.03
Genes with 3-5 fold regulation between the cell lines								
ITGA2	88	98	413	459	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	5q23-q31	4.7	0.04
SFRP1	70	60	315	246	secreted frizzled-related protein 1	8p12-p11.1	4.3	0.10
EDNRA	71	78	350	277	endothelin receptor type A	4q31.22	4.2	0.09
NETO2	74	56	289	248	neuropilin (NRP) and tolloid (TLL)-like 2	16q11	4.2	0.04
RAB27B	52	49	228	187	RAB27B, member RAS oncogene family	18q21.2	4.1	0.08
HLA-DMA	53	47	215	190	major histocompatibility complex, class II, DM alpha	6p21.3	4.0	0.04
SPRY2	132	107	522	427	sprouty homolog 2 (Drosophila)	13q31.1	4.0	0.07
TUBA4A	53	57	179	239	tubulin, alpha 4a	2q35	3.8	0.12
NPAS2	87	86	345	308	neuronal PAS domain protein 2	2q11.2	3.8	0.05
STC1	216	137	619	585	stanniocalcin 1	8p21-p11.2	3.6	0.03
ARHGDIB	138	100	455	339	Rho GDP dissociation inhibitor (GDI) beta	12p12.3	3.3	0.10
EMP1	268	243	884	740	epithelial membrane protein 1	12p12.3	3.2	0.07
ITGB3	67	54	219	150	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	17q21.32	3.0	0.16
HSPA1A	744	641	256	195	heat shock 70kDa protein 1A	6p21.3	0.3	0.03
COL1A2	1832	1775	592	578	collagen, type I, alpha 2	7q22.1	0.3	0.01
LOX	841	875	275	277	lysyl oxidase	5q23.2	0.3	0.02
ITGAV	505	519	170	160	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	2q31-q32	0.3	0.00
NEK7	644	642	201	212	NIMA (never in mitosis gene a)-related kinase 7	1q31.3	0.3	0.01
LAMA4	182	163	69	39	laminin, alpha 4	6q21	0.3	0.03
CYP1B1	555	458	195	119	cytochrome P450, family 1, subfamily B, polypeptide 1	2p21	0.3	0.03
GABARAPL1	141	134	55	29	GABA(A) receptor-associated protein like 1	12p13.2	0.3	0.07
ST20	164	149	47	47	suppressor of tumourigenicity 20	15q25.1	0.3	0.04
IGFBP3	1202	1130	382	320	insulin-like growth factor binding protein 3	7p13-p12	0.3	0.00
FMN2	264	234	79	70	formin 2	1q43	0.3	0.04
ABI3BP	473	400	146	114	ABI family, member 3 (NESH) binding protein	3q12	0.3	0.04
CREG1	124	134	36	41	cellular repressor of E1A-stimulated genes 1	1q24	0.3	0.02
IL8	147	118	49	31	interleukin 8	4q13-q21	0.3	0.05
COL4A2	300	279	90	81	collagen, type IV, alpha 2	13q34	0.3	0.02
IFITM1	376	327	119	87	interferon induced transmembrane protein 1 (9-27)	11p15.5	0.3	0.02
SDC2	323	289	92	86	syndecan 2	8q22-q23	0.3	0.04
DNAJB4	213	259	87	44	DnaJ (Hsp40) homolog, subfamily B, member 4	1p31.1	0.3	0.03
ALPK2	253	210	92	44	alpha-kinase 2	18q21.31-q21.32	0.3	0.04
MFGE8	691	610	204	164	milk fat globule-EGF factor 8 protein	15q25	0.3	0.02
COL1A1	2186	2168	622	588	collagen, type I, alpha 1	17q21.33	0.3	0.00
PPIC	139	144	35	43	peptidylprolyl isomerase C (cyclophilin C)	5q23.2	0.3	0.01
IDH2	257	234	79	56	isocitrate dehydrogenase 2 (NADP+), mitochondrial	15q26.1	0.3	0.01
SLC14A1	549	496	207	80	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	18q11-q12	0.3	0.07
ACTA2	2655	2404	759	588	actin, alpha 2, smooth muscle, aorta	10q23.3	0.3	0.01

