Reports

Enhanced isolation of fibroblasts from human skin explants

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Here we describe a method for growing fibroblasts from human skin explants that increases the number of cells obtained by up to two orders of magnitude, thus increasing the amount of material available for research and diagnostic purposes and potentially for cell-based therapies. Explants can be transferred sequentially up to 80 times, if required, at which point the explants appear to be completely depleted of fibroblasts. Utilizing skin samples obtained from 16 donors, aged 18-66 years old, the first 20 transfers produced cultures with lifespan and growth characteristics that were all very similar to each other, but the cultures derived from later transfers had a decreasing replicative capacity. Final cumulative population doublings did not correlate with donor age, but correlated positively with the telomere length at early passage. We also demonstrated that explants can be transduced directly by lentiviral infection, and that cryopreserved tissue can be explanted successfully using this procedure.

The ease with which normal human diploid fibroblasts can be cultured has meant that for many decades they have been an invaluable resource for studying cellular and molecular biology. They have a limited replicative lifespan (1), which has made them ideal for investigations of the normal controls on proliferative potential and the processes of cellular senescence (2-3). However, their limited life span also means that they are a finite resource. This limitation can be partly overcome by maximizing the number of fibroblasts obtained initially from tissue explants.

Techniques currently used to obtain fibroblasts from human tissues, such as explant outgrowth and mechanical or enzymatic disaggregation procedures, are relatively inefficient. Only a minority of cells grow out from the explants, and tissue disaggregation usually results in loss of a large proportion of the cells (4). We describe a technique that permits a substantial increase in the number of human skin fibroblasts (HSFs) that can be obtained from human skin tissue explants.

Material and methods Donor history

Human skin specimens were obtained, with both informed donor consent and Human Research Ethics Committee approval, from donors undergoing reduction mammoplasty. Tissue samples were obtained from 17 female donors between 18 and 66 years of age (Table 1); one of these samples (Fre148s) was used for lentiviral infection studies.

Sequential outgrowth of human fibroblasts from skin biopsy specimens

Skin specimens were placed in a 10 cm Petri dish and washed in F12–10 carrier medium, consisting of F-12 medium supplemented with 10% fetal bovine serum (FBS), 50 μ g/mL gentamicin and 50 units/mL fungizone; all components were obtained from Gibco, Life Technologies (Grand Island, NY, USA). The skin specimens were cut to approximately 1 cm² pieces, then 4 or 5 explants were placed into a 50 mL tube containing 10–15 mL of 0.2% trypsin (Sigma-Aldrich, St. Louis, MO, USA) made up in F-12 medium without FBS, and then left to digest for 2–4 days at 4°C. After digestion, the trypsin was neutralized by transferring the explants to a Petri dish containing carrier medium, and washed at least twice with fresh carrier medium.

For the first transfer (indicated by -1 at the end of the donor number), 3-5 trypsinized explants were placed into an empty 10 cm Petri dish for approximately 10–15 min to promote attachment, then sufficient growth medium DMEM-10, consisting of DMEM (Life Technologies) supplemented with 10% FBS and gentamicin (50 µg/mL) was gently added before the dishes were incubated in a humidified incubator at 37° C and 5% CO₂ in air. The medium was exchanged every 2–3 days to remove cell debris and

Table 1. Donor history, age, and final lifespan at early transfers.

