Common Delayed Senescence of Melanocytes from Multiple Primary Melanoma Patients

AUTHOR ACCEPTED VERSION*

Jaskaren S Kohli[1], Elena Tolomio[2], Simona Frigerio[3], Andrea Maurichi[2], Monica Rodolfo[3], Dorothy C Bennett[1]

[1]Cell Biology & Genetics Research Centre, Molecular & Clinical Sciences Research Institute, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK

[2]Department of Surgery, Melanoma and Sarcoma Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy

[3]Dept of Experimental Oncology and Molecular Medicine, Immunotherapy Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy

Correspondence to:

Dorothy C Bennett
Molecular & Clinical Sciences Research Institute, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK
Phone: (+44) 20 8725 5202/5918
Email: dbennett@sgul.ac.uk

Short title: Delayed cell senescence in multiple melanoma

Abbreviations: MPM: multiple primary melanoma, SPM: single primary melanoma

ORCIDS:

M Rodolfo orcid.org/0000-0002-9196-0298

DC Bennett orcid.org/0000-0002-3639-7527

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TO THE EDITOR

Approximately 5% of cutaneous melanoma patients acquire at least one additional independent melanoma, a phenomenon known as multiple primary melanoma (MPM). Genetic factors are implicated since a family history is one of the strongest risk factors for MPM (Ferrone et al., 2005).

One study identified germline mutations in *CDKN2A* in approximately 15% of MPM patients (Monzon et al., 1998), and another reported these mutations to be four times more prevalent in MPM than in single primary melanoma (SPM) patients (Pastorino et al., 2008). *CDKN2A*, the commonest known familial melanoma gene, encodes p16, a broad-spectrum tumour suppressor and mediator of cell senescence (Aoude et al., 2015a; Bennett, 2016). Senescence is a permanent cellular arrest following extensive proliferation and telomere shortening/dysfunction, or other genotoxic stresses. p16 induces senescence by inhibiting cyclin-dependent kinase 4 (CDK4)-mediated phosphorylation of retinoblastoma (RB)-family proteins, resulting in RB proteins binding and repressing E2F transcription factor activity, needed for S-phase entry in the cell cycle (Bennett, 2016). Human cells may senesce through either the p53 pathway, the p16 pathway, or both. p53 arrests cells by upregulating another CDK inhibitor, p21 (CDKN1A). However, human melanocytes seem to senesce predominantly through p16. Oncogene (usually *BRAF*) activation in an epidermal melanocyte leads to an initial proliferation followed by senescence, generating a mole or nevus (Bennett, 2016).

Cell lifespan *in vitro* is the number of population doublings completed by a cell strain before replicative senescence. p16-null melanocytes display an increased lifespan but still senesce, through a mechanism involving p21 (Sviderskaya et al., 2003). Similarly, *CDKN2A* mutation carriers tend to have more large nevi than normal (representing more divisions before senescence) (Bennett, 2016), as do individuals with longer telomeres (Bataille et al.,

2007). Moreover mutations in other senescence-related genes have been identified in familial melanoma: *TERT*, *CDK4* and genes encoding components of the telomeric cap shelterin, e.g. *POT1, TERF2IP* (Robles-Espinoza et al., 2014; Shi et al., 2014; Aoude et al., 2015b). These observations suggested that MPM may commonly be associated with genetically defective or delayed melanocyte senescence.

This hypothesis has been tested by explanting melanocyte cultures from biopsies of sun-protected normal skin from MPM or SPM patients wild-type for known melanoma-associated mutations other than in *MC1R* (Table S1). Biopsies were taken with written, informed consent and institutional ethical approval. The use of SPM patients as controls excluded any confounding effect of simply developing melanoma. Melanocytes were serially passaged until they reached replicative arrest. Cumulative growth curves for all cultures are shown (Figure 1a; individual donor information in Figure S1 and Table S2). Melanocyte culture lifespans for each patient are included in Table S1. Senescence was confirmed by β-galactosidase immunocytochemistry in all lines (Figure 1b). The final number of population doublings (lifespan) per culture was compared between the groups.

