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Complementation Assays Adapted for DNA Repair–Deficient Keratinocytes

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Summary

Genetic alterations affecting nucleotide excision repair, the most versatile DNA-repair mechanism responsible for removal of bulky DNA adducts including ultraviolet (UV) light-induced DNA lesions, may result in the rare, recessively inherited autosomal syndromes xeroderma pigmentosum (XP), Cockayne syndrome (CS), or trichothiodystrophy (TTD). Classical approaches such as somatic cell fusions or microinjection assays have formalized the genetic complexity of these related but clinically distinct syndromes, and contributed to the determination of seven, five, and three complementation groups for XP, CS, and TTD, respectively. XP patients are highly susceptible to photoinduced cutaneous cancers of epidermal origin. To better study the responses to UV irradiation of XP keratinocytes, and to objectively determine the extent to which cutaneous gene therapy may be realized, we set up experimental procedures adapted to ex vivo genetic complementation of keratinocytes from XP patients. We provide here detailed rationales and procedures for these approaches.

Key Words: Complementation; DNA repair; gene therapy, keratinocytes; ultraviolet radiation; xeroderma pigmentosum.

1. Introduction

1.1. Clinical Aspects and Rationale

Xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) are rare human disorders inherited as autosomal recessive traits with an estimated frequency of 1 per 250,000 and 1 per 40,000 newborns in North America/Europe and Japan/North Africa, respectively ***(1)***. All XP and some CS and TTD patients exhibit some degree of photosensitivity, and addi-

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tional clinical hallmarks are distinctive to each syndrome. Most dramatically, XP patients, but not CS or TTD patients, are highly susceptible to skin cancers on areas of the body exposed to sunlight. These diseases are life threatening and even for those XP forms exhibiting the simpler phenotype (restricted to skin) no curative treatment is available. For these reasons, XP and more specifically certain complementation groups of XP patients (*see* **Subheadings 1.7.** and **1.8.**), are good candidates for cutaneous gene therapy. Together with the general purpose of assigning a patient to a specific complementation group, the focus of this chapter is the application of genetic complementation to epidermal keratinocytes. These skin cells are the primary targets of ultraviolet (UV) irradiation, leading to their mutagenic transformation and the development of basal and squamous cell carcinomas, which constitute about 30% of human cancers.

1.2. Molecular Aspects

XP and photosensitive CS and TTD patients exhibit impaired capacity in the most versatile DNA repair mechanism, nucleotide excision repair (NER) ***(2)***. Following UV irradiation, residual NER capacity of a patient cell line can thus be determined by quantifying the extent of unscheduled DNA synthesis (UDS), as measured by incorporation of [^{3}H]thymidine into the DNA (**Fig. 1**). Somatic cell fusions followed by UDS have contributed to the definition of complementation groups into which each patient falls. Seven complementation groups called XP-A to XP-G have been identified for classical XP, five in the case of CS (CS-A, CS-B, XP-B, XP-D, XP-G), and three in the case of TTD (XP-B, XP-D and TTD-A). These findings have pointed out some genetic overlap between these syndromes since alterations to the same gene (e.g., *XPD*) can give rise to XP (XP-D), CS (CS/XP-D), or TTD (TTD/XP-D) ***(3)***.

1.3. Significance of Complementation Group Determination

Determination of complementation group is obviously essential for refining clinical diagnosis. It may also aid in genetic counselling and perhaps eventually lead to improvements in patient treatment. In addition, determination of complementation group may contribute to:

1. Knowledge of genotype–phenotype relationships (which specific mutations result in which disease?).
2. Dissection of the molecular events underlying expression of the phenotypic traits characteristic of each disease (what function[s] of the mutant protein is impaired?).
3. Elaboration of targeted pharmacological treatments.
4. Furthering prospects for gene therapy.

