Our research convincingly argues that:
1: the best indicator of age is the abundance of short telomeres,
2: even weak Telomerase inducers such as TA-65 will increase the abundance of short telomeres,
3: that family 5 compounds are safe for use on skin.
4: Telomerase has an anti-aging effect on skin.
5: Family 5 compounds are much stronger than TA-65.

Several years ago Sierra Sciences created and patented what is the world's most effective telomerase inducing compounds. This compound is perfect for immediate sale to the cosmetics industry. There are 6 of these molecules, all very similar in structure and function. We have named them Family 5. We believe Family 5 will be of great value to a multi-national skincare company which can claim, with proven scientific evidence, to exclusively have the only telomerase inducing ingredient in their products. Our Family 5 molecules have been tested on human cells. In fact, all of the data presented is the result of testing on human cells both to determine effectiveness at inducing telomerase and to prove safety and suitability for use in topical application. This is why we are certain that these compounds will be a valuable asset to a skincare company.

Sierra Sciences have been basically sitting on a goldmine without realizing it. It was not until the recent buy out of Sierra Sciences By Andrews Inc and a more forward thinking way of evaluating things that the right questions were asked and the conclusion reached that not only are these molecules commercially viable they are VERY profitable due to the concentration at which they are most effective with just 1g of our chemicals delivering 100litres of full potency topical applications. These molecules are seriously for sale today! With the option for exclusive rights, too!

Sierra Sciences is looking to sell these molecules to a large, successful, science-based skincare company. Our goal is to sign an exclusive agreement with a major skin care company with sales of over $200,000,000 per annum and to have a similar structure as we do with Isogenix whereby we receive a percentage of the retail value of their products as payment for and exclusive rights to our molecules.

	Non GMP			GMP grade		
	500g	**1kg**	**10kg**	**500g**	**1kg**	**10kg**
CO314813						

Lead time (weeks)	**4-6**	**6-8**	**8-10**	**8-10**	**8-10**	**12-15**
CO314814						
Lead time (weeks)	**5-7**	**8-10**	**10-12**	**10-12**	**10-12**	**15-17**
CO314817						
Lead time (weeks)	**4-6**	**6-8**	**8-10**	**8-10**	**8-10**	**12-15**
CO314818						
Lead time (weeks)	**5-7**	**8-10**	**10-12**	**10-12**	**10-12**	**15-17**
CO314272						
Lead time (weeks)	**5-7**	**8-10**	**10-12**	**10-12**	**10-12**	**15-17**
CO314280						
Lead time (weeks)	**5-7**	**8-10**	**10-12**	**10-12**	**10-12**	**15-17**

UNDERSTANDING THE DATA

Our cells undertake distinct steps to express a gene (that is, use our genetic blueprint to create a protein, like telomerase). The first step is “transcription”, where messenger RNA is made from the DNA blueprint. The final step is “translation,” where the cells make a protein using the instructions in the mRNA. If we can confirm that we have both the first and last step working, it is a reasonable assumption that the compound is doing exactly what we want it to do.

Our hTERT mRNA assay is a way for us to observe transcription initiation. We isolate the messenger RNA and make millions of copies of it using PCR, a technique that amplifies DNA or RNA, so that it can be measured. We run PCR until the telomerase mRNA reaches a specific, measurable threshold. Then, by counting the number of PCR amplifications we had

to do, we can mathematically extrapolate how much there was to begin with.

The first chart shows that the telomerase gene was transcribed and processed correctly. When we ran PCR, we indeed saw a strong signal of telomerase mRNA. On the y-axis, we're measuring mRNA based on a unit called "Ct." That is the number of times we had to amplify the mRNA before it reached our specific threshold. The more mRNA that was there to begin with, the fewer Ct it takes to reach that threshold. So, when the Ct is a smaller number, that means there was more mRNA present to begin with.

In a nutshell, the y-Axis shows how much mRNA our compound induced. The taller the bar, the stronger the compound when it comes to inducing transcription initiation.

The yellow bars show how toxic the compound was by showing what percent of living cells were counted after exposure to the compound. The lower the bar, the more toxic the compound is. As you can see, C0314818 is not very toxic at all. On the right of the graph is our positive control, C0057684, the first telomerase inducer we discovered, and the one we've most thoroughly studied. The chart shows that C0314818 is about three times stronger than C0057684.

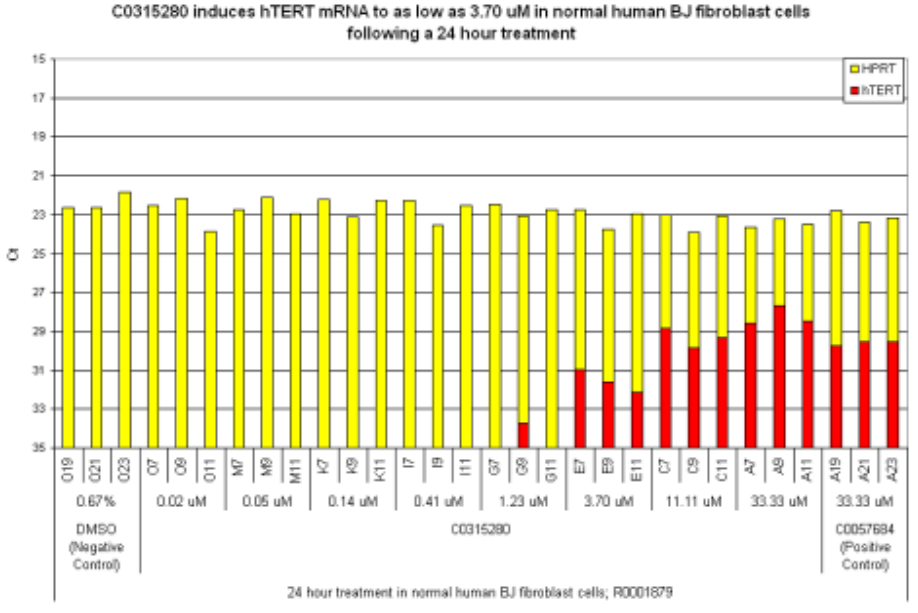
The second chart is TRAP data. TRAP measures protein activity and shows that translation, the final step in gene expression, successfully occurred. In TRAP, we're directly measuring how much telomerase protein is present. So, we know that our compound both began and finished gene expression. It presumably went through all the steps.

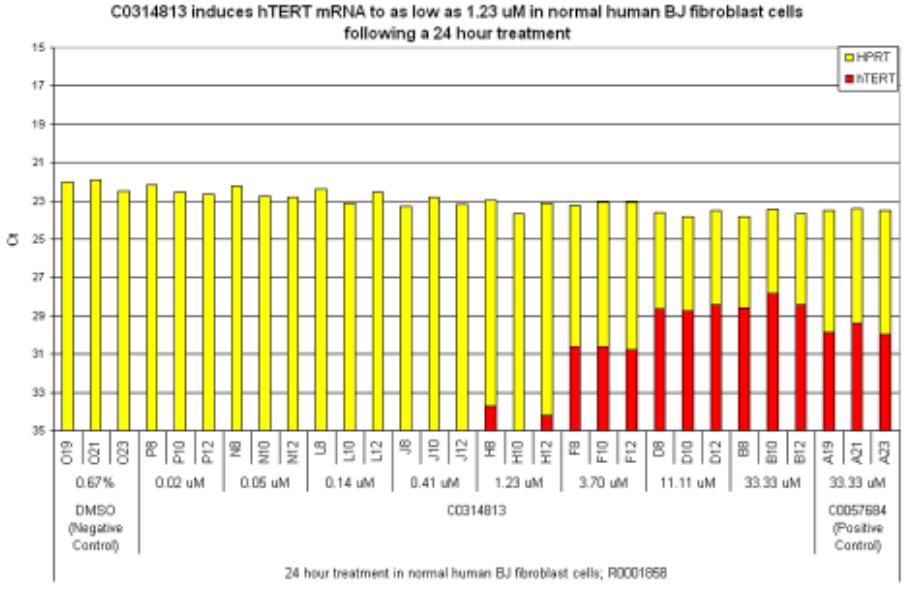
TRAP works using a technique called gel electrophoresis. Gel electrophoresis can sort DNA molecules based on size, and display them as distinct bands on a gel. Telomerase is a unique enzyme, in that it lengthens telomeres 6 bases at a time, so when you sort the molecules into bands on the gel, you see a repetitive sequence of 6 basis, forming a "ladder" pattern. If you see a ladder, you know you have telomerase. And the darker the ladder is, the more telomerase is present.

The TRAP data on C0314818 shows that we see activity at concentrations from 3.70 micromolar up to 33.33 micromolar. Again, TRAP confirms that C0314818 is about three times stronger than C0057684 at a concentration of 33.33 micromolar. We measure the intensity of the bands with a tool called a spectrophotometer.

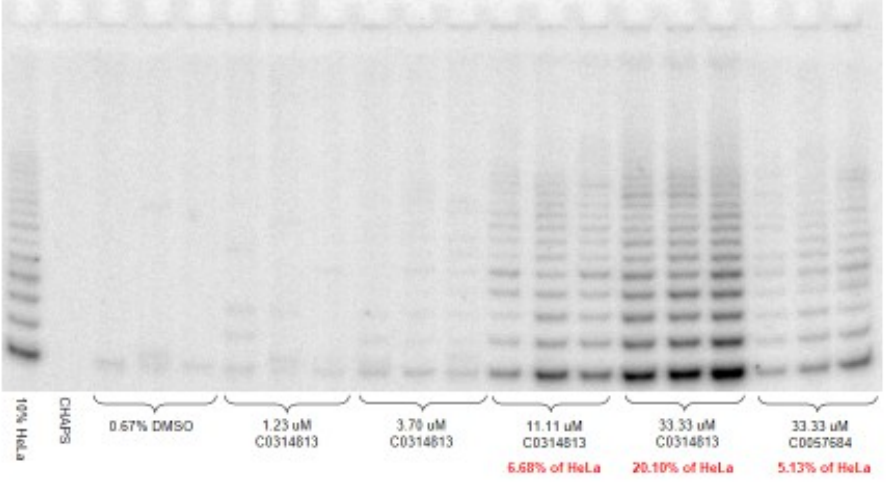
Our control for TRAP is a cell line called HeLa, which produces what we believe to be the minimum amount of telomerase necessary to make a human cell immortal. The bands

created by C0314818 are 1.6 times as strong as bands created by measuring a mixture that is composed 10% of HeLa cells. Therefore, we conclude that C031818 causes a cell to produce about 16% of the telomerase that it would take to make that cell immortal.