The mean lifespan for MPM patients' melanocytes (16.3 doublings, n = 10) was over 4-fold higher than the mean lifespan for SPM patients' melanocytes (3.7 doublings, n = 8), confirming our hypothesis ($p = 0.0057$). Normal adult melanocytes, grown in similar conditions, are reported to have lifespans ranging up to a maximum of 10 population doublings (Graeven and Herlyn, 1992). The maximum lifespan we observed among melanocyte cultures from SPM patients was 9 doublings, suggesting little difference from normal adult donors.

Substantial heterogeneity was observed among lifespans, especially in the MPM group. We examined the role of donor age, since telomere length shortens with age in some

cell types. Conclusions have varied on relations between donor age and lifespans of skin fibroblasts (Schneider and Mitsui, 1976; Cristofolo et al., 1998); not studied in melanocytes to our knowledge. Surprisingly, MPM cultures showed a significant negative correlation of lifespan with donor age ($p = 0.0003$) whereas SPM cultures showed no correlation ($p = 0.59$) (Figure 2). Slopes of the two regression lines differed, highly significantly ($p = 0.0046$), concluding that the significant increase in melanocyte lifespan from MPM patients occurs independently of donor age.

Although there was no significant difference in the mean donor age between MPM and SPM patients ($p = 0.66$), there were two MPM patients younger, and one older, than all SPM patients. To ensure the difference in correlation was not due to this greater range, these three were excluded from a separate analysis (Figure S2). Slopes were still significantly different ($p = 0.028$), and a significant negative correlation of lifespan with donor age was still seen only in melanocytes from MPM patients ($p = 0.027$). The mean melanocyte lifespan from MPM patients was still significantly higher than from SPM patients ($p = 0.0064$).

The lack of decrease of SPM melanocyte lifespan with donor age agrees with reports that adult epidermal melanocytes rarely divide, implying little telomere shortening. Regarding the MPM cultures, one notes that the skin biopsies from MPM patients were by definition taken after diagnosis of a second (or more) melanoma. Thus, in this group, young donors were patients who were young when their second melanoma arose, and these individuals tended to have melanocytes with longer lifespans. This again suggests genetic factors that increase both MPM susceptibility and melanocyte lifespan.

These data clearly support a frequent association of MPM with a genetic tendency for extended melanocyte lifespan. Telomere length is associated with melanoma risk (Burke et al., 2013), as well as nevus size (Bataille et al., 2007). Likewise the specific melanoma-

associated mutations in shelterin genes are predicted to increase telomere length (Robles-Espinoza et al., 2014; Shi et al., 2014; Aoude et al., 2015b). Individuals with longer telomeres would be expected to have melanocytes with greater replicative potential, resulting in greater culture lifespans, and larger nevi following an oncogenic mutation, as seen with *CDKN2A* defects. This would yield more cells per nevus, increasing the risk of further mutations and progression to melanoma. MPM patients would make good candidates for elucidating novel germline melanoma-susceptibility genes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Figure Legends

Figure 1: Delayed cellular senescence in melanocytes from MPM patients.

(a) Cumulative growth curves of melanocytes from MPM (black points, n = 10) and control SPM patients (red points, n = 8). The first subculture when cells were first counted was designated day 0. At each subsequent subculture, the fold increase in number of cells was converted to cumulative population doublings. Each point represents one subculture. See Supplementary Data for Materials and methods. (b) Confirmation of cellular senescence with β-galactosidase immunocytochemistry. Bright-field optics. Left and right images respectively show representative growing and senescent (high-passage) cells from one MPM patient. Senescent cells display positive β-galactosidase activity (blue), also melanin (grey to black). Scale bar for both images = 100 μm.

Figure 2: Relationship of culture lifespan to donor age

Scatter plot illustrating relationship of culture lifespan with donor age from MPM (black points) and control SPM patients (red points). Color of regression line matches color of data points.