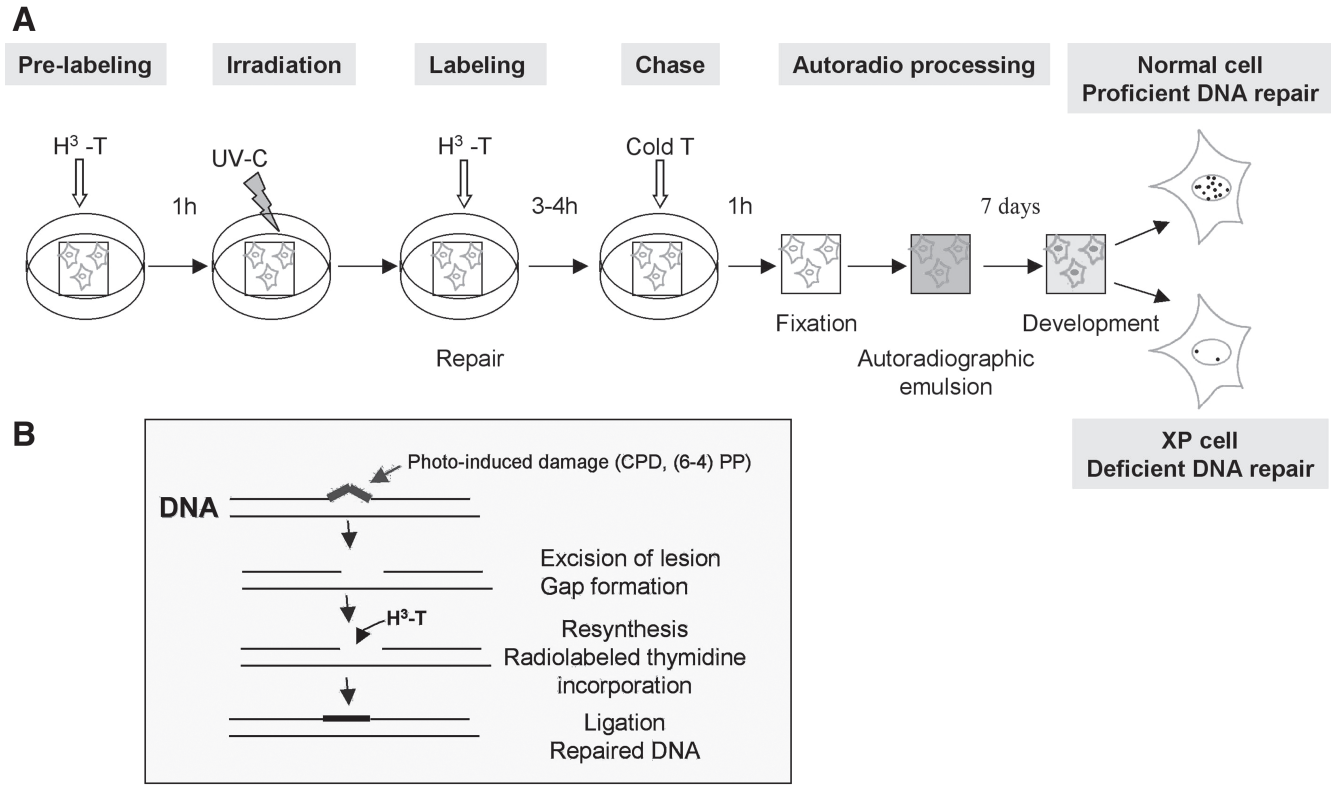


Fig. 1. Unscheduled DNA Synthesis (UDS). **(A)** Schematic representation of the experimental procedure. **(B)** Simplified view of molecular steps occurring upon UDS. *See* text and **ref. *4***.

1.4. Historical Methods for Determining the Complementation Group of NER-Deficient Cells

Molecular cloning of DNA repair genes involved in NER has led to considerable improvement of classical methods for complementation group determination. Reintroduction of the appropriate cloned DNA repair gene to a patient's cells using conventional expression vectors can be performed by either microinjection or classical transfection followed by assessment of repair capacity after UV irradiation and UDS. Microinjection, however, requires specific material and technical skills, and efficiency of transfection in primary cells remains quite poor. A fourth method, called host–cell reactivation, involves the cotransfection of a DNA repair gene together with a reporter vector (luciferase, chloramphenicol acetyltransferase) previously inactivated by exposure to UV light. In each case, the presence of the appropriate DNA repair gene will restore normal levels of either UDS or reporter gene activity. These methods are helpful for determining the complementation group of fibroblasts or transformed cells and have been described in detail previously ***(4)***.