Donor	Age (Years)	Lifespan (PDs)
Fre-129s-2	18	46.1
Fre-114s-2	19	52.6
Fre-107-2	20	53.8
Fre-96s-2	21	67.2
Fre-101s-2	21	63.8
Fre-105s-2	22	65.3
Fre-71s-1	23	60.8
Fre-120s-2	24	43.6
Fre-92s-2	26	63.4
Fre-74s-1a	27	60.8
Fre-119s-2	30	51.7
Fre-76s-2	34	57.7
Fre-88s-2	36	47.5
Fre-102s-3	42	47.9
Fre-148s-2	53	42.3
Fre-110s-2	58	36.4
Fre-115s-1	66	36.4



Figure 1. Serial transfer of skin explants permits the isolation of HSFs with decreasing lifespan. (A and B) Growth curves and histogram for Fre96s over 70 transfers. Cells were collected at the second and every 10th transfer until the 70th transfer and used for lifespan determination. Cumulative lifespan decreased substantially with increasing transfers. (C) Telomere length decreases with increasing serial transfer. Telomere length of Fre96s was determined by TRF analysis at early PDs (5-7 PDs) for transfers 2-70.

maintain a physiological pH. In order to minimize fungal contamination that might occur in the later transfers, parallel dishes were supplemented with an antimycotic: either fungizone (50 U/mL), or voriconazole (2.5 μ g/mL) (Sigma-Aldrich).

The first outgrowth from the explants was usually heterogeneous, comprising skin keratinocytes and HSFs. The second and subsequent transfers were performed every 2-3 weeks, when sufficient cells had migrated from the explants, producing an HSF outgrowth of about 20 mm from the explants. HSFs were collected from such small outgrowths for two reasons: first, to produce homogeneous cell populations and secondly, to obtain HSFs at early population doublings (PDs). The skin explants were then transferred aseptically to a new Petri dish, by inverting the explant before placing it into the dish, leaving it without medium for 10-15 min before adding DMEM-10 to the second transfer. Meanwhile, the remaining HSFs in the original dish from the first outgrowth were removed by trypsinization, counted and either cryopreserved or used to determine the lifespan. Skin explants were then transferred until the desired amount of HSFs was obtained or until no further outgrowth was observed.

Cryopreserving tissue specimens

Transfer Number

HSFs were grown for lifespan determination from skin specimens that were explanted after cryopreservation in liquid nitrogen for up to two years. In preparation for cryopreservation, the skin specimen was cut into approximately 5 $mm \times 20$ mm pieces, and treated with trypsin as described above. After washing the skin in DMEM-10, it was transferred into a cryovial with freezing medium [10% dimethyl sulfoxide (DMSO) + 90% FBS] and left for 5–10 min at room temperature, to allow the explant to equilibrate before freezing at -80°C. The cryovial was left at -80°C for no more than one week before transfer to liquid nitrogen. Tissues were reconstituted by thawing at 37°C, and then transferred to a Petri dish with DMEM-10 to wash out the DMSO before being used for serial transfer experiments.

Authentication of individual donor outgrowths

Short tandem repeat (STR) profiling was used to authenticate all cell cultures, comparing STR profiles to the original breast tissue specimen. These analyses were conducted by CellBank Australia (Westmead, Australia). All HSF cultures were authenticated as arising from the correct donor tissue.



Figure 2. Studies on HSFs isolated from 16 donors at early transfers. (A and B) Growth curves and histogram of HSFs isolated from 16 donors aged 18–66 years, at either the first, second, or third transfer. No association was observed between lifespan (cumulative PDs) and donor age. (C) Telomere length is strongly associated with lifespan. Telomere length was determined by pulsed field gel electrophoresis of terminal restriction fragments. The first 16 lanes represent donors in order of increasing age. Data are shown for one of two experiments. (D) Association between telomere length and lifespan. Telomere length was estimated by using the ImageQuant program and plotted against lifespan for the 16 donors.

Cell culture and treatments

For lifespan determination, all HSFs were grown in DMEM-10 until confluent and sub-cultured at a split ratio of 1:8 or 1:16 for young cultures, and then at lower split ratios for later passages when the cultures were approaching senescence. The number of cells was determined at each sub-cultivation using an electronic counter, Coulter Counter Z1 (Beckman Coulter, Luton, UK). The accumulated PDs were determined according to the formula PDs = $\log n / \log 2$ where n =total number of cells/cell number seeded. Cultures were terminated when the fibroblasts were unable to reach confluency after four weeks.