Figure 1

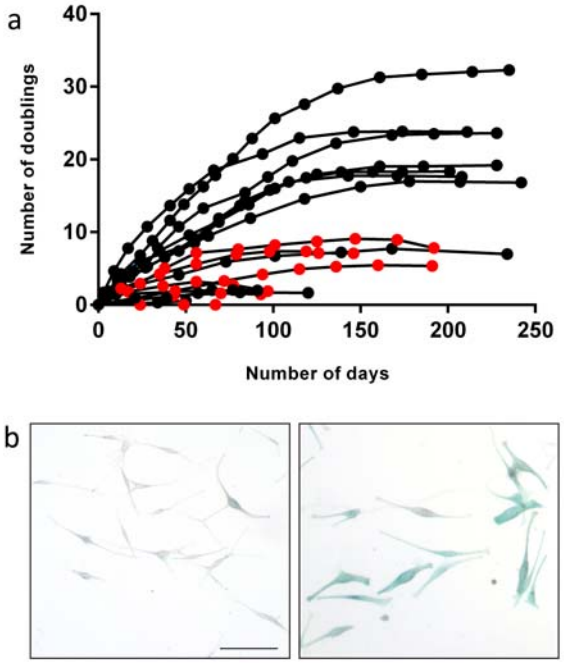
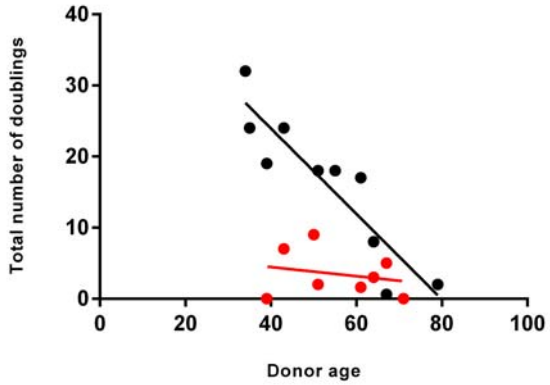

a
Number of doublings
40
30
20
10
0
0
50
100
150
200
250
Number of days
b

Figure 2


Total number of doublings
40
30
20
10
0
0
20
40
60
80
100
Donor age

SUPPLEMENTARY INFORMATION

METHODS

Primary culture and propagation of human melanocytes

Normal skin biopsies were obtained from patients with multiple or with a single melanoma upon informed consent in the Surgery Day Hospital at the Fondazione IRCCS Istituto Nazionale Tumori. A 5-mm punch biopsy of skin was taken under local anaesthetic and under aseptic conditions from the medial lower part of the arm. The biopsies were immediately placed in 15 ml sterile tubes full of chilled culture medium. Under sterile conditions, the biopsies were then transferred into another 15 ml tube with culture medium at pH 7 and sent on ice by next day delivery to St George's, University of London, where they were processed for cell culture.

Under sterile conditions, subcutaneous fat was removed from skin biopsies using a scalpel. Biopsies were briefly washed in penicillin/streptomycin (Sigma) and PBSA (Dulbecco's PBS without $MgCl_2$ and $CaCl_2$). Biopsies were then placed in two 5 ml volumes of 50 μg/ml gentamicin (Sigma) for 10 minutes each. Biopsies were then placed, epidermal side down, in 5 ml of 10 mg/ml dispase (Sigma) in Hank's balanced salt solution (Sigma) and kept overnight at $4^{O}C$.

The epidermis was peeled from the dermis the next day using sterile forceps. To ensure no possible contamination of dermal fibroblasts, forceps used to peel the epidermis never came into contact with the dermis. The epidermal sheet was cut into smaller fragments with a sterile blade and placed in 2.5 ml of 500 μg/ml trypsin (Sigma) in PBSA at $37^{O}C$ for 10 minutes. An epidermal suspension was made by continual shearing of the epidermis with a 21 gauge needle, attached to a 1 ml syringe. The suspension was centrifuged at 1,500 rpm for 15 minutes and the pellet was resuspended in 5 ml of growth medium. Primary cells were plated on to $2.5x10^5$ mitomycin C inactivated XB2 keratinocyte feeder cells in a T25 flask. Generation of mitomycin C inactivated XB2 feeder cells were carried out exactly as previously described (Soo et al., 2011). Components of melanocyte medium, as well as

protocols for passaging and generation of growth curves were exactly as previously described (Soo et al., 2011).