1.5. Specifications for Keratinocyte Complementation

The epidermis constitutes the external skin compartment. It is mainly composed of keratinocytes organized in stratified cell layers distinguishable by their proliferation and differentiation capacity. Some keratinocytes forming the basal (innermost) layer are stem cells ***(5)***. In vivo, the presence of stem cells and transient amplifying cells (i.e., rapidly proliferating cells exhibiting a limited [<15 cell divisions] lifespan) sustains permanent epidermal replenishment according to a stepwise program of differentiation initiated in the basal layer and completed with the formation of dead, outermost keratinocytes that form horny layers. Under appropriate procedures, primary epidermal keratinocytes can be cultured in vitro from a very small (generally 2–5 mm diameter) punch skin biopsy (*see* **Subheading 3.**). Adequate culture conditions allow the maintenance of stem cells and the preservation of their growth and differentiation potential. Under these circumstances, it is possible to: (1) genetically (and phenotypically) modify stem cells and transient amplifying cells ex vivo; and (2) reconstruct skin *in vitro* from these cells ***(6,7)*** or regenerate genetically modified skin in vivo, for instance, after engraftment of epithelial sheets onto an athymic mouse ***(8)***. These specifications depend on strict cell culture conditions as described in **Subheading 3.** Further refinements concerning control of expression (duration, level, regulation) of the corrective gene and the topology (i.e., stratum specificity) of its expression within the epidermal compartment then relies on the type and design of the vector utilized. Ideally, the gene of interest should be placed under transcriptional control of its own DNA regulatory elements, but many difficulties related to cargo capacity of the vectors

and their specific efficiency of gene transfer into host DNA constitute limiting parameters.

1.6. New Complementation Methods Adapted to Keratinocyte Complementation

Besides classical retrovirus-based gene transfer, methods of nonviral transfer have been developed. Nonviral gene transfer methodologies such as direct injection or electroporation of naked DNA or topical gene delivery using a liposomal–DNA mixture have the advantage of being simple and easy to perform. However, they have the disadvantages of poor efficiency of epidermal gene delivery and generally drive only transitory expression of the transgene *(9–11)*. Integration of large DNA sequences is now possible using the C31 bacteriophage integrase-based gene transfer, but the quantity of cells showing stable, long-term expression of the transgene remains lower than with retroviral vectors *(12)*.

Virus-based methodologies rely on the use of adenoviral vectors, adenovirus-associated vectors, retrovirus and lentivirus (**Table 1**). Adenoviral vectors remain episomal leading to short-term expression of the gene of interest, because the viral construct is lost in proliferating cells. Adenovirus-associated vectors are able to integrate into genomic DNA but exhibit a rather small carrying capacity, which can be limiting for the transfer of large genes. Recombinant retroviral vectors (RRV) are highly efficient at gene transfer and gene integration, making them the most utilized vehicle in gene therapy approaches. One obstacle to transduction by retroviral vectors, however, is the requirement for replication of target cells at the time of infection *(13)*. Consequently, only ex vivo gene therapy can be realized using RRV. In other respects, lentiviral vectors have demonstrated efficient delivery and integration and stable expression of genes in nondividing cells. Consequently ex vivo as well as in vivo gene transfer can be envisaged with lentiviral vectors.

1.7. Retrovirus-Mediated Transduction of DNA Repair Genes into Keratinocytes

Among the vectors briefly described above, as a first attempt we used the backbone of a retroviral vector derived from Moloney murine leukemia virus (LXSN) encoding the bacterial *neomycin phosphotransferase* selection gene *neo*. As described previously, cDNAs of repair genes were inserted downstream of the 5′ long terminal repeat (LTR) of the proviral DNA which contains regulatory elements of transcription *(14)*. Production of infectious retroviral particles was performed using the packaging Crip cell line, a derivative of NIH-3T3 cells genetically engineered for the production of GAG, POL, and ENV retroviral proteins that are necessary for the production of infectious retroviral particles *(4)*.

Table 1
Comparison of Properties of Viral Vectors Used in Gene Transfer Approaches in Keratinocytes

	Vector capacity	Viral titer (cfu/mL)	Efficiency of transfer	Genomic integration	Long term expression in vivo	Disadvantages	Advantages	References
Adenoviral vector (AV)	>30 kb	10^{11}–10^{13}	Transduction of replicating and nonreplicating cells.	No	No	Transient expression.	High cargo capacity.	***17, 18***
Adeno-associated viral vector (AAV)	5 kb	~ 10^{10}	Transduction of nonreplicating cells possible but transduction of replicating cells is more efficient.	Yes, when not coinfected with helper virus, at one specific site in human cells (19q13-qter).	Possible	Small cargo capacity.	Possibility of specific integration.	***19, 20***
Retroviral vector (RV)	10 kb	~ 10^{6}–10^{7}	Transduction of replicating cells uniquely.	Yes	>40 wk	Random integration: insertional mutation. Influence of LTR on the expression of the transgene.	High efficiency of transduction. Long term expression.	***13, 21–23***
Lentiviral vector (LV)	10 kb	>10^{9}	Transduction of replicating and nonreplicating cells.	Yes	> 6 mo	Random integration: insertional mutation.	High efficiency of transduction. Long term expression. Adapted to in vivo transduction.	***24, 25***