C0314813 induces telomerase activity to as low as 11.11 uM in normal human BJ fibroblast cells following a 48 hour treatment



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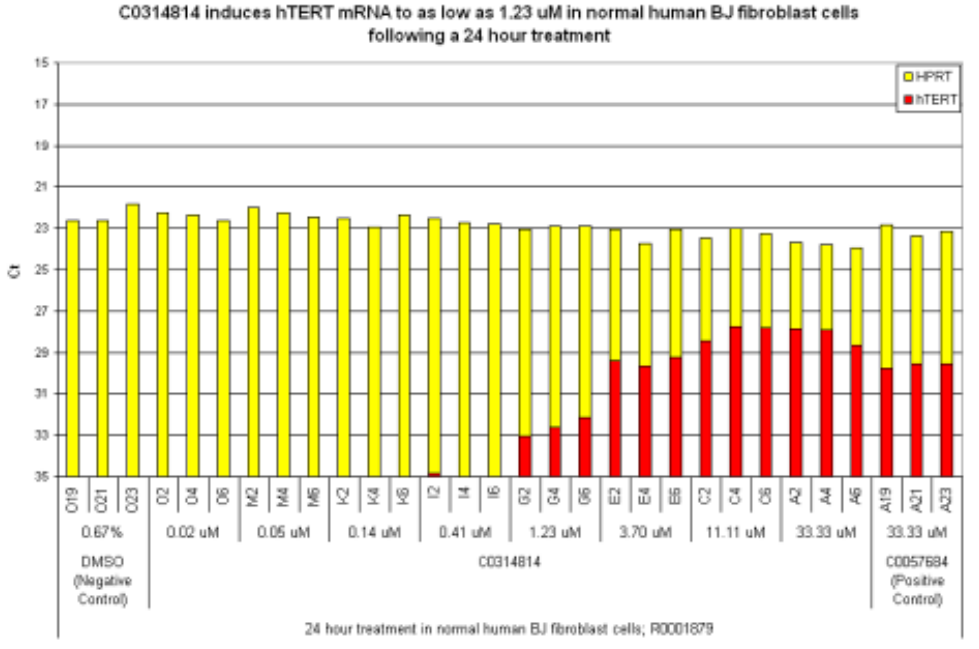
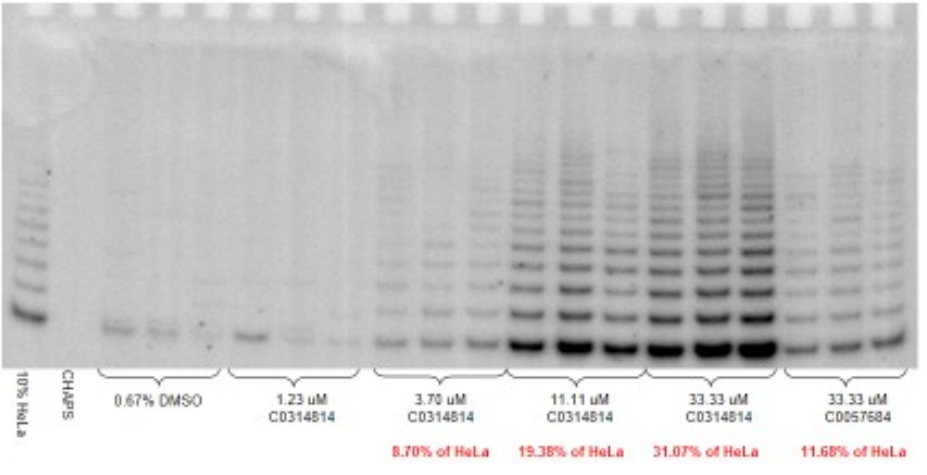


C0314814 induces telomerase activity to as low as 3.70 uM in normal human BJ fibroblast cells following a 48 hour treatment



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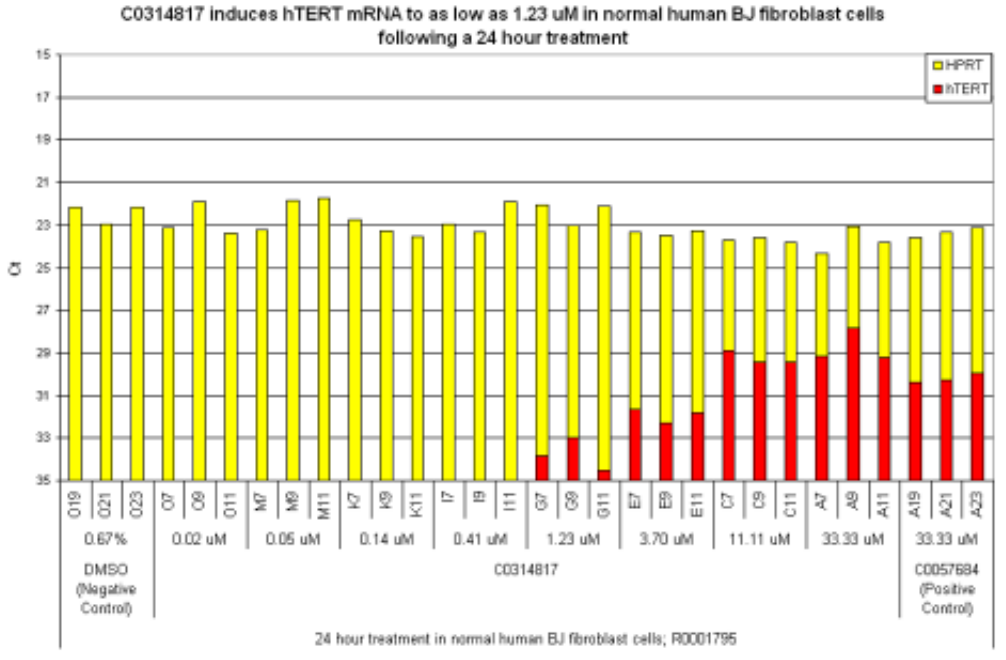
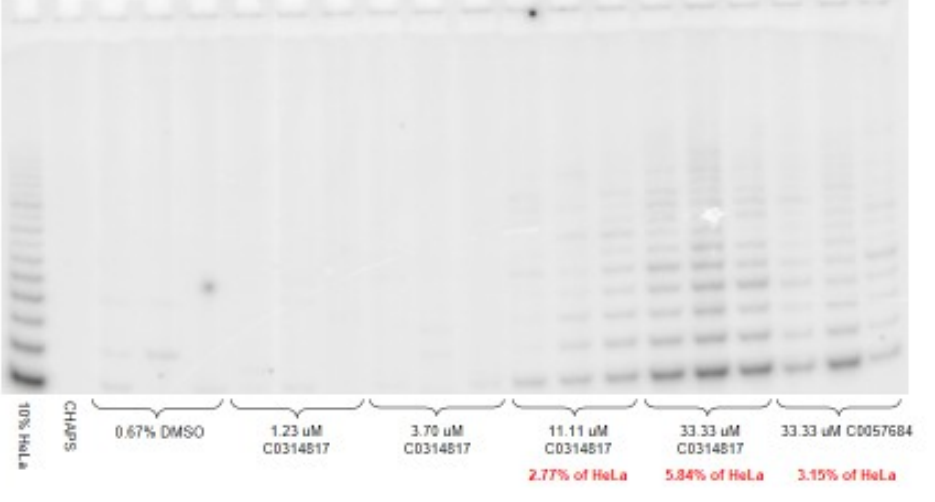


C0314817 induces telomerase activity to as low as 11.11 uM in normal human BJ fibroblast cells following a 48 hour treatment



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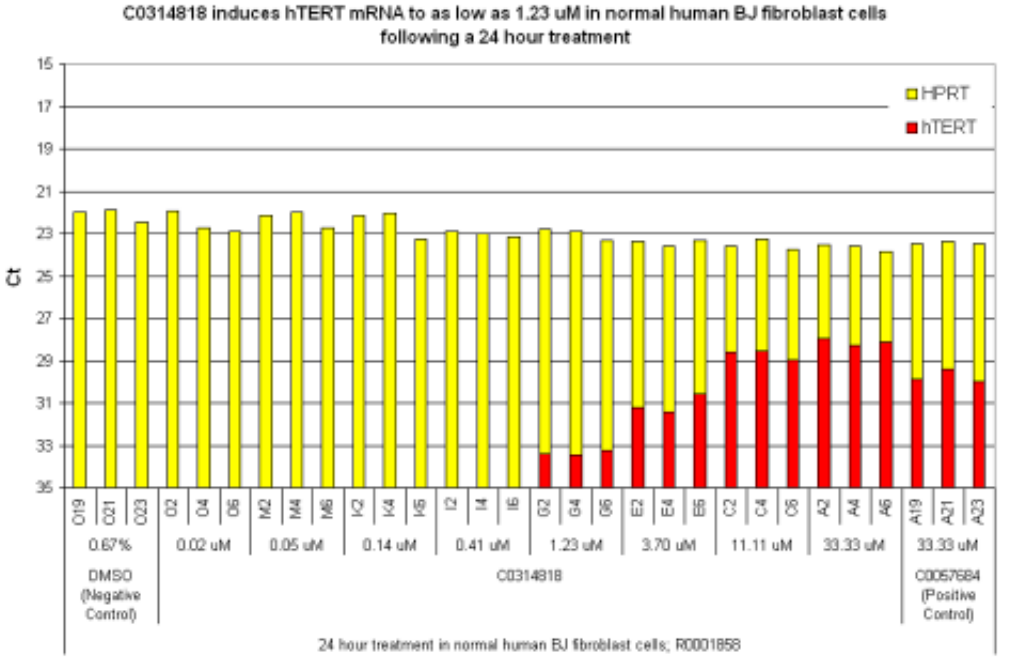