(Continued)

Table I. *(Continued).*

Gene Symbol	TERT4 control	TERT4 irradiation	TERT20 control	TERT20 irradiation	Gene Description	Cytoband	Fold difference	p-value
THY1	570	479	133	139	Thy-1 cell surface antigen	11q22.3-q23	0.3	0.07
LEPREL1	287	319	79	79	leprecan-like 1	3q28	0.3	0.05
TAGLN	579	522	167	121	transgelin	11q23.2	0.3	0.01
ADAMTS2	545	539	146	132	ADAM metallopeptidase with thrombospondin type 1 motif, 2	5qter	0.3	0.00
KRTAP1-1	181	191	48	47	keratin associated protein 1-1	17q12|17q12-q21	0.3	0.02
TLE4	244	235	55	60	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	9q21.31	0.2	0.00
COL3A1	237	213	55	53	collagen, type III, alpha 1	2q31	0.2	0.04
COL4A1	160	137	36	35	collagen, type IV, alpha 1	13q34	0.2	0.06
SPOCK1	247	244	61	53	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	5q31	0.2	0.01
ADAM12	453	409	102	97	ADAM metallopeptidase domain 12	10q26.3	0.2	0.04
LGMN	350	334	74	82	legumain	14q32.1	0.2	0.00
EDIL3	777	783	196	149	EGF-like repeats and discoidin I-like domains 3	5q14	0.2	0.02

Genes showing at least a three-fold difference in up or down regulation between the samples. Those genes displaying a cell line specific or treatment response regulation are listed. The fold score for the radiation responding genes are based on the best defining ratio. CD44, CDKN1A and TERT are included as known genes with similar expression levels (based on previous reports).

on a large number of samples. In our study, PAWR was down-regulated, while PLAU and PLAUR were up-regulated in TERT20. PAWR is a tumour suppressor gene encoding a protein that selectively induces apoptosis in cancer cells [12]. The PLAU and PLAUR genes encode proteins that are part of the plasminogen activation system [13] which interact with the Akt protein kinase involved in apoptosis [14]. Their normal function involves extracellular matrix degradation and tissue remodelling, and reports have implicated an up-regulation in tumour cells showing increased tumour migration, proliferation and metastases [15]. Thus, TERT20 could evade the apoptotic signals from the Akt pathway by up-regulating PLAU and PLAUR and by a reduced expression of the tumour suppressor PAWR.

The second Ingenuity network listed in Table II consists of genes coding for components of the connective tissue and basal membrane, such as ELN, DCN, NID1 and several collagen variants. All were found to be down-regulated in TERT20. The genes MMP1, MMP14, LOX and TIMP3 all affect the balance between creating and degrading connective tissue. The RNA levels of these four genes were also verified by q-RT-PCR. TIMP3 normally inhibits MMP1 and MMP14 degradation of the connective tissue [16], but in TERT20 TIMP3 is down-regulated and MMP1 and MMP14 are up-regulated. MMP1 facilities tumour growth through the extracellular matrix and is seen as a tumour-derived growth factor [17]. It is derived from the surrounding tissue/fibroblasts and can alter the tumour microenvironment in favour of cancer cell migration and invasion through PAR1 cleavage [18]. The gene cluster containing MMP1 is indicated as important for cancer development and progression [19]. We also found that LOX, which establishes cross-linking between the components of the connective tissue [20] was down-regulated in TERT20. This is in line with the observation of PLAU and PLAUR up-regulation and indicates that TERT20, contrary to TERT 4, actively disrupts connective tissue rather that creating it.