Psi-Crip cells are transfected with the recombinant proviral DNA encoding the DNA repair gene of interest together with the *neo* selectable marker. Neomycin selection of transfected cells permits the isolation of clones exhibiting a high titer of infection ($\geq 10^6$ plaque-forming units per milliliter) of target cells. Alternatively, infectious retroviral particles can be produced by transient cotransfection of human embryonic cell line 293 using, on the one hand, two helper vectors, one encoding GAG and POL proteins and the other encoding ENV protein, and on the other hand, the proviral DNA encoding the DNA-repair gene ***(15)***. Collected retroviral particles can then be used to transfer the DNA repair gene into patient cells. After G418 selection of transduced cells, production and function of the DNA repair protein is checked by challenging the transduced cells with UV light. Examples of recovery of normal levels of cell survival following UV-irradiation and UDS in *XPC*-transduced XP-C primary keratinocytes are shown in **Fig. 2**.

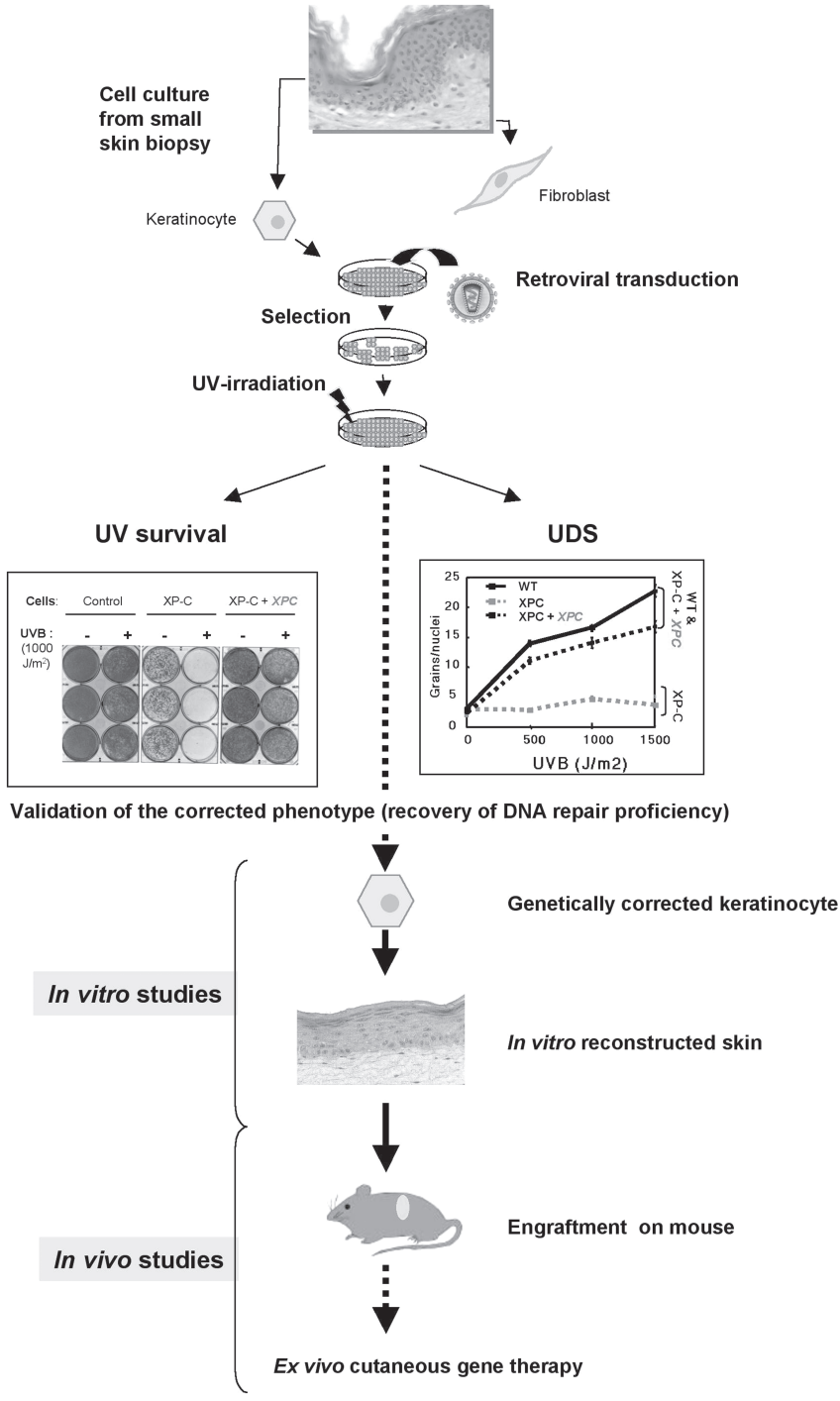
1.8. Contribution of Keratinocyte Complementation to Cutaneous Gene Therapy of XP