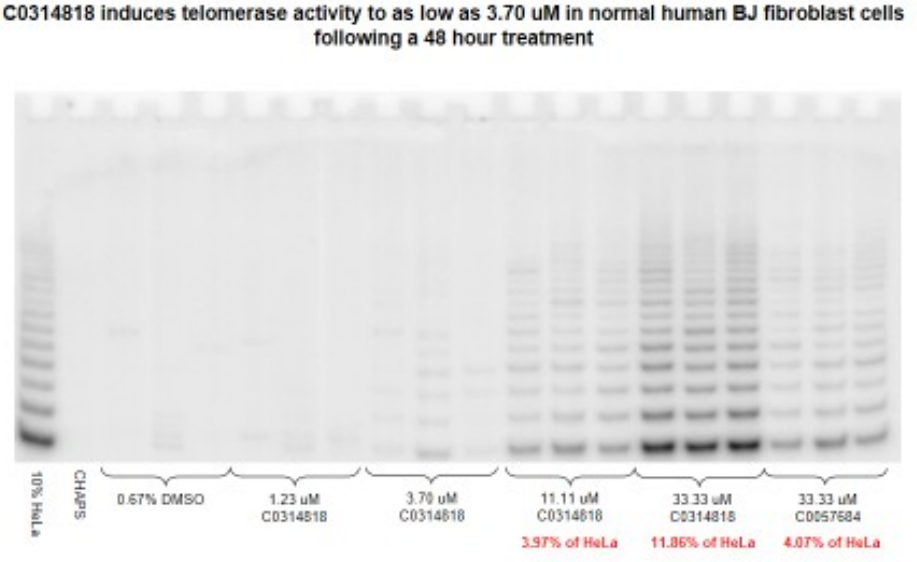

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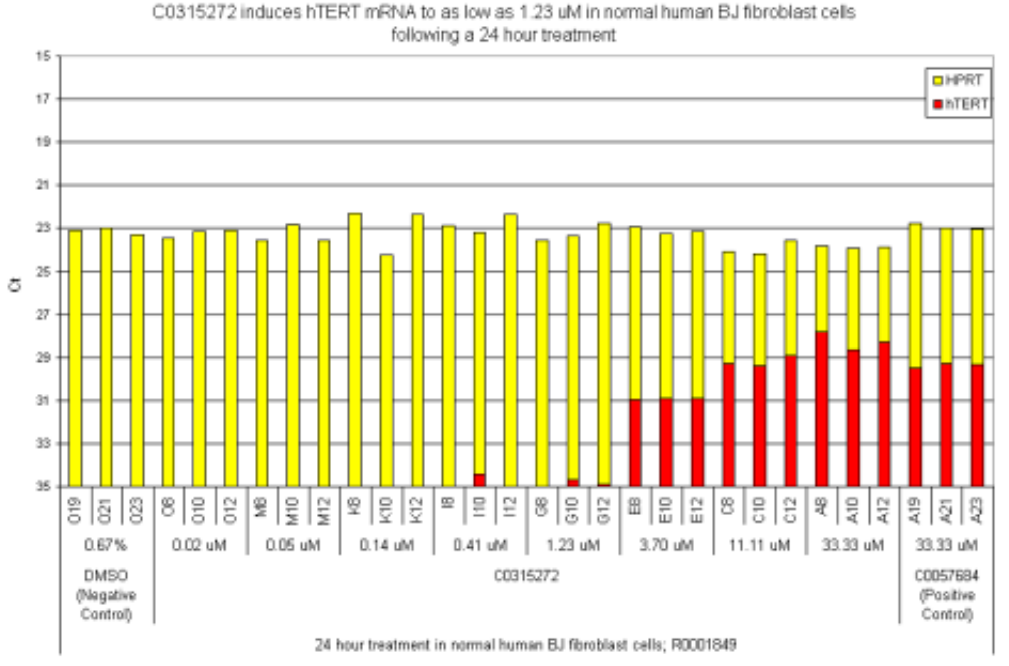
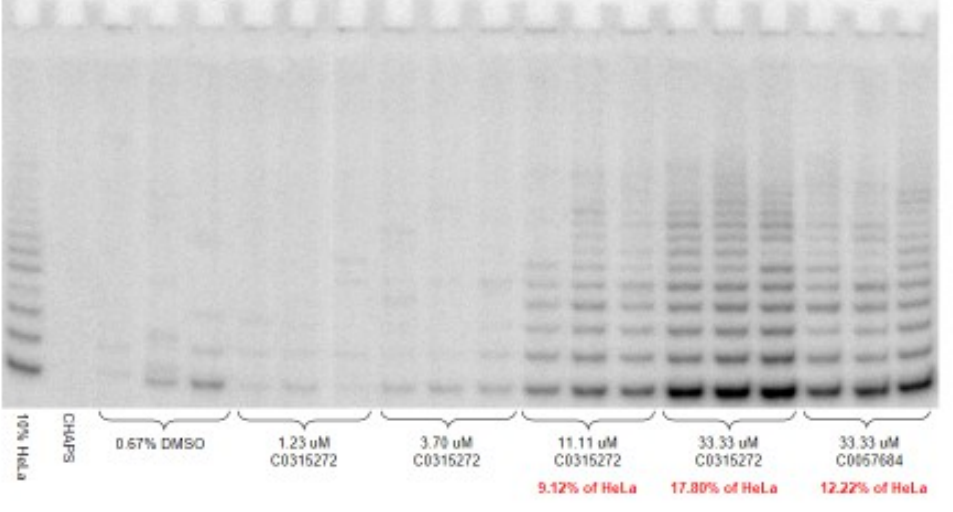


C0315272 induces telomerase activity to as low as 11.11 uM in normal human BJ fibroblast cells following a 48 hour treatment



Assay: 11/22/11, CE/Lane: 700, CE/Assay: 1000; 32P reference date: 11/12/11, TS label: 11/17/11 LKB, Taq Pol: Platinum Taq; Lot# 1038844 ; Cat# 10966-034; Invitrogen, PCR: 27cycles, PhosphorImager exposure: Overnight, 12.5% PAGE, 0.8mm spacers, run: 300 V for .30h 400V for 1.15h. Grey Scale: 699.93. CMPD QC0056

We commissioned Abich laboratories S.r.l. - based in Lago Maggiore. Northern Italy. 150 grams of each sample to Dr Elena Bocchietto (Abich Laboratories) for the following evaluations:

SYNOPSIS

TESTING of NEW COMPOUNDS (SMALL MOLECULES)

There are proper procedures for determining proof of concept - Lead compounds go through a series of tests to provide an early assessment of the safety of the lead compound. Scientists test Absorption, Distribution, Metabolism, Excretion and Toxicological (ADME/Tox) properties, or "pharmacokinetics," of each lead. Successful compounds (drugs) must be:

- Absorbed into the bloodstream,
- Distributed to the proper site of action in the body,
- Metabolized efficiently and effectively,
- Successfully excreted from the body and
- Demonstrated to be not toxic.

In this instance, as a starting point, very rudimentary safety profile and risk assessment testing was carried out

SAMPLE PREPARTION

The compounds were used at 0.25% w/w. This value of 0.25% was selected randomly but on the lower side first and foremost because of its unknown nature. Stereotypically topical treatments have active components loaded at anywhere from 0.01% to 2% to achieve efficacious functionality.

1. Synthetic compounds as supplied by Federico Gaeta - L031272-02 & L0314813-02

2. **L031272-02**: Appears to be only partially water-soluble. There are various options for achieving improved product stability and skin delivery. In this situation we selected liposomal delivery.
   The active was encapsulated into a multilamellar vesicle liposome then added to the emulsion as per normal production parameters.

   - The Liposomal ingredients consisted of Phosphatidylcholine, Hydrogenated Lecithin, Glycerin and Alcohol.

   - Encapsulation was achieved by microfluidisation – Although this was not measured or sized a milky solution without separation was realized during the process. This normally is evident of liposome formation.

- Loading at 10% min (~ 12-15%)

3. **L0314813-02:** This compound appears to be water dispersible or at least predominately soluble – This was pre-dispersed in water at 30°C then immediately added to the emulsion at the final stage under homogenization.

4. Both Actives were then introduced to their respective emulsion systems at temperatures below 30°C

5. **The Emulsion system** used in both test cases was a Lipid Glucoside system. Which could be deemed as an advanced emulsion system – it provides a liquid crystal structured emulsion (Anisotropic fluids). From experience we know this system provides 1-5 micron size droplets with excellent stability and superior dermal delivery.

6. **The Emulsion Ingredient List:** Water – (emulsifiers) Cetearyl Glucoside - Arachidyl Glucoside – Behenyl, Arachidyl, Cetearyl Alcohols - (Emollients') C12-15 Alkyl benzoate – Diethylhexyl succinate – Jojoba Oil – Almond Oil – (Humectant) Glycerin – (Preservatives) Caprylhydroxamic Acid - Glyceryl Caprylate

7. 200 gram samples prepared with the active compound added to ensure 0.25% w/w of respective compounds
- L0314813-02 ➔ When added a drop in viscosity was evident but did not require adjustments – Final pH 5.6
- L031272-02 ➔When added no dramatic change was noted – Final pH 5.7

- T023777-02 is a PLACEBO/BLANK – Cream Base only - minus any active compounds

8. **Stability** – At this stage a small 20-gram sample of each was retained for real time stability testing or observation. Currently there are no test protocols developed to determine the compound behavior or quantitative measurement in emulsion systems. Obviously it is too early to comment on stability.
In the cosmeceutical arena we only measure emulsion stability by microbiological and chemical parameters. Normally physical and rheological changes are evidence of emulsion instability – Be it preservative failure or

internal physical and chemical structural failure.
Once commercial products are developed a dedicated stability program would be required – Guidelines would be based on PAO (Period after opening) This would require 30 months shelf life

BACK GROUND INFORMATION

As discussed previously – Initially the products are to be promoted as cosmeceuticals, and not medicine/medical devices, which basically mean far less stringent testing and regulatory constraints. Where applicable or used, a normative reference has been provided.

However: As per the Council Directive 76/768 and the Regulation (EC) no 1223/2009, any cosmetic product marketed or sold within the Community must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use.
To this purpose specific studies are performed to protect both the manufacturer and the end consumer.
After performing toxicological evaluations and specific in-vitro preliminary tests, a competent specialty laboratory can assess the tolerability of a cosmetic product with tests on human subjects.
These tests are normally conducted under strict supervision of an investigator on panelists who are enrolled according to the following inclusion criteria:
(a) good state of health,
(b) no skin disease,
(c) no pharmacological treatment in progress,
(d) promise not to change one's daily routine
(e) negative anamnesis for atopy.

All the partnership facilities that we work with follow the Ethical Principles for Medical Research Involving Human Subjects written in the "World Medical Association Declaration of Helsinki 2008"

We commissioned Abich laboratories S.r.l. - based in Lago Maggiore. Northern Italy. 150 grams of each sample to Dr Elena Bocchietto (Abich Laboratories) for the following evaluations:

- Cytotoxicity (in-vitro) UNI EN ISO 10993-5: 2009
- Dermal Irritancy & Sensitisation,
- Patch Testing,

Please note: ALL testing facilities require disclosure of active compounds being tested – I did not disclose details or any information as to the source of these compounds – For the purposes of this testing, they were registered as "Peptides"

Where applicable or used, a normative reference has been provided.
CYTOTOXICITY - UNI/ENISO10993-5:2009
Aim of the test is to evaluate the cytotoxicity of finished products or raw materials intended to be used on the skin or on the mucosae following the UNI EN ISO 10993-5 standard concerning the biological evaluation of medical devices.

The MTT assay determination assay is simple, accurate and yields reproducible results. Mossman originally developed this method.

The cytotoxicity assay performed in this study was designed to evaluate the cytotoxic potential of the tested product on the fibroblasts/ keratinocytes. .
This method is customized for improved result interpretation based on historical results obtained in this particular laboratory with an improved non-standard keratinocyte cell line.

The key component is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
or MTT. This product is of yellowish colour in solution. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, leading to the formation of purple crystals that are insoluble in aqueous solutions. The crystals are re-dissolved in isopropanol and the resulting purple solution is measured spectrophotometrically.
An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

Cells have been seeded in 96 wells plates (10000cells/well) and allowed to grow for 24 h at 37°C and 5% CO2.
The second day fresh medium is added, supplemented with 6 scalar dilutions of the tested product ranging from 5.00 mg/ml µl/ml and 0.16 mg/ml µl/ml. The sample has been dissolved directly in the medium culture. The test is carried out in three replicas for each test dilution. At the end of the incubation period, the cells are tested for their viability with the cytotoxicity (MTT) assay. Cells treated with a known irritating surfactant (Sodium Lauryl Sulfate – SLS) in concentration ranging from 0.5mg/ml to 0.03mg/ml are used as positive control. Untreated cells are used

as blank control. The MTT assay allows to evaluate the toxic impact of the tested compound on the cells viability.

After 24h exposure of the cells to the test material, the culture medium is removed and the cells incubated for 2 h in 100 µl/well of 1mg/ml MTT solution at 37°C. The solution is then removed and replaced with 200 µl/well of isopropanol, with further 30' incubation at room temperature under medium speed shaking.
The absorbance at 570 nm is measured with a microplate reader (Tecan modello Sunrise remote), deducting background at 650 nm. The absorbance values are corrected by subtracting the reading of the blanks, with the diluent only.

The results are expressed in terms of viability:
% **Of cell viability** = [OD(570 nm - 650 nm) test product / OD(570 nm - 650 nm) negative control] x 100
In that case it is possible to calculate the IC50 value (Inhibiting Concentration 50), which indicates the concentration of the test compound, which inhibits the cell viability of 50%.
For finished products, IC50 values higher than 1mg/ml (or 1µl/ml) may be considered as irritating, values lowest than 3mg/ml (or 3µl/ml) show a good biocompatibility. For detergents and rinsing off products, the eventual dilution factor should be taken in account. For raw materials, the use concentration should be taken in account.
(Translated from Italian)

RESULTS

Compound	**IC50/ IC50 values**	**Explanation**
L031272-02	> 5mg/ml	Very good biocompatibility-not cytotoxic
L0314813-02	> 5mg/ml	Very good biocompatibility-not cytotoxic
T023777-02	> 5mg/ml	Very good biocompatibility – not considered cytotoxic

SKIN IRRITATION
OECD/OCDE: Guidelines for the testing of chemicals
In-vitro Irritation on reconstituted human epidermis test method
Skin irritation and skin sensitization are different types of reactions… the first is caused by irritating agents (such as SLS) and it may happen after the first contact with the

irritating substance. The sensitization is a more complex reaction in which immune system is involved (repeated exposure to the allergens are needed to evoke the activation of the immune system).