HLA class II genes dominate the third network and all are up-regulated in TERT20. These genes play an important role in the immune system [21]. HLA-DP and HLA-DR display the antigens to T-cells. Both have alpha and beta subunits, the levels of which were increased. HLA-DM is a chaperon encoding gene that helps with antigen presentation. Several other genes implicated in cellular immune response were up-regulated in TERT20. This includes CD74, HMGA2 and TGFB2, all of which interact with the HLA class II genes. These effects on immune related genes were only seen in TERT20, even though both cell lines were handled in parallel. This may be

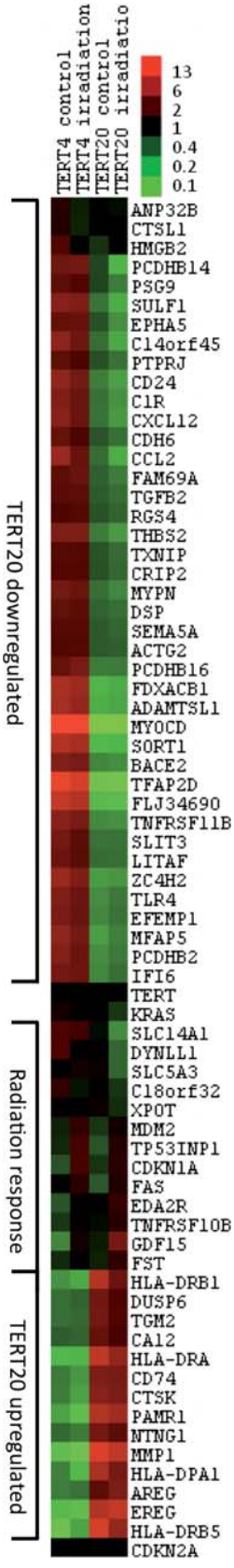

Figure 2. Heat map of those genes showing more than a five-fold altered regulation of expression levels, measured using the Affymetrix Human Exon array chip. Clustering analysis was achieved with Gene Cluster 2.11 using Pearson's correlation and complete linkage and visualised with Java Treeview. Hierarchical clustering of the data with median centred genes and complete linkage; red is up-regulated and green is down-regulated compared to the mean of the individual gene's expression levels. The genes TERT, CDKN2A and K-RAS were included as controls with known expression patterns.

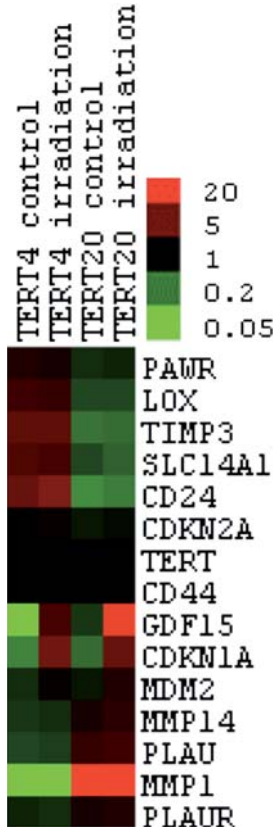

Figure 3. Heat map of the genes verified by q-RT-PCR. Clustering analysis was achieved with Gene Cluster 2.11 using Pearson's correlation and complete linkage and visualised with Java Treeview. Hierarchical clustering of the data with median centred genes and complete linkage; red is up-regulated and green is down-regulated compared to the mean of the individual gene's expression levels. The genes TERT and CDKN2A were included as controls with known expression patterns.

linked to the tumourigenic process of TERT20, since some tumours have previously shown an increase in expression of HLA II genes [22].

The fourth network implicates cell morphology, and connective tissue development and function. The most important genes encode for heat shock proteins, caspases, and proteins involved in cell cycle regulation. Lastly, the fifth network consists of genes implicated in cell death, hair and skin development, and cancer. Here the most critical genes involve several integrins and genes related to the regulation of ERK1/ERK2, although the expression of these latter genes was not affected.

Table II. Gene interaction networks.