Except for strict sun avoidance and surgical resection of epidermal tumors, no effective curative treatment is available to XP patients. At least for some complementation groups of XP (in particular XP-C), cutaneous gene therapy may thus provide a promising approach for improving the quality of patient life. In this respect, it is necessary to ensure long term expression of the correcting gene and absence of immunogenicity of both the DNA repair protein and selection marker. Using a novel generation of RRV, our preclinical assays based on regeneration of genetically corrected skin onto laboratory animals are currently addressing some of these questions.

2. Materials

2.1. Cell Culture

1. Keratinocyte culture medium, CFAD: three volumes of Dulbecco's minimal essential medium (DMEM) (Gibco/Invitrogen), one volume of F10 (Ham's) (Gibco), 5 µg/mL of insulin (Sigma), 2×10^{-9} M triiodothyronin (T3, Sigma), 0.4 µg/mL of hydrocortisone (Calbiochem), 10^{-10} *M* cholera toxin (ICN), 1.8×10^{-5} *M* adenine (Sigma), 5 µg/mL of transferrin (Sigma), 1% nonessential amino acids (Gibco), 1% sodium pyruvate (Gibco), 1% penicillin–streptomycin (Gibco), 1% glutamine (Gibco), 0.2% fungizone (Gibco), 10% fetal bovine serum. CFAD supplemented with 10 ng/mL of epidermal growth factor (EGF; Chemicon).
2. Swiss 3T3 J2 mouse fibroblasts were a kind gift of Dr. James Rheinwald (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA).
3. 3T3 J2 and Psi-Crip culture medium: DMEM (Gibco), 1% penicillin–streptomycin (Gibco), 0.2% fungizone (Gibco), 10% bovine calf serum (HyClone).
4. Phosphate-buffered saline (PBS), pH 7.4: 137 m*M* NaCl, 2.7 m*M* KCl, 4,3 m*M* Na_2HPO_4 (Gibco).


Cell culture from small skin biopsy
Keratinocyte
Fibroblast
Retroviral transduction
Selection
UV-irradiation
UV survival
UDS
Cells:
Control
XP-C
XP-C + XPC
UVB : (1000 J/m²)
- + - + - +
Grains/nuclei
25
20
15
10
5
0
WT
XPC
XPC + XPC
WT & XP-C + XPC
XP-C
0
500
1000
1500
UVB (J/m2)
Validation of the corrected phenotype (recovery of DNA repair proficiency)
Genetically corrected keratinocyte
In vitro studies
In vitro reconstructed skin
Engraftment on mouse
In vivo studies
Ex vivo cutaneous gene therapy

5. 0.1% Trypsin solution: 1X PBS, 0.1% glucose, 0.1% trypsin, 0.0015% phenol red, 1% penicillin–streptomycin (Gibco). Adjust the pH to 7.45 with 2 *N* NaOH, filter the solution and store at –20°C.
6. 0.02% EDTA in 1X PBS.
7. 35-, 60- and 100-mm tissue-culture dishes, 25-, 75-, and 150-cm^2 tissue-culture flasks (T25, 75, and 150).
8. 37°C, 10% CO_2 humidified incubators.
9. Sterile 15-mL polypropylene centrifuge tubes (Falcon).

2.2. Transduction of Cells

1. Psi-Crip cells producing the retrovirus of interest.
2. Psi-Crip culture medium (*see* **item 3**, **Subheading 2.1.**).
3. SFM-keratinocyte medium, containing 0.2 ng/mL of EGF, 0.25 µg/mL of bovine pituary extract (BPE; Gibco), 0.1 m*M* $CaCl_2$.
4. 10 mg/mL of polybrene (Sigma).
5. 0.45-µm filter.
6. Neomycin (G418) at 50 mg/mL (Gibco).