All isolated HSFs were routinely monitored for mycoplasma contamination after growth in antibiotic-free medium for 2 weeks. Mycoplasma testing was performed by CellBank Australia using the MycoAlert Mycoplasma Detection Kit (Lonza Group, Basel, Switzerland) and by PCR. No mycoplasma was detected in any of the tested samples. The stress response in HSFs was assessed using gamma irradiation. HSFs from 16 donors were plated at 500,000 cells per T-25 tissue culture flask (BD Falcon, BD Biosciences, Bedford, MA, USA) and irradiated 24 h later with 5 Gy using a Gammacell 3000 Elan (MDS Nordian, Ottawa, Canada). The protein levels of p53 and its downstream targets were analyzed by Western blot at 0, 4, and 12 h after irradiation.

To assess the efficacy of genetic manipulation, a skin explant was transduced with lentivirus encoding RRLsin. cPPT.CMV.EGFP.WPRE (a gift from Dr. Inder Verma). After the tissue was trypsinized, the tissue was cut into approximately $5 \text{ mm} \times 5 \text{ mm}$ pieces, and placed into one well of a 12-well tray with medium containing 8 µg/mL of polybrene and 1× 10⁸ transducing units of the lentivirus. The outgrowing HSFs were visualized seven days after infection using an inverted microscope (Model DMIRB, Leica Microsystems, North Ryde, New South Wales, Australia). Images were captured with a Spot TR-SE6 monochrome camera (Model TRSE, Diagnostic Instruments, SciTech Pty Ltd, Preston, VIC Australia). HSFs were monitored for green fluorescent protein (GFP) expression by flow cytometry as described (5).

Western blot analysis

Western blots were performed as previously described (6). The following antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA: p53 (DO1); p53 (ser15); p21^{WAF1/} ^{CIP1} (F-5); and Mdm2 (SMP14). Actin (A2066) antibody was obtained from Sigma-Aldrich. Western blot analyses were quantitated using MultiGauge (Fujifilm, Brookvale, New South Wales, Australia).

Terminal restriction fragment (TRF) analysis

Genomic DNA was isolated using the QIAmp blood DNA extraction kit (Qiagen, Doncaster, VIC, Australia) and digested using *Hin*fI and *Rsa*I restriction enzymes (New England Biolabs, Arundel, QLD, Australia). Digested DNA was separated by pulsed field gel electrophoresis (Biorad, Gladesville, New South Wales, Australia) and hybridized in-gel to a γ -³²P-ATPlabeled (TTAGGG)₄ telomere probe. A PhosphorImager screen was exposed to the gel and scanned on a Typhoon Trio (GE Healthcare, Rydalmere, New South Wales, Australia). Mean telomere length was calculated using ImageQuant (GE Healthcare) as described (7).

Results and discussion

Serial transfer of explants permits the increased production of HSFs

Standard protocols for harvesting HSFs use skin explants for up to two transfers before discarding the explanted tissue (4). In the current study, the tissues were digested in 0.2% trypsin to enable the fibroblasts to migrate more efficiently from within the extracellular matrix when transferred to a Petri dish. After two to three weeks when sufficient fibroblasts had migrated, the explant was transferred into a new dish while the remaining outgrowth was collected by trypsinization for freezing or lifespan determination. This protocol permits the explant to be transferred until all HSF outgrowth ceases. Explants from two donors were transferred >70 times, and from one donor >80 times, over a period of more than three years.

This protocol was also used to obtain fibroblasts from adult mouse tissues, including skin, tail, bone and liver (data not shown). These experiments were terminated after 10 transfers.

The replicative lifespan of HSF cultures decreases slowly with increasing transfers

We first tested whether trypsin digestion affected the replicative capacity of fibroblasts at the first, second and 9th transfers, by comparing two and four days of digestion to no trypsin treatment. The overall cumulative lifespan for undigested explants was shorter (Supplementary Figure 1), indicating that there was no detrimental effect of trypsin digestion on the final cumulative lifespans.