1. The test chemical is applied topically to a three-dimensional RhE model, comprised of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human *epidermis*. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*.
2. Chemical-induced skin irritation, manifested by erythema and oedema, is the result of a cascade of events beginning with penetration of the *stratum corneum* and damage to the underlying layers of keratinocytes. The dying keratinocytes release mediators that begin the inflammatory cascade that acts on the cells in the *dermis*, particularly the stromal and endothelial cells. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema (24). The RhE-based test methods measure the initiating events in the cascade.
3. Cell viability in RhE models is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1], into a blue formazan salt that is quantitatively measured after extraction from tissues (25). Irritant chemicals are identified by their ability to decrease cell viability below defined threshold levels (*i.e.* ≤ 50% for UN GHS Category 2). Depending on the regulatory framework and applicability of the Test Guideline, chemicals that produce cell viabilities above the defined threshold level, may be considered non-irritants (*i.e.* > 50%, No Category).

RESULTS

Compound	Results
L031272-02	Potential threshold > 50%
L0314813-02	Potential threshold > 50%
T023777-02	> 50%

SKIN SENSITISATION

Monocyte-derived human Immature Dendritic Cells as *IN VITRO* Models for predicting potential sensitizing of chemicals.

It is now well established that dendritic cells (DC) play pivotal roles in the initiation and orchestration of adaptive immune responses, including cutaneous immune responses to chemical allergens that drive the acquisition of skin sensitization. It is not unexpected; therefore, that a large number, and wide variety, of proposed approaches for the identification of skin sensitizing chemicals *in vitro* are based upon

the use of cultured DC or DC-like cells. The use of DC in this context is legitimate. However, with our rapidly increasing understanding of the diversity of cutaneous DC with respect to both phenotype and function, it is timely now to review briefly the potential limitations and interpretive difficulties that are associated with the use of DC-based assays. Among the important considerations are the fact that chemical-induced changes in the characteristics and function of cultured DC will not necessarily reflect accurately the events that that support the development of skin sensitization *in vivo*. In addition, most DC-based assays are predicated on a view that cutaneous DC have as their primary function the initiation of adaptive immune responses. However, it is now appreciated that cutaneous DC, and in particular epidermal Langerhans cells (LC), may also play important immunoregulatory roles that serve to limit and contain skin immune responses. Notwithstanding these considerations there is reason to believe that at least some *in vitro* DC-based assays are of value, and indeed some are currently the subject of a formal validation process. However, it is appropriate that such assays are configured and interpreted carefully, and with an appreciation of the complexity of DC biology.

RESULTS

L031272-02	As tested deemed not Sensitizing
L0314813-02	As tested deemed not sensitizing
T023777-02	As tested deemed not sensitizing

Read More: http://informahealthcare.com/doi/abs/10.3109/15569527.2012.692135?journalCode=cot

Human skin equivalent / reconstituted epidermis cultures

In vitro three-dimensional cultures of living human skin generally fall into two categories: epidermal or skin equivalents. Epidermal equivalents consist of keratinocytes which, when cultured on a filter or matrix at the air-liquid interface, develop into a fully differentiated epidermis with a stratum corneum. Kubilus et al. (1996) examined cell viability, histological changes and the release of IL-1 and prostaglandin E2 (PGE2) by the EpiDerm model following treatment with irritants and allergens. They found that the dose response curves for the release of IL-1 and PGE2 following treatment with contact irritants reflected the cytotoxicity of the dose (i.e. the higher the cytokine release, the greater the cytotoxicity).
However, following treatment with contact allergens, the amount of cytokine released did not correspond directly to the degree of cytotoxicity as more IL-1 and PGE2 were produced at non-cytotoxic doses of the chemical and varied with the

allergen. The SkinEthic model was used by Coquette et al. (1999) to measure IL-1 and IL-8 mRNA and protein levels as well as cytotoxicity following irritant and allergen treatment. More recently, Coquette et al. (2003) have suggested that determination of IL-8, with IL-1, and MTT conversion shows potential to discriminate and classify irritants and allergens in a single assay. Like Kubilus et al., they report that IL-1 release increases with cytotoxicity after treatment with the irritants Triton X100 and BC, but had no effect on IL-8 levels. In contrast, the allergen DNCB did not induce IL-1 but instead, elicited elevated IL-8 levels. However, when mRNA expression was measured, BC, Triton X100 and DNCB all induced an up-regulation in message for IL-8 while only BC up-regulated IL-1 mRNA.

The results of a number of studies conducted to assess the ability of irritants and contact allergens to affect cytokine mRNA levels in skin equivalents have been reviewed by Gerberick and Sikorski (1998). Data from those studies indicated that while cytokine message could be modulated by chemical treatment, each chemical appeared to produce changes in cytokine mRNA expression that was unique to the chemical and was also concentration and or time-dependent. No single cytokine or profile of cytokines was identified which were predictive for sensitization potential. However, Corsini et al. (1999) recently reported that interleukin-12 (IL-12) is selectively induced in the Episkin model by treatment with chemical allergens. Following a 3 hour exposure to test chemicals, mRNA levels for both IL-12 p40 and IL-12 p35 were found to be up-regulated by contact allergens (oxazolone, eugenol and DNCB) but not the irritants (SLS and BC).

1. References - See body of the text
2. Developer of the method - Not applicable
3. Known users - None
4. Status of validation and/or standardisation – No validation of test methods to date.
5. What efforts are needed to complete validation of the method not applicable

PATCH TESTING

SKIN IRRITATION TEST - Patch Test

(Irritation and delayed-type hypersensitivity)

The evaluation of a potential irritant effect of a cosmetic product according to the amended Draize method. The scope of this test is to evaluate the tolerability of a cosmetic product by determining and classifying its potential irritant effect.
This test may support the 'dermatologically tested' claim.

- 20 healthy volunteers – Female and Male between the age of 18 and 65
- Over a 72 hour period

RESULTS

Compound	MII (Mean Irritation Index)	Classification
L031272-02	≤ 0.4 (> 80%)	Non Irritant
L0314813-02	≤ 0.4 (> 70%) ≤ 0.5 – 1.9	Non irritant
T023777-02	≤ 0.4 (>70%) ≤ 0.5 – 1.9	Non Irritant

Human Repeated Insult Patch Tests (HRIPT)

Short description, scientific relevance and purpose

The HRIPT is performed in at least four different forms. The concentration of material chosen for induction and challenge in the HRIPT is determined by considering the following factors: previous human experience, previous sensitisation tests in guinea pigs and irritation studies in humans. It is common practice to test multiple substances simultaneously, because it saves time and cost, but the scientific basis for multiple simultaneous inductions is not substantiated.

a) Draize test: ten consecutive induction patches are applied to new skin sites on the arms or back for 24 hours every other day 3 times a week. Each induction site is evaluated for erythema and edema after removal of the patch. Two weeks after the last induction, a challenge patch is applied for 24 hours and subsequently read. The response after challenge is compared to the responses reported after the early induction patches.

b) Shelanski-Shelanski test: it is comparable to the original Draize HRIPT but employs 15 consecutive induction patches to the same site and if erythema and/or edema develops during induction the following patch should be moved to an adjacent untreated area. 2-3 weeks after the last induction a challenge patch is applied for 48 hours and scored. The induction patch responses are also noted and interpreted as evidence of cumulative irritation.

c) Voss-Griffith test: it is also like the original Draize HRIPT with nine 24-hour patch tests conducted over a 3 weeks period and challenge is performed 2 weeks later with duplicate patches applied to the induction skin site and to the opposite arm. This assay allowed testing of four materials simultaneously. Repeated challenge is recommended in case of dubious reactions.

d) Modified Draize test: it differs from the original Draize test by subjecting the volunteers to a continuous induction period with patch exchange 3 times a week until a total of 10 patches have been applied. The patches are reapplied to the same site, and only if moderate inflammation has developed, the next patch is moved to an

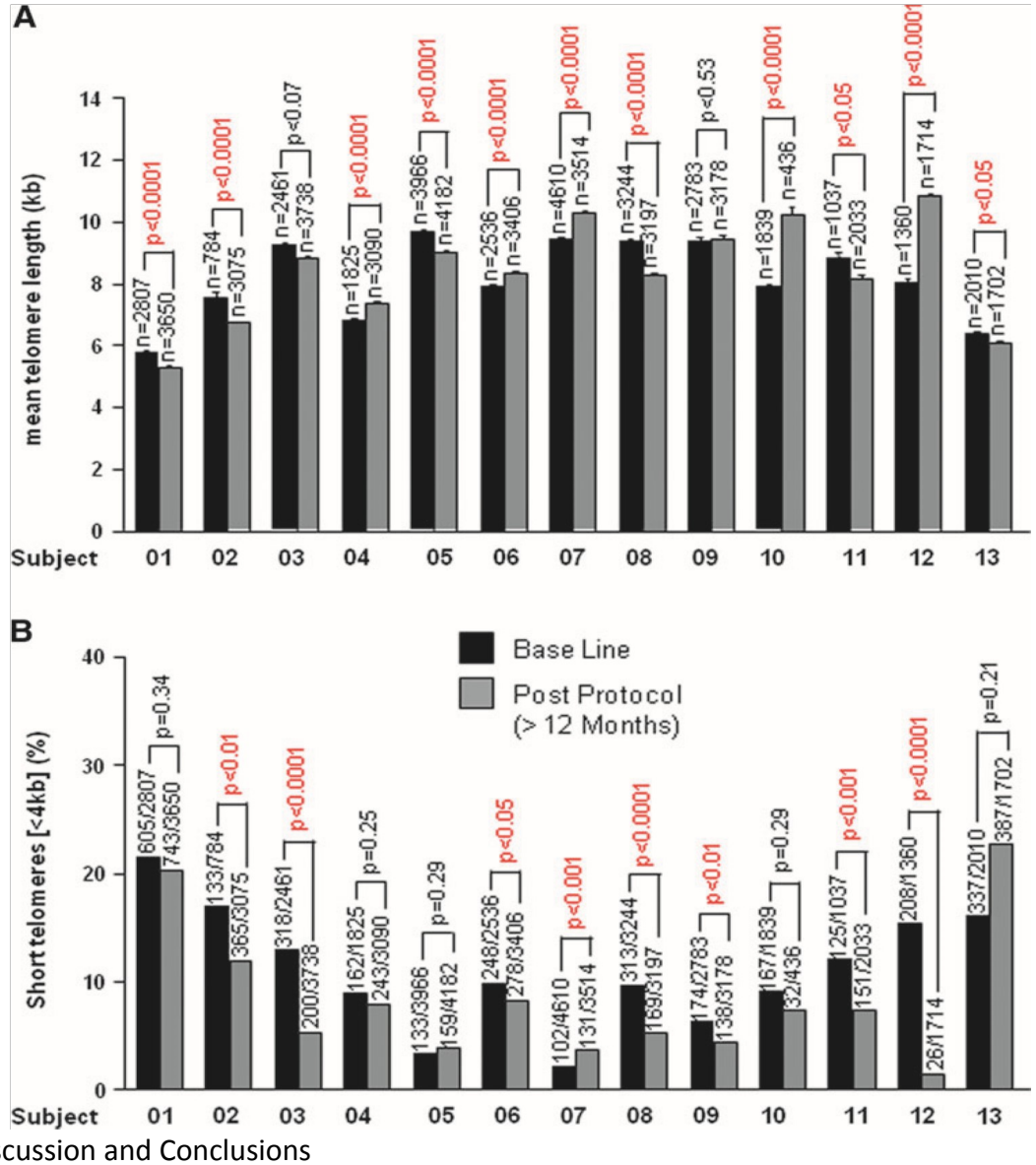
adjacent skin site. Challenge is performed on naive skin two weeks later with a 72-hour patch test with a non-irritating concentration of the substance.
Nowadays, the HRIPT is usually only performed to confirm the safe use of potentially
sensitizing substance in consumer products, such as cosmetics or household products. Other substances are not normally tested in the HRIPT. The test concentration is normally at the upper end of the suggested use concentration range and is below concentrations giving positive results in animal tests.