2.3. Measurement of DNA Repair

2.3.1. UDS

1. UV-B radiation source: Spectroline transilluminator, model TR-312 (Spectronics Corporation, Westbury, NY), equipped with a cutoff filter, WG 305 (Schott). Fluence is measured using a VLX312 radiophotometer (Vilbert-Lourma). A UV-C radiation source (254 nm) can also be used. (*See* **Note 1**.)
2. Fetal bovine serum dialyzed with PBS (DFBS) and sterilized by filtration.
3. F10 (Ham's) medium (Gibco).
4. [^{3}H]thymidine (SA of 50 Ci/mmol).
5. 1 *M* Unlabeled thymidine.
6. 10 µ*M* Fluorodeoxyuridine.
7. 5% Trichloroacetic acid (TCA) diluted in water, kept at 4°C.
8. 100% Ethanol.

Fig. 2. *(Opposite)* From biopsy to cutaneous gene therapy. Fibroblasts and keratinocytes are isolated from small skin biopsies taken from patients suspected of having a DNA repair deficiency. Keratinocytes are transduced with retroviruses expressing a wild-type copy of the DNA repair gene (e.g., *XPC* or *XPD*) together with the selection gene, such as the G418 resistance gene *neo*. Cells are then selected using G418. Phenotypic correction is checked by both cell survival and UDS assays following UV irradiation. Only cells transduced by the appropriate DNA repair gene exhibit the corrected phenotype in both assays. In vitro studies can be done and it is possible to reconstruct skin in vitro using genetically corrected keratinocytes. Engraftment of genetically corrected skin onto a laboratory mouse constitutes a validation step for the principle and methodologies of cutaneous gene therapy.

9. PBS (*see* **item 4**, **Subheading 2.1.**).
10. Glass microscope slides (2.5 × 7.5 cm^2) and sterile glass coverslips (2 × 2 cm^2).
11. Mounting medium for microscopic preparations (Eukitt).
12. Kodak emulsion NTB2, Kodak D19 developer, Kodak 3000 fixer.
13. Mayer's hematoxylin staining solution (Merck).

2.3.2. Clonal Survival Following UV Irradiation

1. UV-B irradiation device and WG 305 Schott cutoff filter; VLX312 UV-B light radiophotometer (*see* **item 1**, **Subheading 2.3.1.**).
2. 60- and 100-mm tissue-culture dishes.
3. PBS (*see* **item 4**, **Subheading 2.1.**).
4. 0.02% EDTA in 1X PBS.
5. Trypsin (*see* **item 5**, **Subheading 2.1.**).
6. Rhodamine blue staining solution: one volume of 1% rhodamine and one volume of 1% Nile blue.
7. 3.7% Formaldehyde in PBS.

2.3.3. Mass Culture Survival Following UV Irradiation

1. Six-well Multidishes.
2. UV-B lamp at 312 nm and corresponding UV light dosimeter (*see* **item 1**, **Subheading 2.3.1.**).
3. Rhodamine blue staining solution (*see* **item 6**, **Subheading 2.3.2.**).
4. 3.7% Formaldehyde in PBS.
5. Fluor-S Max MultiImager (Bio-Rad).
6. Quantity One software (Bio-Rad).

3. Methods

3.1. Isolation of Keratinocytes from Skin Biopsy

1. Seed 3T3 J2 cells at 1500 cells/ cm^2 in a T150 flask (*see* **Notes 2** and **3**).
2. Two days later, 3T3 J2 cells are trypsinized and γ-irradiated at 45 Gy in CFAD medium (*see* **Note 4**). Seed the γ-irradiated 3T3 J2 cells in 35-mm plates at 15,000 cells/cm^2 in CFAD medium and incubate at 37°C, 10% CO_2 from 2 h (time necessary for the cells to adhere to tissue culture plastic) to 24 h.
3. Obtain skin biopsies from nonexposed (to sunlight) and normally pigmented sites from consenting patients. Disinfect the biopsy area with 0.1% chlorexidine, obtain the biopsy material and immediately place it in DMEM medium, 10% FCS, 1% penicillin–streptomycin.
4. Wash the biopsy three times with 25 mL of PBS for 3 min.
5. Place the biopsy in 0.1% trypsin solution and mince the tissue into 1-mm^2 fragments with a scalpel. Transfer to a 12.5-cm^2 flask.
6. Repeat **step 5** until the volume of trypsin reaches 1 mL.
7. Add 1 mL of 0.02% EDTA solution, resuspend and transfer the remaining tissue to the 12.5-cm^2 flask.
8. Incubate for 2 h at 37°C

9. Add 8 mL of CFAD to the 12.5-cm^2 flask, mix and settle the suspension for 5 min.
10. Transfer the supernatant to centrifugation tube 1.
11. Add 10 mL of CFAD to the 12.5-cm^2 flask containing the remaining skin fragments, mix vigorously again and let the suspension settle for 5 min.
12. Transfer the second supernatant to centrifugation tube 2.
13. Repeat **step 11** and transfer the supernatant to centrifugation tube 3.
14. Centrifuge tubes 1, 2, and 3 at 400*g* (1500 rpm) for 10 min.
15. Resuspend the pellets in CFAD and seed the cells in plates previously coated with γ-irradiated 3T3 J2 feeder cells.