We determined the effect of serial transfer on replicative lifespan by calculating the total cumulative PDs of cells obtained from outgrowths at approximately every 10 transfers. In order to attain the maximum replicative lifespan for each donor culture, a variable split ratio was used because this strategy avoids the loss of prolifer-



Figure 3. DNA damage response is normal after gamma irradiation. The expression of p53, p53-serine-15, Mdm2, p21^{CIPIWAF1}, and actin was determined for 16 donors by Western blot analysis at 0, 4, and 12 h after 5 Gy irradiation. The data for three donors (Fre88s-2, Fre92s-2, and Fre96s-2) are shown. All donors showed an increase in the levels of p53, p53-serine15 and p21^{CIPIWAF1} at 4 h and 12 h after gamma irradiation. Mdm2 levels peaked at 4 h for all donors. These analyses were obtained in two independent experiments.





ative capacity that results from seeding cells at constant density (for example, see Figure 3 in reference 8, where it was shown that seeding adult skin fibroblasts at a density of 1000 cells/cm² shortened the lifespan by 10 PDs). In Figure 1A the final cumulative lifespan of Fre96s over 70 transfers is shown. For the first 20 transfers, the final lifespans were similar but with subsequent transfers there was an overall trend for the replicative capacity of Fre96s cultures to decrease (Figure 1B). This was also the case for other donors examined (Supplementary Figure S2). The observed reduction in replicative capacity of the HSFs most likely indicates that the fibroblasts continue dividing within the explants during the course of the experiment. However, the lifespan of the culture obtained from the final Fre96s-70 explant outgrowth was greater than those for the 40^{th} to the 60^{th} transfers, which may reflect outgrowth of different sub-populations from within the explant. The final cumulative PDs for each transfer can be used to obtain a more accurate estimate of the lifespan by regression analysis of final lifespans of all explanted cultures (Figure 1B). The lifespan calculated by regression analysis is used in the remainder of this study.

Although transferring explant cultures for three years is unlikely to become routine, our results demonstrate the capacity of this protocol to increase the number of early passage cells obtained from explants at least 100-fold. At later transfers the HSF cultures had shorter lifespans, but for the first 20 transfers the decrease in lifespan was modest (see Figure 1B, where the first 20 transfers show approximately 10% reduction in overall lifespan). Therefore HSFs isolated from the first 20 transfers would provide a large supply of HSFs with similar growth potentials for experimentation. The lifespan of cultures obtained after 50 to 60 transfers dropped by about 50% compared with the first transfer (Figure 1B; data not shown). Because the flask of cells obtained from tissue outgrowth is usually regarded as PD0, the size of the explanted tissue has an effect on the apparent culture lifespan because more cell generations are required to obtain the first flask of cells growing out from smaller pieces of tissue. In agreement with this, a study using a different explant technique found that a 1mm² skin biopsy per 25cm² flask could be transferred four times; the lifespans from the first three transfers were similar, but the replicative capacity of the cultures from the last transfer dropped by 72%,

presumably because cells had been depleted in the tissue fragment by the fourth transfer (8).

Characterization of HSFs isolated using the serial transfer protocol

HSFs obtained from donors of different chronological ages were used to determine whether age correlated with proliferative potential or cellular stress response. HSFs were obtained from early transfers (first, second, or third) from 16 donors aged 18 to 66 years. The final cumulative PDs ranged from 36.4 to 67.2 and did not correlate with donor age (Table 1 and Figure 2A). The relationship between HSF replicative capacity and donor age has been contentious. Early studies showed a negative correlation between donor age and lifespan (9), but this has not been confirmed by later studies (10, 11). Our study, which analyzed a relatively small cohort, supports the conclusion that there is no correlation between the in vitro growth potential of fibroblasts and donor age (Figure 2B).