Genes in Ingenuity Networks	Score	Top Functions
Akt, *ASAH1*, ***CEP170***, Collagen Alpha1, *CREG1*, ***DUSP6***, ***EMP1***, ***EREG***, FSH, ***GDF15***, hCG, *IGFBP3*, Lh, LRP, ***MARCH3***, N-cor, *PAWR*, ***PLAU***, ***PLAUR***, *PPAP2A*, *PTPRJ*, SAA, ***SERPINB2***, *SH3BP4*, ***SLC20A1***, *Smad2/3-Smad4*, *SPARC*, ***SPRY2***, ***STC1***, *SULF1*, *TCF4*, *THBS2*, ***TMEM158***, *TPM1*, Vegf	41	tumour Morphology, Small Molecule Biochemistry, Cell Death
ADAM12, *COL12A1*, *COL1A1*, *COL1A2*, *COL3A1*, *COL4A1*, *COL4A2*, collagen, Collagen type IV, Complement component 1, Creatine Kinase, ***CTSK***, *DCN*, *EFEMP1*, Elastase, *ELN*, *FBLN1*, Fibrinogen, *HSPG2*, *Igfbp*, *IGFBP7*, IL17R, Laminin1, *LOX*, *MFAP5*, ***MMP1***, ***MMP14***, *NID1*, PI3K (complex), *SDC2*, *SERPINH1*, ***SFRP1***, ***SH2B3***, STAT1/3/5 dimer, *TIMP3*	40	Connective Tissue Disorders, Genetic Disorder, Cellular Assembly and Organisation
ANKRD1, *ATP6AP2*, ***CD74***, ***DUSP5***, ***ENPP1***, ***HLA-DMA***, ***HLA-DPA1***, ***HLA-DPB1***, ***HLA-DR***, ***HLA-DRA***, Hla-Drb, ***HLA-DRB1***, ***HLA-DRB5***, ***HMGA2***, *HMGB2*, ***HSP***, IFN Beta, Ifn gamma, IL23, *LGMN*, *LITAF*, MHC, Mhc class ii, MHC Class II (complex), MHC II-Î², Mhc2 Alpha, *MYPN*, NFkB (complex), Notch, peptidase, *PLK2*, *TGFB2*, Tlr, *TLR4*, *TXNRD1*	31	Dermatological Diseases and Conditions, Immunological Disease, Inflammatory Disease
Actin, Alpha catenin, ***ARHGDIB***, Cadherin, Caspase, *CDH6*, *CDH11*, ***CDH20***, *CTNND2*, Cyclin A, Cytochrome c, *DOCK5*, *DSP*, E2f, GOLT1B, Hdac, *HIST1H3A* (includes others), Hsp27, Hsp70, *HSPA1A*/HSPA1B, Jnk, ***KCNJ2***, MAP2K1/2, ***MAPKAPK3***, MIR1, *MMD*, *MYBL2*, *NEO1*, ***NETO2***, NfkB1-RelA, ***PREX1***, Pro-inflammatory Cytokine, Rb, Tnf, Tnf receptor	25	Cell Morphology, Cellular Development, Connective Tissue Development and Function
ACTA2, *ACTG2*, *C5orf13*, *CD24*, Collagen type III, Collagen(s), Eotaxin, ERK1/2, ***FERMT2***, Fibrin, G-Actin, Integrin, Integrin alpha 3 beta 1, Integrin alpha 4 beta 1, Integrin alpha 5 beta 1, Integrin alpha 5 beta 3, Integrin alpha V beta 3, IntegrinÎ², ***ITGA2***, *ITGAV*, ***ITGB3***, *LAMA4*, Laminin, *LIMA1*, *LPP*, ***LPXN***, ***Metalloprotease***, *MFGE8*, *MYOCD*, *NTN4*, Pdgfr, Smooth Muscle Actin, *TAGLN*, Tenascin, *TNFRSF11B*	24	Cell Death, Hair and Skin Development and Function, Cancer

Pathway analysis of the altered genes showing the most likely affected networks in TERT20 as opposed to TERT4. Genes with two-fold or more up-regulation are marked in bold, and those with a two-fold or more downregulation are underscored.

Overall, the Ingenuity pathway analysis indicated that the most likely biological function of those genes that were altered in TERT20 play a role in cancer development; the genes expressed in TERT20 seem more involved in disassembling the basal membrane and connective tissue and less implicated in pathways leading to cell death. Other networks lower on the Ingenuity pathway analysis list also had functions which implicated cancer, tissue morphology or mesenchymal disorders.

In response to radiation treatment with 2 Gy the expression of some 16 genes was altered. CDKN1A, EDA2R, FAS, MDM2, TP53INP1 and TNFRSF10B were up-regulated in both cell lines and all these

Table III. Genes verified by q-RT-PCR.