3.2. Culture of Keratinocytes

1. D 1: Seed 3T3 J2 cells at 1500 cells/ cm^2 in a T150 flask.
2. D 4: Irradiate 3T3 J2 at 45 Gy and plate at 15,000 cells/cm^2. After 2–18 h, thaw primary keratinocytes and seed at 10,000 cells/cm^2 on the γ-irradiated 3T3 J2 feeder cell layer.
3. D 6: Change CFAD medium and replace with CFAD containing 10 ng/mL of EGF. From this date, change CFAD + EGF (10 ng/mL) every 2 d.
4. Preconfluent keratinocytes can be frozen or passed onto freshly γ-irradiated 3T3 J2 cells. Cocultures are treated with EDTA (0.02% in PBS) for 5 min at room temperature in order to remove 3T3 J2 feeder cells. Keratinocytes are then trypsinized and either frozen (*see* **Note 5**) or reseeded.

3.3. Transduction of Cells with Retrovirus

1. D 1: Seed keratinocytes to be infected at 10,000 cells/cm^2. Seed the stably retrovirus producing cell line (i.e., psi-crip) at 13,000 cells/cm^2 in a 75-cm^2 flask (*see* **Note 6**).
2. D 3: Place the keratinocytes and the producer cells overnight in SFM–keratinocytes medium supplemented with 0.2 ng/mL of EGF, 0.25 µg/mL BPE, 0.1 m*M* $CaCl_2$, 50,000 U of penicillin–streptomycin, and 250 U of fungizone (*see* **Note 7**).
3. D 4: Collect the virus-containing medium, filter through a 0.45-µm filter and add Polybrene at 5 µg/mL. Remove the keratinocytes medium. Refeed keratinocytes using virus-containing medium. Change the medium with CFAD + EGF 8–10 h following infection.
4. At 72 h after infection, trypsinize the infected keratinocytes and seed onto 3T3 J2 cells resistant to neomycin, that is, 3T3 J2 cells that have been transduced using retroviral particles expressing the *LXSN* proviral genome. Add neomycin at 200 µg/mL to select the transduced cells.

3.4. Unscheduled DNA Synthesis

1. D 1: Seed keratinocytes at 10,000 cells/cm^2 on sterile glass coverslips in a 35-mm dish.
2. D 4: When keratinocyte colonies contain about 20–30 cells, wash the cells once with serum-free DMEM, and refeed the cells with F10 (Ham's), 1% DFBS. Incubate keratinocytes for 16 h at 37°C in F10, 1 % DFBS.

3. D 5: a. Prelabeling: Remove the F10, 1% DFBS medium and refeed the keratinocytes with 1.5 mL of F10, 1% DFBS containing 10 μCi/mL of [^{3}H]thymidine and 10 μ*M* of fluorodeoxyuridine. Incubate for 1 h at 37°C (put the plates in plastic boxes to avoid contaminating the incubator with ^{3}H). (*See* **Note 8**.)
   b. UV irradiation: Remove the prelabeling medium and wash the cells with PBS (37°C). Irradiate control and "of interest" keratinocytes using three UV-B doses (0, 1000, 1500, and 2000 J/m^2). Doses should be measured using a UV-B photoradiometer. (*See* **Note 1**.)
   c. Labeling: Refeed the keratinocytes with F10, 1% DFCS containing 10 μCi /mL of [^{3}H]thymidine and incubate at 37°C for 3 h.
   d. Chase: Remove the labeling medium and refeed the keratinocytes with 1.5 mL of F10, 1% DFCS containing 10^{-5} *M* of unlabeled thymidine. Incubate for 1 h at 37°C.
4. D 7: Remove the medium and wash the cells twice with PBS.
5. Fix the cells with 100% methanol under a fume hood for 10 min and TCA-precipitate nucleic acids using ice cold 5% TCA twice for 15 min.
6. Dehydrate the cells once in 70% ethanol for 5 min and twice in 100% ethanol for 5 min.
7. Air-dry and mount the cover slips on a glass microscope slide with mounting medium. Position the cover slips so that the cellular side is up. Air-dry overnight.
8. D 8: In the dark, prewarm two volumes of photographic emulsion at 42°C and dilute it in three volumes of prewarmed water. Plunge the slide with cover slips into the emulsion for 5 s and air-dry for 4 h. Put the slide in a dark box, wrap it with aluminum foil, and keep at 4°C for 5 d.
9. In the dark, develop the slides with Kodak D19 at 14°C for 5 min, stop the reaction with 2% acetic acid for 30 s and fix with diluted Kodak 3000 solution (1 part fix: 4 parts water) for 10 min.
10. Gently wash the slides in tap water for 10 min.
11. Stain the cells with Mayer's hematoxylin for 5 min and wash in tap water.
12. Dehydrate cells in successive ethanol solutions, 70%, 90%, 95%, and 100%, for 1 min each.
13. Air-dry the slides and mount another cover slip on the cells with mounting medium.
14. Using a microscope (×100 magnification), count the grain number in each nucleus. DNA repair synthesis rate is expressed by mean grain number of at least 30 nuclei.