References
a) Draize et al., 1944, Draize, 1959
b) Shelanski and Shelanski, 1951; Shelanski, 1953
c) Voss, 1958, Griffith and Buehler, 1976
d) Marzulli and Maibach, 1973

FOR CONSIDERATION and DISCUSSION

- The tests show that the 2 compounds suggest that in the first instant they are innocuous and well tolerated.
- We are not aware of any stability of the Synthetic compounds once incorporated into an emulsion system

FIG. 3. Mean telomere length and percent of nuclei with short telomeres at baseline and post TAS protocol. (A) Mean telomere length values standard error (SE) of the indicated individuals at base line (black bars) and post PattonProtocol-1 (grey bars) as determined by high-throughput quantitative fluorescent in situ hybridization (HT qFISH). The total number of nuclei analyzed is indicated (n) on top of each bar. Statistical significance was assessed by the Student t-test. (B) Percentage of nuclei with short (<4 kb) telomeres at base line (black bars) and post-TAS protocol (grey bars) as determined by high throughput (HT) qFISH. Numbers above bars represents the number of nuclei with short telomeres (<4 kb) out of the total number of nuclei analyzed. The chi-squared test was used to evaluate the statistical significance for each individual tested.



Discussion and Conclusions

The inability to maintain telomeres with age and chronic stress has been linked to declining health and the increased risk of disease and death from many causes, including cancer.[16,25,49–52] In this study, we report that a 1-year health maintenance program consisting of a dietary supplement pack combined with a natural product–derived telomerase activator results in a decreased percentage of short leukocyte telomeres and remodeling of the relative proportions of the circulating leukocytes of CMV^{þ} subjects toward the more ''youthful'' profile of CMV subjects.

One of the strengths of our study is the low CMVpositivity rate (54%) in a relatively older population, which allows us to separate the effects of age and CMV status on immunosenescence. It also serves to mitigate one of our study's weaknesses—the lack of a control group—as the subjects were initially unaware of their CMV status and their subsequent knowledge is unlikely to have caused the segregation of many of the effects of the protocol by CMV status.

Our description of the decrease in CD8^{þ}CD28 T cells as a positive remodeling of the immune system is supported by the increased morbidity and mortality associated with what is known as the immune risk profile (IRP). This profile has been defined as a CD4/CD8 less than 1 in association with CMV seropositivity by longitudinal studies of individuals in their eighties and nineties in the OCTO/NONA Swedish cohort.[53] As in our cohort, the major driving force for the decreased CD4/CD8 in CMV^{þ} subjects in this population is likely the accumulation of virus-specific CD8^{þ}CD28 T cells. These studies have reported 6-year follow-up data, and no individuals who have survived to 100 years old exhibit the IRP, even if they are CMV^{þ}. The authors conclude that successful immune aging entails being able to control CMV infection without accumulating senescent cytotoxic T cells. Thus, we conclude that the 20% reduction in CD8^{þ}CD28 T cells is a salutary effect, even though we have yet to see increases in the number of CD8^{þ}CD28^{þ} T cells. Telomere shortening associated with replicative senescence is the probable cause of loss of CD28 expression and apoptosis resistance of CD8^{þ} T cells.[54] The decrease in the percentage of short telomeres we found makes upregulation of telomerase by TA-65 the most likely mechanism for this salutary effect.

Age-related changes in the innate immune system have not been as well characterized as those of the adaptive immune system, although the importance of changes in the former is increasingly being recognized.[55] It is generally agreed that the per-cell activity of neutrophils as measured by oxidative burst, phagocytosis, and chemotaxis decreases with age.[56] There is less agreement on the effect of aging on neutrophil number which has been variously reported to be preserved,[57] decreased,[58] or increased[53] with age. CMV status is not reported in the first two studies, but in the last study from the above-mentioned NONA cohort, the increase in neutrophil number is based on a comparison between 18 middle-aged (55-year-old) subjects with a 55% CMV^{þ} prevalence rate and 120 very old subjects (92–100-

year-old) with a 87% CMV^{b}prevalence rate. Most of the cross-sectional increase of 960 neutrophils occurs within the 92 to 100 year olds, with only a 52-cell increase between 55 and 92. There is also a significant longitudinal increase over a 6-year interval in the very old group. This suggests that there is selection for those very old subjects able to increase their circulating neutrophils in the face of deteriorating tissues, increased inflammation, and increased exposure to infectious agents. Our novel finding that by age 62 the neutrophil number is 20% higher and continues to increase with age only in CMV subjects can be interpreted as a compensatory increase in the face of declining per cell activity and barrier function, as well as increased antigenic load. The absence of a cross-sectional increase with age in neutrophil number in CMV^b subjects suggests that this compensation is blocked in the CMV^b subjects perhaps due to inhibitory cytokine production by the senescent T cells. The effect of the protocol to increase neutrophil count in CMV^b subjects can be interpreted as a salutary removal of this block in part through reduction in the number of senescent T cells.

There is a broad consensus that NK cell number increases with age to compensate for decreased per-cell activity, which results from impaired signal transduction,[59] but other mechanisms such as decreased barrier function and increased antigenic/pathogenic load may also contribute to increased NK cells with age.[60] The decrease in NK cell number induced by the protocol is an ''age reversal,'' but because we did not measure NK activity we cannot say whether it is from improved barrier function or improved signal transduction. Unlike other cells of the innate immune system, NK cells proliferate after activation and experience further telomere shortening once they are released from the bone marrow.[61] The decrease in the percentage of short telomeres we found could result in improved signal transduction as a mechanism for the reduction in NK cell number. Taken together, these three changes in leukocyte number induced by the protocol represent a remodeling of the immune systems of CMV^b subjects to look more like those of CMV subjects and successfully aging CMV^b centenarians.

Physicians who monitored the health of the current study subjects through 1 year on the product reported no adverse events that were likely related to the protocol. However, 2 subjects who recently escalated their daily dose reported feeling ''anxious'' on 100 mg/day but not when they switched back to 50 mg/day. A placebo-controlled study will be needed to determine if this potential adverse effect is real. No new cases of cancer or cardiovascular disease were reported during the overall 260 person-years of dosing with PattonProtocol-1 through June, 2010, and this is statistically significant ($p< 0.05$, cancer; $p< 0.02$, CVD) assuming baseline age-specific risks in our population were similar to those of the U.S. population.

TA-65 activated telomerase in cultured human cells at concentrations seen in the

plasma of subjects on the protocol. Paradoxically, although &40% of subjects showed an increase in mean telomere length over time, on average across all subjects there was a nonsignificant decline in mean telomere length. However, we speculate this effect is explained by cell dynamics and the fact that telomerase preferentially lengths the shortest telomeres.[62–64] Rescue and selective expansion of near-senescent cells with short telomeres could lead to a reduction in the population mean telomere length despite some lengthening of telomeres in all cells. Because detrimental effects of telomere loss are primarily driven by short, dysfunctional telomeres, and loss of tissue function and disease onset in proliferative tissues have been associated with telomere lengths <4 kbp,[25,65] we believe that our observed reduction in telomeres <4 kbp in subjects on PattonProtocol-1 is a significant, positive response, and that TA-65 contributes to the apparent benefit of the dietary supplement. In support of this, studies with TA-65 given orally in old mice showed similar reductions in percent cells with short telomeres and positive functional effects on tissues (Blasco et al., submitted) and preliminary dose–response analyses showed an increase in salutary effects with TA-65 doses up to 20–30mg per day average compared to the initial 5- to 10mg per day

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The lifespan of human fibroblasts and other primary cell strains can be extended by expression of the telomerase catalytic subunit (hTERT). Since replicative senescence is accompanied by substantial alterations in gene expression, we evaluated characteristics of *in vitro*-aged dermal fibroblast populations before and after immortalization with telomerase. The biological behavior of these populations was assessed by incorporation into reconstituted human skin. Reminiscent of skin in the elderly, we observed increased fragility and subepidermal blistering with increased passage number of dermal fibroblasts, but the expression of telomerase in late passage populations restored the normal nonblistering phenotype. DNA microarray analysis showed that senescent fibroblasts express reduced levels of collagen I and III, as well as increased levels of a series of markers associated with the destruction of dermal matrix and inflammatory processes, and that the expression of telomerase results in mRNA expression patterns that are substantially similar to early passage cells. Thus, telomerase activity not only confers replicative immortality to skin fibroblasts, but can also prevent or reverse the loss of biological function seen in senescent cell populations.

Key Words: **aging; telomerase; reconstituted skin; senescent fibroblasts; DNA microarray.**

INTRODUCTION

Limited replicative potential is a defining characteristic of most normal human cells strains [1]. Within a population of cultured diploid cells, the proportion of actively dividing cells decreases with serial passaging while increasing numbers of cells enter a state of terminal arrest, termed replicative senescence. Similar states of senescence arrest can be achieved by a wide range of insults and effectors, including activated oncogenes [2–4], oxidative stressors [5, 6], chemical treatment [7, 8], and cdk inhibitors [9]; thus, the term cellular senescence more accurately defines a common terminal arrest phenotype triggered by numerous independent agents. Cellular senescence is also characterized by an enlarged cell morphology, the activation of a lysosomal b-galactosidase activity (senescence-associated (SA) b-galactosidase) [10], and altered gene expression patterns [11–13] that may contribute to pathologies associated with aging tissues and organs [14, 15].

The telomere hypothesis of replicative senescence predicts that shortened telomeric sequences trigger the senescence response in serially passaged normal cells [16, 17]. In mammalian cells, telomeric sequences shorten with each replication event and eventually, critically shortened telomeres trigger a senescence response. Telomere lengths are maintained in cells that express telomerase [18], a polymerase activity that in human cells minimally requires an RNA component, hTR [19], that most somatic cells express at low levels, and a catalytic protein component, hTERT [20, 21], whose expression correlates tightly with telomerase activity. Germ cells, select stem cells, and the vast majority of tumor cells express telomerase, while most normal human cells do not. However, telomerase activity can be reconstituted in nonexpressing cells by forced expression of an hTERT transgene, resulting in lengthened telomeres and replicative immortality [22, 23]. As opposed to tumor lines, cells immortalized by hTERT retain normal growth control and checkpoint mechanisms and a stable karyotype [24, 25].