Symbol	Fold	p-Value	Response	TaqMan probe ID
Genes supporting apoptosis evasion				
PAWR	0.5	≤0.001	TERT4 upreg.	Hs01088574_m1
PLAUR	2.1	≤0.001	TERT20 upreg.	Hs00959822_m1
PLAU	3.2	≤0.001	TERT20 upreg.	Hs01547054_m1
Genes supporting matrix remodelling				
TIMP3	0.2	≤0.001	TERT4 upreg.	Hs00165949_m1
LOX	0.3	≤0.001	TERT4 upreg.	Hs00184700_m1
MMP14	2.4	≤0.001	TERT20 upreg.	Hs01037009_g1
MMP1	105.317108835908	≤0.001	TERT20 upreg.	Hs00899658_m1
Genes responding to radiation				
MDM2	1.9	≤0.05	Radiation	Hs00234753_m1
CDKN1A	7.2	≤0.05	Radiation	Hs00355782_m1
GDF15	12.6	≤0.001	Radiat. TERT20	Hs00171132_m1
Genes with specific expression				
CD24	0.1	≤0.05	TERT4 upreg.	Hs00273561_s1
CD44	1.0	≥0.1	No difference	Hs00153304_m1
TERT	0.8	≥0.05	No difference	Hs00972656_m1
CDKN2A	n.a.	n.a.	No expression	Hs00923894_m1

Changes in gene expression verified by q-RT-PCR using the listed TaqMAM probes. CDKN1A and TERT were included as controls with known similar expression levels. The gene GDF15 showed up-regulation in TERT20 and after irradiation for both cell lines.

genes are implicated in either cell cycle inhibition or apoptosis induction [23–27]. An altered RNA level of the two most radiation responsive genes, CDKN1A and MDM2, was verified by Q-RT-PCR. The FST gene was also up-regulated, but this gene does not seem to be involved in either apoptosis or DNA damage pathways. C18orf32 and XPOT were down-regulated in both cell lines after irradiation. All the above listed had the same starting level in TERT4 and TERT20. However, GDF15 is clonally different since the levels in TERT20 were higher than that in TERT4, both before and after irradiation. But GDF15 was up-regulated in both cell lines following irradiation. This pattern was also seen in the q-RT-PCR verification. GDF15 is implicated as a tumour suppressor gene, but has also been reported to be involved in metastasis and tumour proliferation [28]. The up-regulation in TERT20 can partly explain its phenotype if GDF15 functions as a proliferation inducer. The remaining genes only showed a response to treatment in one of the cell lines. SLC5A3 was down-regulated after irradiation in TERT20 only, while CTSL1, ANP32B, and HMGB2 had reduced levels of expression in TERT4 after irradiation. For these three genes the level before irradiation was high and following the radiation treatment was reduced to a level that was similar to that seen in TERT20, regardless of whether the TERT20 were irradiated or not. None of this latter group of genes seem directly implicated in DNA damage or apoptosis.

Among the genes with the greatest differential expression between TERT4 and TERT20 we found that SORT1 was some 20-fold lower in TERT20 compared to TERT4. Since SORT1 is involved in differentiation of mesenchymal stem cells to osteogenic cells and mineralisation [29], then the higher levels in TERT4 could indicate that this TERT4 cell line is being primed for differentiation; the original cell line from which these TERT4 and TERT20 cell lines originated is still in use as a bone formation model and TERT4 seems to have retained the potential to differentiate into osteoblasts.

Conclusion

This study showed that the tumourigenic mesenchymal stem cell line TERT20 has a greater resistance to radiation treatment than the nontumourigenic TERT 4 cell line. Such a finding is consistent with the current theory of enhanced radiation resistance of CSC over normal cells of the same origin. Whether this phenomenon is true for all types of CSC is not known. Our investigation of the tumourigenic TERT20 and nontumourigenic TERT4 cell lines revealed differences in the gene expression that implicate down-regulation of apoptosis and disruption of connective tissue and cellular development. None of the induced gene expression differences fully explain the difference in radiation resistance between the two cell lines, suggesting that there may be an indirect influence from several events related to tumourigenesis that play a role in the observed radiation resistance.

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