The study was reviewed and approved by the Institutional Review Board and the Independent Ethics Committee at Istituto Nazionale Tumori (ref. INT124/13), and by the West London & GTAC Research Ethics Committee (ref. 06/Q0803/39) for the cell culture and storage procedures.

β-galactosidase immunocytochemistry

Detection of β-galactosidase activity was carried out exactly as previously described (Soo et al., 2011).

Statistical analysis

Significance of the difference between total lifespans of melanocytes from MPM and SPM patients was tested using a one-tailed Mann-Whitney U test. Presence of a significant relationship between culture lifespan and donor age was tested using an F test for linear regression. An analysis of covariance was used to test whether slopes of regression lines were significantly different. Significance of the difference between the mean ages of MPM and SPM patients was calculated with a two-tailed t-test. All significance tests were calculated using GraphPad Prism version 6 software.

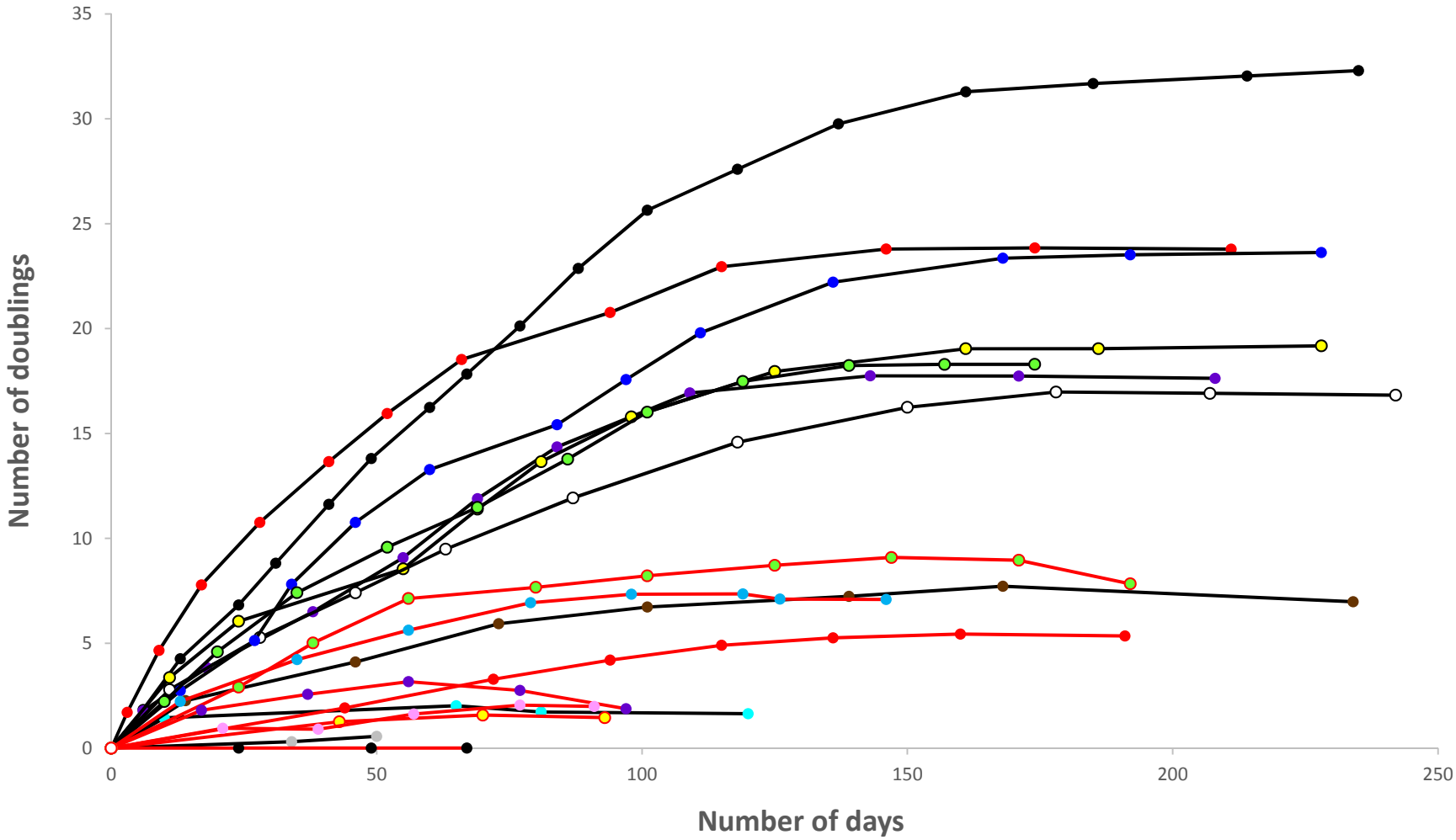


Figure S1. Cumulative growth curves of melanocytes from MPM and SPM patients. The same data as in Figure 1a, but in higher resolution, and here symbols are color coded and individual patient origin for each curve is identified in Table S2.

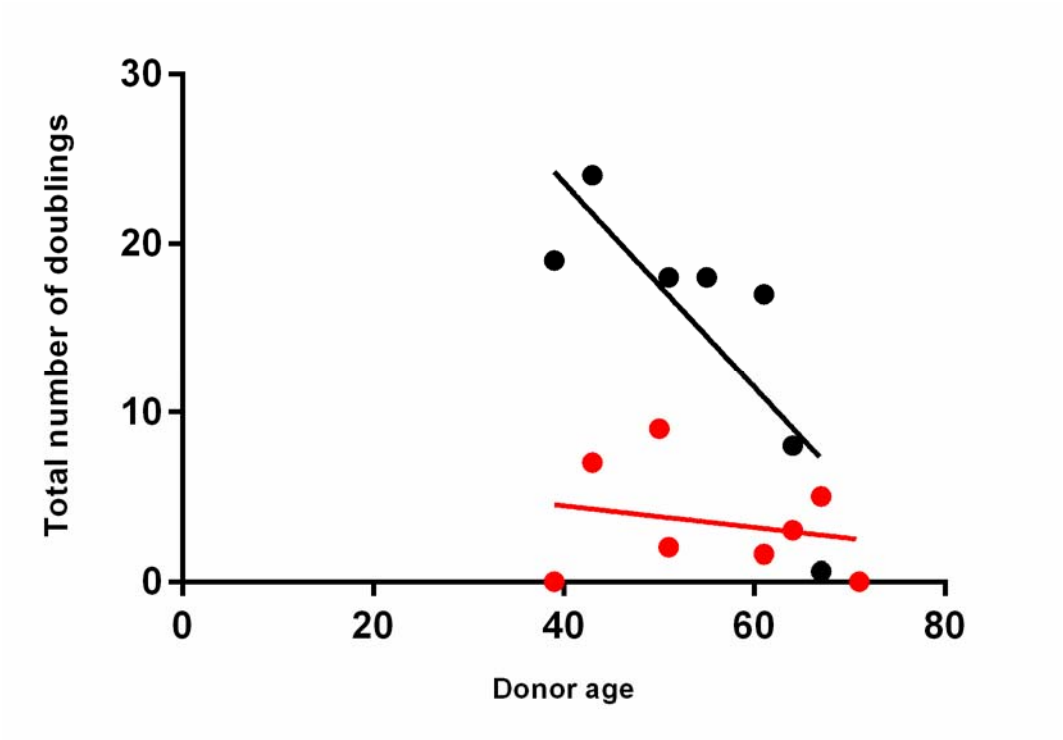


Figure S2. Culture lifespans from MPM patients within the same age range as SPM patients.