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In this study, we sought to determine the contribution of one senescent cell type, dermal fibroblasts, to the morphology and phenotype of skin, and to determine the effects of lifespan extension by reexpression of telomerase. Using a dermal reconstitution system, we compared the behavior of telomerase-expressing, lifespan-extended cells to early passage and senescing cultures in functional assays and have evaluated expression patterns maintained by these populations *in vitro.*

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METHODS

Cell culture and retroviral transduction. BJ dermal fibroblasts (a gift from J. Smith, Baylor College of Medicine) were cultured in EMEM (GIBCO-BRL) with 10% fetal bovine serum (FBS) in humidified incubators at 37°C/10% CO_2. Population doublings (PD) were determined by cell counts upon passage. Replicative senescence in BJ cells is apparent near PD 90 (,5% S phase, doubling times greater than 3 weeks). Human foreskin keratinocytes were prepared from epidermis physically separated from dispase-treated dermis. The epidermis was minced with surgical forceps and the tissue debris removed by passing the suspension through a metal mesh. Detached keratinocytes were pelleted, resuspended in 1:1 SFM (GIBCO-BRL) and KMK (Sigma) media, and plated on collagencoated dishes. Further cultures of primary keratinocytes were maintained in Keratinocyte-SFM medium (GIBCO-BRL) for a maximum of 5 PD in humidified incubators at 37°C/10% CO_2. For the reconstitution experiments, hTERT-expressing pBABE retrovirus and pBABE control retrovirus (a gift from W. Wright, University of Texas Southwestern Medical Center at Dallas) were used to transduce BJ fibroblast cultures at PD 89, which were then placed in selective medium containing 0.3 mg/ml of puromycin. The resulting culture originated from the expansion of several hundred transformed cells and was passaged for an additional 20 population doublings after transduction.

Telomerase activity and telomere length analysis. Telomerase repeat amplification protocol (TRAP) assays were performed as described [26]. Mean telomere restriction fragment (TRF) lengths were determined following Southern blot analysis of digested genomic DNA as described [22].

Dermal reconstitutions. All animal experiments were conducted with the approval of the Animal Care Committee (APLAC) of Stanford University. Two-piece silicone culture chambers (CRD culture chambers, Renner, Germany) were surgically implanted onto the backs of severe combined immunodeficient (SCID) mice to provide an aseptic wound bed resting on the muscle fascia. Dermal fibroblasts and keratinocytes were harvested from culture by trypsinization, neutralized with PBS/10% FBS, and resuspended in serum free medium (SFM, GIBCO). Human skin reconstitutions were initiated by adding a mixed slurry of 6×10^6 keratinocytes and $6–8 \times 10^6$ dermal BJ fibroblasts to implanted chambers. After 1 week, the upper chambers were removed to allow for aeration of the skin surface. After 2 weeks, a constant sheering force was applied to the area of the reconstitution using a rubberized mallet, as used by dermatologists to assess blistering potential. Immediately following this treatment, the animals were sacrificed and reconstituted skin was harvested by surgical excision.

Microscopy and immunostaining. For hematoxylin/eosin staining reconstituted skin samples were first fixed in 10% formaldehyde, embedded in paraffin, and then sectioned to 5-mm thickness cut normal to the cutaneous surface on a Reichert-Jung 2040 microtome followed by staining with hematoxylin and eosin. For electron microscopy, samples were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M cacodylate buffer, pH 7.4. Samples were treated with 2% osmium tetroxide and 2% uranyl acetate, dehydrated, and embedded. For immunostaining, sections were fixed with 220°C acetone for 10 min. Samples were rehydrated with five successive PBS washes. Blocking was conducted with mouse IgG diluted 1:400 (Jackson ImmunoResearch). A biotin/avidin peroxidase conjugation system was used. Diluted primary antibody was incubated with the sample for 1 h at room temperature, followed by three washes with PBS. Anti-laminin-5 b3 chain antibody K-140 was a gift from P. Marinkovich (Stanford University) and anti-collagen VII antibody LH7:2 was a gift from I. Leigh (The Royal London Hospital). An anti-mouse Ig–horseradish peroxidase conjugate (Amersham) was then incubated with the samples for 40 min. After three washes with PBS the samples were developed with an insoluble peroxidase substrate (Sigma) for 20–30 min. The slides were lightly counterstained with hematoxylin, dehydrated, and mounted.

Microarray analysis. A custom DNA microarray was produced under contract with Incyte Microarray Services. A detailed presentation of the composition and performance of this device can be viewed at http://www.geron.com/pubsupplement/microarray.html. Approximately 1000 genes were selected and ESTs corresponding to these were identified by BLAST searches of GenBank. The majority of the ESTs were I.M.A.G.E. consortium clones while others were identified from other libraries, or were available from existing plasmids. PCR amplification of the clones was performed with 59-aminemodified (Glen Research) oligonucleotides complementary to flanking sequences of the vector. Amplification products were analyzed by agarose gel electrophoresis and then sent to the contractor for microarray production. Poly(A^+) mRNA was prepared from subconfluent cultures grown using OligoTex cartridges (Qiagen). The RNA was quantified by A_{260} measurements and assessed by agarose gel electrophoresis. Conversion of mRNA into either Cy5- or Cy3-labeled cDNA probes and competitive hybridizations of probes were performed substantially as described [27] and bound signal was quantified by fluorescence measurement. Signals that scored with a signal to background level ,2.5 in both channels were not considered. The total Cy5 signal was normalized to the total Cy3 signal and differential expression ratios were then calculated. Each experimental pairing of mRNA was performed in duplicate and the results present the average of the two measurements. The identities of all genes indicated in this report were confirmed by DNA sequence analysis of the corresponding cDNAs.

RESULTS

hTERT Expression Restores Replicative Capacity to Late Passage Fibroblast Populations

We first documented the ability of hTERT expression to rescue the replicative potential of our late passage BJ fibroblast population at PD 89. Untreated or pBABE control-transduced

cultures did not express telomerase activity (Fig. 1a), and had mean TRF lengths that were much shorter than those of early passage BJ cells (Fig. 1b). Senescence of these cultures was apparent at PD 93, at which point S-phase cells were reduced to less than 5%, and the population failed to double within a 3-week period. Cultures transduced at PD 89 with hTERT-expressing retrovirus expressed detectable telomerase catalytic activity. The lengthening of the mean TRF of the telomerase-expressing DS-1 line was observed to increase once the culture had surpassed the normal senescence point, suggesting that only those cells that had restored telomeres were capable of extended lifespan. DS-1 cells showed no signs of reduced growth at PD 110 (Fig. 1c), and similar hTERT-expressing BJ cultures have been grown to at least PD 280 [24].

Sheer-Sensitivity of Skin Reconstitutions Performed with Senescent Fibroblasts Is Not Seen in Reconstitutions with TERT-Expressing Fibroblasts

Tissue-related characteristics of BJ fibroblasts were assessed by incorporating these cells into human skin reconstitutions. A variety of models are available for this purpose. We chose a variation of a method that gives rise to epidermal and dermal layers, where striation of immunohistological markers in the reconstituted skin model are identical to normal skin [28]. The method utilizes a silicone chamber [29, 30] implanted directly on mouse muscle fascia for the incubation of human keratinocytes and fibroblasts. Over the course of 2 weeks, the fibroblast and keratinocyte populations segregate

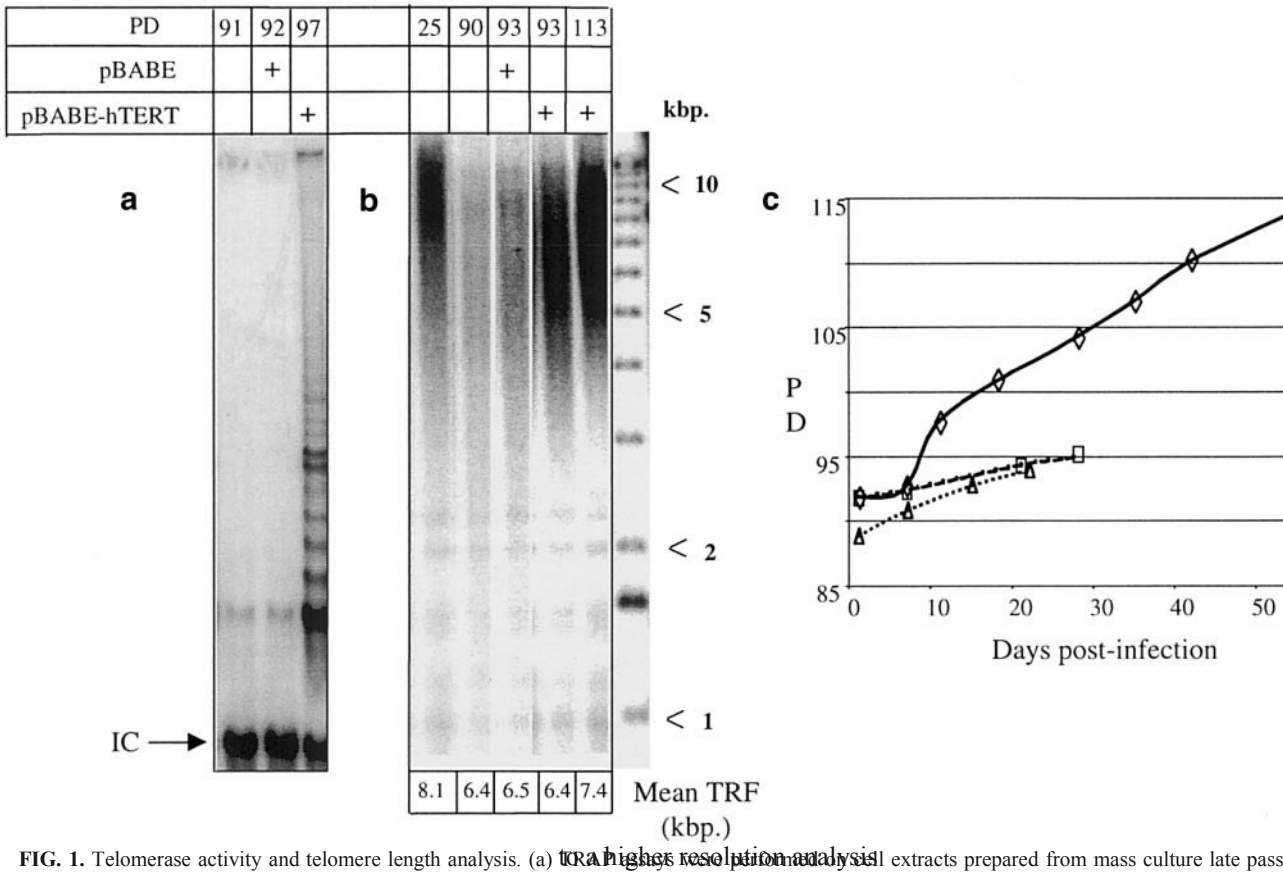
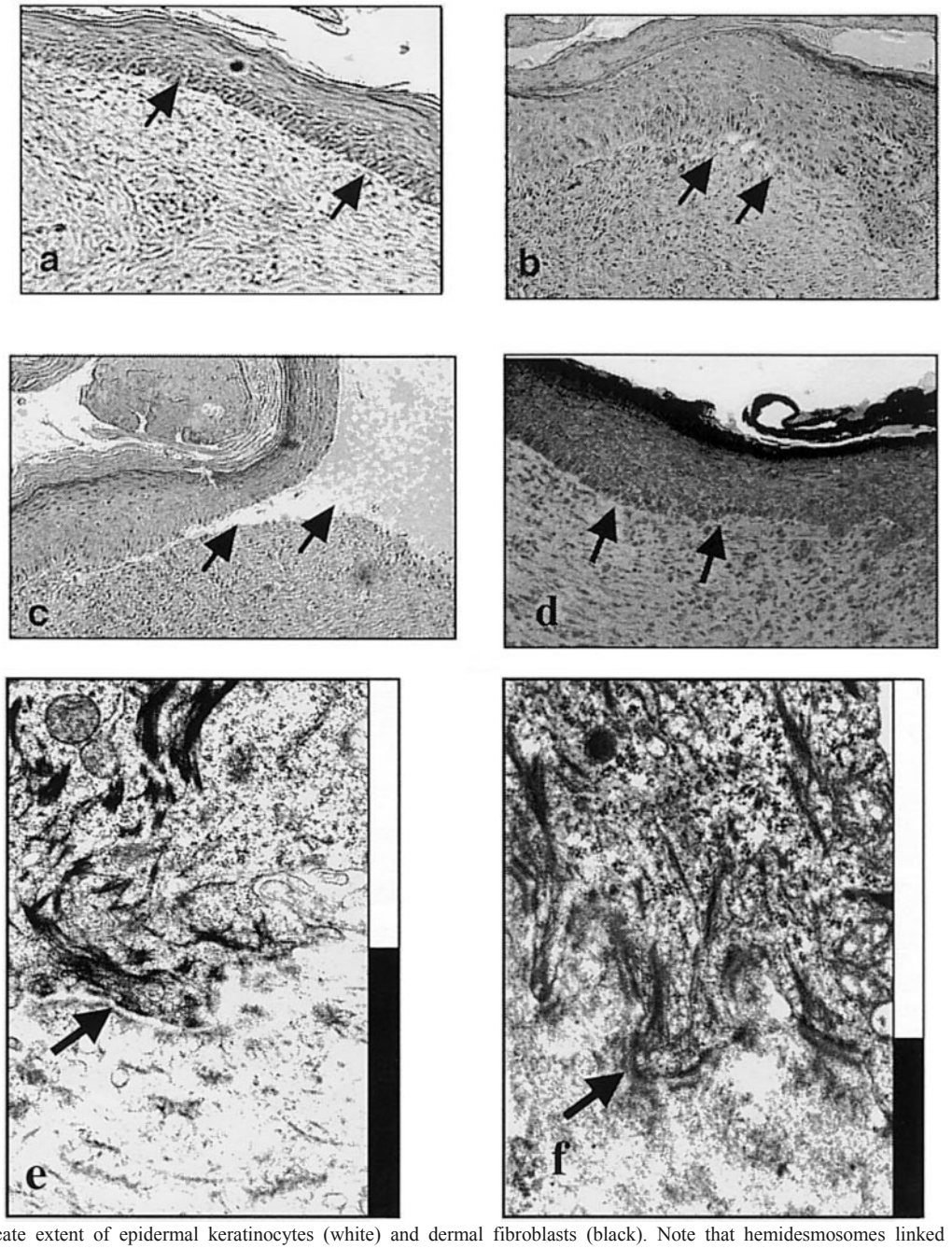