3.5. Cell Survival Assay Following UV Irradiation

3.5.1. Clonal Analysis

1. D 1: For each cell line (control and complemented cell lines), seed four 60-mm dishes with keratinocytes at 10,000 cell/cm^2 on γ irradiated 3T3 J2 cells.
2. D 5: a. Irradiation: Wash the keratinocytes with PBS and immediately irradiate with UV-B. Usually, one dish of each cell strain previously pre-

pared is exposed to one of the following UV-B doses: 0, 500, 1000, and 1500 J/m^2.

b. Immediately after irradiation, trypsinize, count, and seed the cells at 35 and 70 cells/cm^2 in 100-mm dishes onto γ-irradiated 3T3 J2 feeder cells. Change the culture medium (CFAD + EGF) every 2 d.

3. D 17: Wash the keratinocytes with PBS and fix for 30 min in 3.7% formaldehyde in PBS. Stain for 1 h in 1% rhodamine blue staining solution.
4. Determine the numbers and types of colonies as described ***(16)***.

3.5.2. Mass Culture

1. D 1: For each cell line (control and complemented cell lines), seed keratinocytes (15,000 cell/cm^2) in six-well multidishes on γ-irradiated 3T3 J2 fibroblasts in CFAD medium.
2. D 4: Irradiate the cells for clonal analysis and grow 6 additional days.
3. D 11: Fix the keratinocytes for 30 min in 3.7% formaldehyde in PBS and stain using 1% rhodamine blue staining solution.
4. Cell survival is determined after scanning the dishes (Fluor-S Max MultiImager, Bio-Rad) and image analysis using Quantity One software (Bio-Rad) ***(7)***.

4. Notes

1. UV-C (254 nm) can also be used. We have shown previously that UV-C and UV-B irradiations result in equivalent UDS values *(16)*. Because UV-B wavelengths are more physiologically relevant, we perform analyses of cell survival after UV-B exposure.
2. Culture of primary keratinocytes from very small skin biopsies has been optimized for growth on γ-irradiated 3T3 J2 Swiss fibroblasts. In our hands, other methods of keratinocyte growth relying on the use of a "defined" medium (e.g., SFM keratinocyte medium, Invitrogen Corp.) are not adapted to very small skin biopsies from XP patients.
3. 3T3 J2 cells can be used as a feeder layer until they reach passage 15 if they have never been cultivated to confluence.
4. Irradiation of 3T3 J2 can be replaced by mitomycin C (MMC) treatment as follows: Prepare a stock solution of MMC 0.5 mg/mL in PBS. Dilute MMC in DMEM without serum to an intermediate concentration of 0.1 mg/mL. Filter through a 0.22-µm filter. Prepare enough medium + MMC to treat all the flasks to a final concentration of 0.01 mg/mL. Rinse cells with PBS twice and replace PBS with DMEM + MMC (0.01 mg/mL). Incubate cells for 2 h in the incubator. Rinse cells with PBS and trypsinize. After counting, seed cells at the same density used for γ-irradiated 3T3 J2 cells. Do this a few hours before seeding the keratinocytes.
5. Keratinocytes are frozen in one volume of freezing solution: DMEM, 25% serum, 10% DMSO.
6. The retrovirus producer cell line Psi-crip must be grown after confluency before the retrovirus containing medium is harvested.

7. Cultivation of keratinocytes in low calcium (0.1 m*M*) SFM–keratinocyte medium changes their morphology and impedes their capacity to attach to each other and form highly cohesive colonies.
8. This step is to label and identify replicative cells.

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