The telomere length of early passage (5 to 10 PDs) cultures from early transfers was also analyzed for the 16 donors. No correlation was observed between the mean telomere length and donor age but there was a significant association $(r^2 = 0.51)$ between the mean telomere length and lifespan (Figure 2C and D). We also tested whether there was a correlation between mean telomere length and lifespan with increasing transfer number, because the lifespan decreases with increasing transfer for all donors examined (Figure 1A and Supplementary Figure 1). Telomere length was analyzed by TRF analysis for Fre96s at early passage over 70 transfers. The telomere length of Fre96s shortened over 70 transfers reflecting a strong association between telomere length and the replicative capacity of HSF cultures (Figure 1C). However, the relationship between telomere length and lifespan was less clear for cultures arising from serial transfers of Fre96s.

Next, we investigated whether the stress response in HSFs was affected by donor age by irradiating the 16 donor cultures at early passage with 5 Gy. The optimal response to radiation stress was determined to be 4 and 12 h after irradiation (data not shown). The levels of p53-serine15 and two downstream targets of p53, Mdm2 and p21^{WAF1/CIP1}, were compared at 0, 4, and 12 h after irradiation (Figure 3; data not shown). All donors showed an increase in the levels of p53, p53-serine15, and $p21^{WAF1/CIP1}$ at 4 and 12 h after irradiation, although the response was variable. The increase in protein levels was 1.4-5.7-fold for p53, 1.4–11-fold for p53-serine15, and 1.5–11-fold for p21^{WAF1/CIP1}. The Mdm2 levels were also elevated after irradiation but maximal at 4 h for all donors. These data indicate that the overall response to radiation stress was normal and there was no observable effect of donor age. This is in contrast to a report for mouse fibroblasts (12). We conclude that HSFs obtained using the protocol described here retain normal characteristics with respect to growth kinetics, telomere shortening and response to DNA damage.

Cryopreservation of tissue samples prior to explant culture

To avoid the necessity for immediate culture of tissue upon its arrival in the laboratory, we developed a procedure for storing tissues in liquid nitrogen and found that explant cultures could be established successfully in 90% of cases after two years of cryopreservation, and that the explants could be transferred many times. The lifespans of frozen and fresh explants were compared using the 2^{nd} , 10^{th} and 20^{th} transfers (Figure 4A).

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The lifespans of cultures from the 2nd transfer of frozen and fresh explants were similar, but at later transfers the frozen explants gave rise to cultures with considerably shorter lifespans. These data indicate that there may have been significant cell death during the freezing procedure (Figure 4B). Nevertheless, the ability to freeze skin samples would enable laboratories to store tissues for later culture and generation of large numbers of cells from patients with rare conditions. In addition, frozen biopsy specimens could potentially be used to produce induced pluripotent stem cells for therapeutic purposes.

Lentiviral infection of explant tissue

One of the limitations of working with normal cells such as fibroblasts is that the number of available PDs is insufficient to permit multiple rounds of gene transfer. We demonstrated here that genes can be transferred into cells that are still inside explant tissues via lentiviral infection. Explant tissue from donor Fre148s-2 was infected with lentivirus encoding GFP. Seven days later, images show the presence of GFP in both the explants and migrating cells (Figure 4C). Approximately 30% of outgrowing cells were positive for GFP as measured by flow cytometry (data not shown). Fibroblasts growing on the plastic surface retain a similar spindle-shaped morphology to those that are located in situ. The ability to transfer genes directly into explant tissue may increase the number of genetic manipulations that may be conducted within the proliferative lifespan of normal cells.

In summary, we have described a technique that allows large numbers of early passage HSFs to be obtained from explants



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of fresh or frozen tissue. This should extend the usefulness of normal human fibroblasts for studies of cell biology and functional genomics involving multiple rounds of genetic manipulations.

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Competing interests

The authors declare no competing interests.

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