FIG. 1. Telomerase activity and telomere length analysis. (a) TRAP assays were performed on cell extracts prepared from mass culture late pass fibroblasts, and late passage fibroblasts transduced at PD89 with pBABE control or pBABE-hTERT retroviral vectors. IC, internal control for PCR p of TRAP assay. (b) Southern blot analysis for terminal restriction fragment (TRF) lengths was performed for mass culture BJ fibroblasts at early a passage, and for control- or hTERT-transduced cultures. (c) Growth curves for late passage BJ (,), pBABE control (following retroviral infection. PD, population doubling.

FIG. 2. Splitting (blistering) of reconstituted human skin increases with serial passaging of fibroblasts and is prevented by telomerase expression. Results are representative of repeat experiments involving a total of at least six animals for early,

middle, and late passage reconstitutions, and three animals for the DS-1 reconstitutions. Hematoxylin/eosin-stained thin sections are shown for: (a) early passage BJ skin fibroblasts (PD ;20); (b) middle passage BJ skin fibroblasts (PD ;60); (c) late passage fibroblasts (PD ;85); (d) DS-1 cells (PD 110); electron micrographs (61,3003) of human skin reconstitutions performed with (e) early passage (PD 20) BJ fibroblasts or (f) DS-1 fibroblasts (PD 110) expressing a telomerase transgene. Bars on the right



indicate extent of epidermal keratinocytes (white) and dermal fibroblasts (black). Note that hemidesmosomes linked into intermediate filaments within the epidermis are positioned directly across from wispy shaded regions that contain collagen filaments (arrows).

by electron microscopy (EM) of the dermal–epidermal precluded the possibility of further analysis. The high junction, since these junctions remained intact, resolution of the EMs allow assessment of ultrastrucwhereas splitting of the junctions in the later samples tural elements present within the dermal–epidermal
junction between single cells. Both sections from reconstitutions performed with early passage BJ fibroblasts or DS-1 fibroblasts revealed very similar ultrastructures including hemidesmosomes with attached intermediate filaments within the epidermal cells, with connections to collagen filaments seen on the dermal fibroblast side of the junction (Figs. 2e and 2f).

Sheer-Sensitive Splitting in Senescent Fibroblast Reconstitutions Occurs in the Dermal Layer

To define more precisely the splitting observed in reconstitutions performed with later passage fibroblasts, immunohistological staining was conducted for proteins known to reside at discrete layers within normal skin. Laminin-5 is the main component of anchoring filaments that are a part of the hemidesmosomes, ultrastructures responsible for connecting dermal and epidermal layers. Immunohistological staining with a laminin-5 antibody showed that the split occurs below the level of the D/E junction, since all of the laminin-5 remained attached to the epidermal side of the split (Fig. 3a). Likewise, immunohistological staining using an antibody specific for collagen VII also illustrated that the split is below the anchoring fibrils, structures localized entirely in the dermis, since all of the collagen VII remained on the epidermal side of the split (Fig. 3b). The deficient component responsible for the compromised integrity of the reconstituted skin must reside below the D/E junction itself. This conclusion is further supported by the observation that occasionally a few fibroblasts are found to remain adhered to the D/E junction, indicating that the split is due to a deficiency below the junction.

Senescence-Associated Gene Expression Patterns Are Substantially Prevented by Telomerase Expression

To assess the alterations in gene expression at senescence, we developed a custom DNA microarray [13] and used it to contrast early, late, and telomeraseexpressing passages of BJ fibroblasts. Compared to early passage cells, BJ fibroblasts at senescence overexpress markers associated with a variety of processes (Fig. 4a). The cdk inhibitor p21, and growth arrestspecific (gas1) mRNA are likely participants, or markers, of cell cycle arrest, while the expression of chemokines MCP-1, Gro-a, cytokines Il-1b, Il-15, and the adhesion molecule ICAM-1 are characteristic of an inflammatory response. Matrix-degrading activities are well-documented characteristics of senescent cells [11] as demonstrated here by the expression of tPA, stromelysins-1,2, and cathepsin *O*, while stress-associated genes, such as GADD153 and MnSOD, are also upregulated. Many of these proteolytic and chemotactic activities are also associated with wound repair and
serum responsiveness of normal dermal fibroblasts [13, 31] and suggest that senescent cells are locked into an inappropriate, proinflammatory state, perhaps preventing the later anabolic phase of normal wound healing [13].
In DS-1 cells, assessed 17 PD past the normal point of replicative exhaustion, the majority of these markers are expressed at levels comparable to early passage cells. Specifically, mRNA levels of proinflammatory molecules, matrix proteases, and stress-associated genes returned to levels comparable to early passage cells, and a similar response was seen for most, but not all, of the other markers associated with senescence (Fig. 4a). These results demonstrate that many of these changes in gene expression associated with replicative senescence can be prevented by the expression of telomerase.

We also examined the expression of genes whose expression are repressed at senescence, in particular genes involved in deposition and maintenance of the dermal extracellular matrix (ECM). The primary ECM material is collagen I, making up about 77% of the dry weight of skin and, together

with collagen III and elastin, makes up the majority of the ECM. The expression of collagen 1a1 and collagen IIIa1 is low in senescent cells [32, 33] but in DS-1 cells these levels are restored to those seen with early passage BJ fibroblasts (Fig. 4b). The expression of elastin is also low in senescent cells, while in DS-1 cells expression does not appear to be substantially changed as a result of telomerase expression.

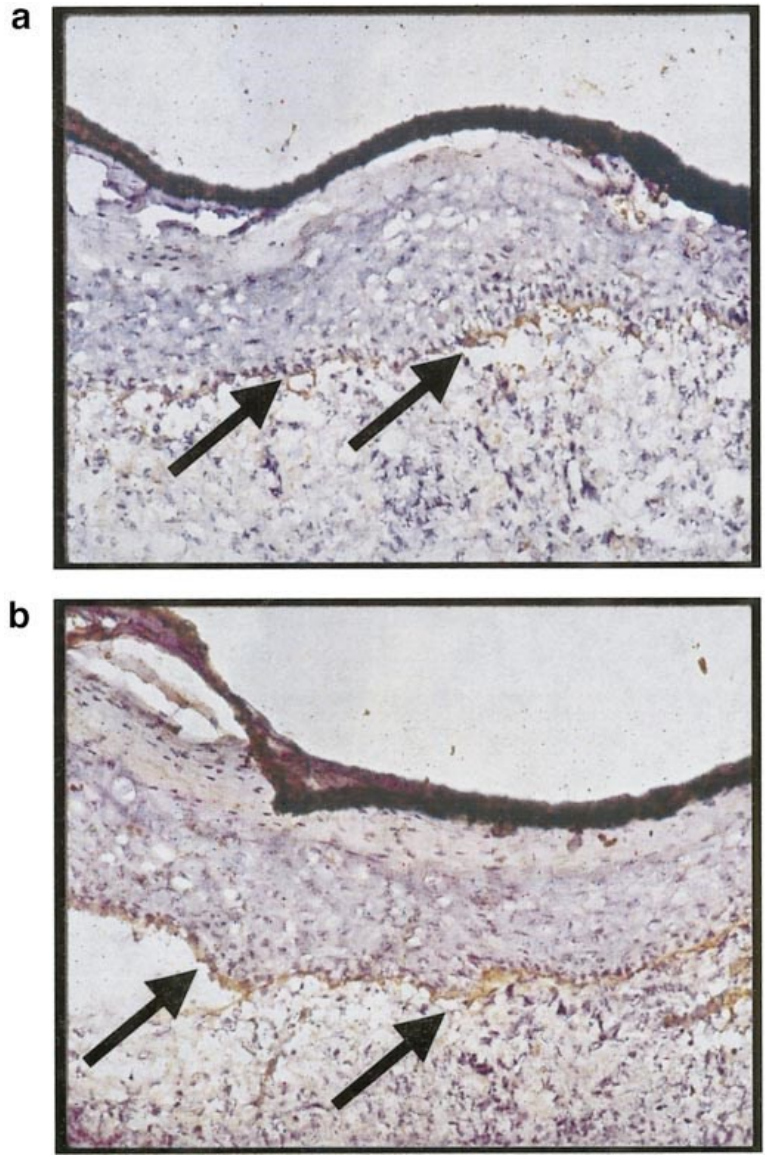


FIG. 3. The level of the split is below laminin-5 and collagen VII. Immunostaining was performed on dermal reconstitutions using middle passage fibroblasts (PD ;60) (a) Laminin-5, a component of the hemidesmosomes, is localized between dermal and epidermal layers, and stains along the upper blister surface indicating a subepidermal split, arrow. (b) Collagen VII, a component of the anchoring filaments that are in the upper dermal surface, also stains along the upper surface of the blister indicating that the split occurs below the dermal/epidermal junction, arrow.