Scatter plot illustrating the relationship of culture lifespan to donor age from MPM (black points) and control SPM patients (red points). Colors of regression lines match color of data points.

Multiple Melanoma

Patient ID	Lifespan (doublings)[1]	Gender	Age	*CDKN2A*				*CDK4*	*MITF*	*MC1R*	*POT1*	*TERT*
				Promoter/5' UTR[2]	Exon 1α-2	Exon 3	Exon 1ß	Exon 2	*E318K*		Exon 10	Promoter
3M5	2	Male	79	-191 A/G	WT	540 C/T	WT	WT	WT	G248V; D294H	WT	WT
3M6	32	Male	34	-493 A/T	WT	WT	WT	WT	WT	WT	WT	-245 T/C
3M14	0.6	Male	67	WT	WT	WT	WT	WT	WT	WT	WT	-245 T/C
3M15	18	Male	55	WT	WT	540 C/T	WT	WT	WT	R160W;(I264I)	WT	-245 T/C
3M16	24	Female	35	WT	WT	WT	WT	WT	WT	R151C	WT	-245 T/C
3M20	19	Female	39	-191 G/G[3]	WT	WT	WT	WT	WT	V60L	WT	-269 G/A
3M22	17	Female	61	-191 A/G; -735G/A	WT	500 C/G	WT	WT	WT	V60L	WT	WT
3M23	8	Male	64	WT	WT	WT	WT	WT	WT	D294H	WT	WT
3M24	24	Male	43	-191 A/G	WT	WT	WT	WT	WT	WT	WT	WT
3M50	18	Male	51	-191 A/G; -735G/A	WT	500 C/G	WT	WT	WT	R151C	WT	WT

Single Melanoma

Patient ID	Lifespan (doublings)[1]	Gender	Age	*CDKN2A*				*CDK4*	*MITF*	*MC1R*	*POT1*	*TERT*
				Promoter/5' UTR[2]	Exon 1α-2	Exon 3	Exon 1ß	Exon 2	*E318K*		Exon 10	Promoter
3M36	9	Female	50	-191 A/G; -735G/A	WT	500 C/G	WT	WT	WT	V92M; R142H	WT	WT
3M37	1.6	Female	61	-735G/A	WT	500 C/G	WT	WT	WT	V92M; R160W	WT	WT
3M38	5	Female	67	WT	WT	WT	WT	WT	WT	V92M; (T314T)	WT	WT
3M51	0	Male	71	- 191 A/G	WT	WT	WT	WT	WT	WT	WT	WT
3M52	7	Male	43	- 191 A/G	WT	WT	WT	WT	WT	V60L	WT	WT
3M58	3	Male	64	WT	WT	WT	WT	WT	WT	R142H; R151C	WT	-245 C/C[3]
3M59	2	Male	51	WT	WT	WT	WT	WT	WT	V60L	WT	WT
3M60	0	Male	39	- 191 A/G	WT	WT	WT	WT	WT	R160W	WT	WT

Table S1. Genotypes of known familial melanoma genes, with gender, age and melanocyte culture lifespan, of MPM and SPM patients in our study.

Genotyping was carried out by sequence analysis according to published protocols (Bruno et al, 2016; Shi et al, 2014; Horn et al 2013).

[1] Lifespan of normal epidermal melanocyte culture from each patient, in population doublings.

[2] Polymorphisms found at positions -493 and -735 are located in the promoter while that at position -191 is located in the 5' UTR.

[3] Polymorphisms are homozygous in these patients.

Multiple Melanoma

Patient ID	Growth curve symbol
3M5	
3M6	
3M14	
3M15	
3M16	
3M20	
3M22	
3M23	
3M24	
3M50	

Single Melanoma

Patient ID	Growth curve symbol
3M36	
3M37	
3M38	
3M51	
3M52	
3M58	
3M59	
3M60	

Table S2. Color key to donor patient for each melanocyte growth curve in figure S1.

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