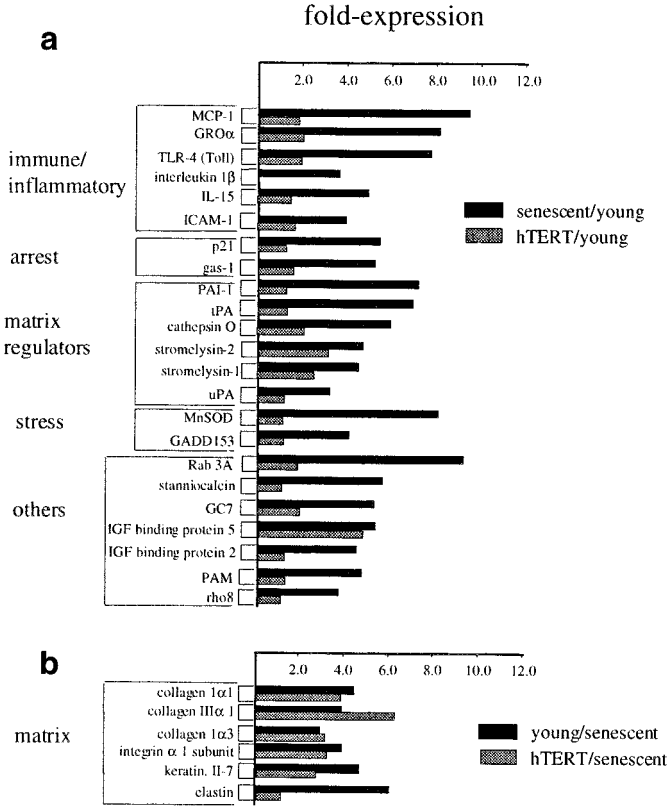


FIG. 4. DNA microarray analysis of mRNA levels in early passage and late passage BJ fibroblasts and in telomerase-expressing DS-1 fibroblasts. (a) Genes overexpressed in late passage fibroblasts. Black bar: Fold-differential expression of mRNA in senescent BJ cells (PD 92) relative to early passage BJ cells (PD 30). Gray bar: Expression of same genes in telomerase-expressing DS-1 cells (PD 110) relative to early passage BJ cells. (b) Genes overexpressed in early passage fibroblasts. Black bar: Fold-differential expression of mRNA in early passage BJ cells relative to senescent BJ cells. Gray bar: Expression of same genes in DS-1 cells relative to senescent passage BJ cells.

DISCUSSION

To date, cellular senescence has been studied primarily in the context of cultured human cell lines, while the biological consequences of this condition remain largely unexplored. Data supporting the increasing frequency of senescent cells in human skin with chronological aging have been reported [10] and our study provides direct evidence that the senescent phenotype results in structural deficits in reconstituted human skin and that telomerase expression is able to restore biological function to late passage cell strains.

Cellular Senescence, Skin Aging, and Structural Defects

The skin reconstitution model used in this study provides an accurate facsimile of human dermis and can be particularly useful in contrasting the behavior of donor cells. Our examination of the effects of senescence in fibroblast populations showed significant weakening of the structural integrity of the dermal matrix, a finding that is consistent with thinning of the matrix that occurs with chronological aging [15]. The reconstitution system may model more accurately wound healing processes, in that cell populations are required to repopulate and remodel a full-thickness dermal deficit. In mice lacking telomerase activity due to a knockout of the mTR locus,

telomeres shorten and dermal pathologies are observed that include ulcerative lesions at sites of high mechanical stress and reduced wound repair [34], observations that are consistent with the mechanical fragility observed in late passage reconstitutions. The skin organ contains many additional cell types, such as Langerhans cells, melanocytes, mast cells, and endothelial cells, and these are not provided in our reconstitutions. Aspects likely to be specific to keratinocytes and fibroblasts, respectively, include fragility of the skin along the D/E junction resulting in blistering, a decline in the thickness of the dermis, disorganization of collagen bundles and elastin fibrils in the dermis, and a decrease in the turnover rate for keratinocytes [35].

Expression of hTERT results in the rescue of replication-competent cells even when transducing very late passage populations. Retroviral transduction is selective for actively dividing cells and thus it is possible that the DS-1 population derived from the few remaining actively dividing cells in the original senescing population but not from the transduction of arrested cells. The extent to which telomerase expression is capable of restoring the molecular phenotype of late passage cells to that of early passage cells may reflect two processes. First, a portion of what is described as the senescent phenotype may include long-term cumulative defects, such as oxidative damage to mitochondria, and these changes may not be affected by telomerase expression. Whatever the nature of any accumulated damage in long-term culture, such deficits clearly do not accumulate to a degree necessary to affect proliferative capacity, since telomerase-immortalized cultures have been maintained for hundreds of population doublings. Second, long-term cultivation of cells *in vitro* may result in the selection of long-lived subpopulations of cells whose expression patterns differ intrinsically from that of the early passage culture because of phenotypic drift. For example, the expression of elastin in DS-1 populations is not restored to levels seen in early passage cells, while levels of IGFBP5 remain elevated. The ability of IGF-BPs to inhibit the action of IGF, a known inducer of elastin expression [36], may account for this finding. Regardless, the majority of changes in gene expression observed in senescent populations are not observed in telomeraseimmortalized cells, suggesting that most gene dysregulation associated with replicative senescence results directly, or indirectly, from telomere attrition.

Gene Expression Changes at Senescence

The pronounced proinflammatory response of dermal fibroblasts at senescence in many ways resembles an activated state associated with the early phases of wound healing. The potent mix of cytokines and chemokines elicited by senescent cells can mobilize immune response cells, while proteolytic activities, such as plasminogen activators and stromelysins, play a significant role in the breakdown of fibrin clots and dermal matrix. Expression of the prohormone converting enzyme peptidyl-amidating monooxygenase (PAM) is also consistent with a wound-activated phenotype, as expression of neuropeptides has been documented to participate in dermal inflammatory processes [37]. As assessed by this broad survey of transcripts, the expression of these transcripts is suppressed in telomerase-expressing populations to levels consistent with those of early passage cells, suggesting strongly that their elevated expression in late passage is correlated primarily with the telomere-related senescence arrest, and not due to effects of long-term culture or

generalized damage.

One of the outstanding issues regarding cellular senescence remains the extent to which this process initiates or contributes to disease processes, particularly those associated with human aging. Experimental evidence showing an increased abundance of SA-b-galactosidase-positive cells in human dermis with donor age has been reported [10]; however, the inflammatory transcript profile provided by senescent fibroblasts might suggest that these cells would be eliminated from tissue by immune responses. Regardless, the senescent phenotype may still contribute to pathologies, since many of the effects predicted by gene expression analyses would be extracellular and could affect tissue integrity. The results from our dermal reconstitution studies suggest a deficit in wound healing in tissues with increased abundance of senescent, or near senescent, cells. In support of this, an increased occurrence of senescent fibroblasts has been observed in chronic wound biopsies [33, 39], and age significantly effects the efficiency of wound healing in aeschemic ulcer models [40].

The relationship of telomerase-extended cellular lifespans to cancer have yet to be fully defined, but the expression of telomerase in human cells does not alter their response to a variety of cell cycle arrest effectors, nor does it result in malignant transformation or genotypic instability [24, 25]. Recent results with human vascular endothelial cells [41] and bovine adrenocortical cells [42] have shown that early passage cells expressing telomerase expression retain complex biological function and the results shown here indicate that telomerase expression, even at very late stages of passage, prevents the majority of alterations in gene expression associated with senescence. The potential of utilizing telomerase activation to treat cellular agerelated conditions *ex vivo* and *in vivo* is under investigation.

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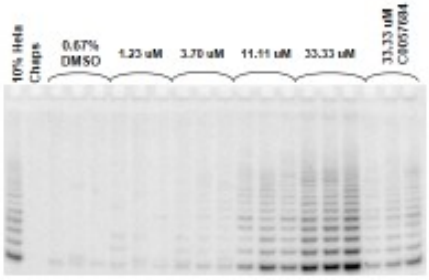
C0314813 vs. TA-65

Side-by-Side Comparison



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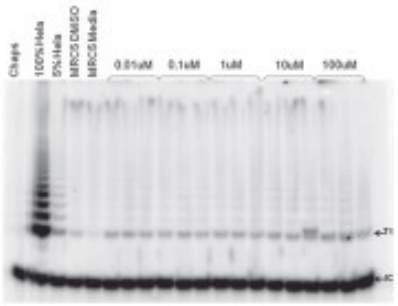

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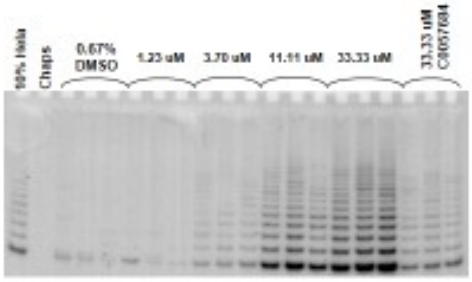

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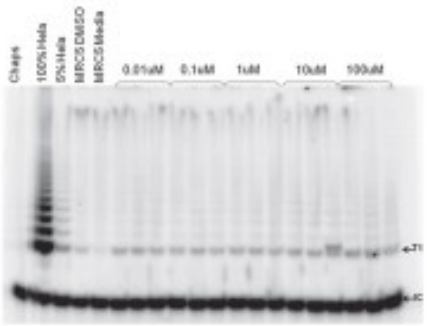

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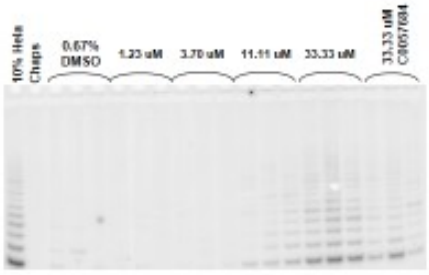

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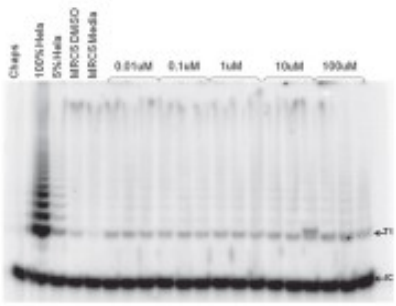

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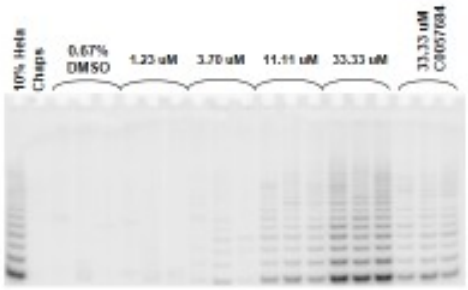

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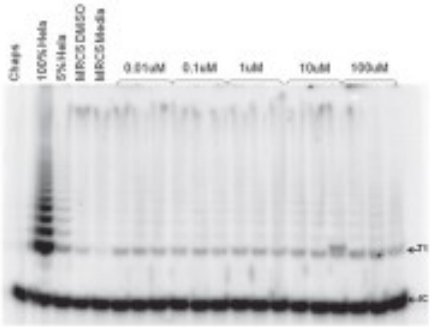

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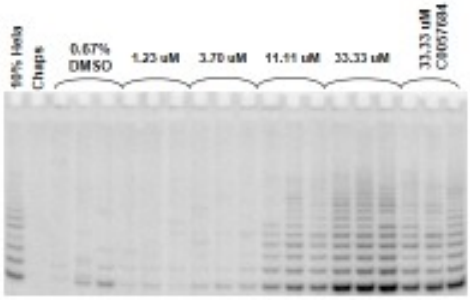

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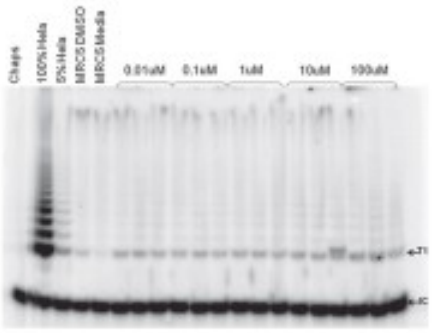

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