Carolina de Barros Reis Quayle

Os papéis específicos das fotolesões de DNA CPDs e 6-4PPs em respostas epiteliais distintas à irradiação ultravioleta em camundongos deficientes em reparo de DNA

Ph.D. Thesis presented to the Microbiology Graduation Program of the Institute of Biomedical Sciences of the University of São Paulo, to obtain the Degree of Doctor of Sciences.

São Paulo
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Concentration area: Microbiology

Supervisor: Prof. Dr. Carlos Frederico M. Menck

Co-supervisor: Prof. Dr. Gijsbertus van der Horst

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS

Candidato(a): Carolina de Barros Reis Quayle.

Título da Tese: Os papéis específicos das fotolesões de DNA CPDs e 6-4PPs em respostas epiteliais à irradiação ultravioleta em camundongos deficientes em reparo de DNA .

Orientador(a): Prof. Dr. Carlos Frederico Martins Menck.

A Comissão Julgadora dos trabalhos de Defesa da Tese de Doutorado, em sessão pública realizada a ...../...../....., considerou

( ) Aprovado(a) **( ) Reprovado(a)**

Examinador(a): Assinatura: .....
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CERTIFICADO

Certificamos que o protocolo registrado sob nº **059** nas fls. **46** do livro **2** para uso de animais em experimentação, sob a responsabilidade de **Carlos Frederico Martins Menck** Coordenador(a) da Linha de pesquisa "***Estudo do efeito das lesões causadas no DNA por irradiação ultravioleta em MEFs e camundongos nocaute para genes de reparo de DNA***" do qual participou(aram) o(s) alunos ***Carolina Quayle, Ricardo Alexandre Leite Melissa Gava Armelini e a pesquisadora Patrícia Gama,*** está de acordo com os Princípios Éticos de Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela *COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL* (CEEA) em **19.06.2007**.

São Paulo, 20 de junho de 2007.

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador
CEEA - ICB/USP

Profa. Dra. PATRÍCIA CASTELUCCI
Secretária
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Profa. Dra. PATRÍCIA CASTELUCCI
Secretária
CEEA – ICB/USP

stichting DEC Dier Experimenten Commissie

Consult

ADVIES DEC Nr. EUR 1758 (EMCnr. 139-09-10)

Vergunninghouder	*Erasmus MC*
Titel onderzoeksplan	*De rol van CPD en 6-4PP DNA beschadigingen in UV-geïnduceerde carcinogenese*
Status	*Continuering van een eerder aan de DEC voorgelegd en uitgevoerd onderzoeksplan EUR 130 (139-03-05)*
Ingediend door	*Prof.dr. G.T.J. van der Horst* g.vanderhorst@erasmusmc.nl
Afschrift aan	*onderzoeker, proefdierdeskundige, leden van de commissie*
Adviestraject	*Vergadering 8 april 2009 Bespreking OZP Advies.*

Doel:

- Wat is de bijdrage van UV-geïnduceerde DNA cyclobutaan pyrimidine dimeren (CPDs) en 6-4 fotoproducten (6-4PPs) aan het ontstaan van verbranding van de huid en het optreden van huidkanker?
- Is er een verschil in het tumorspectrum (carcinoma vs papilloma)?

Maatschappelijk en/of wetenschappelijk belang:
In de laatste decennia is de incidentie van huidkanker sterk gestegen. Huidkanker tast de kwaliteit van leven aan en vormt een steeds hogere kostenpost voor de maatschappij. Onderzoek met de gegenereerde genetisch gemodificeerde muizen, zoals in dit protocol beschreven, levert fundamentele kennis op over het ontstaan van UV-geïnduceerde huidkanker. De verwachting is dat deze kennis in sterke mate bijdraagt aan de ontwikkeling van methoden voor therapie en preventie van huidkanker. Hierbij valt te denken aan het opruimen van DNA schade in aan de zon blootgestelde huid d.m.v. cremes/lotion met liposomen die het enzym fotolyase bevatten.

Aangevraagde dieren en verantwoording daarvan: [*aantallen, M/V, diersoort, stam(men); verdeling over aantal groepen, groepsgrootte; herkomst*]
Er worden totaal 70 muizen (transgene fotolyase muizen (met of zonder *Xpa* DNA-herstel defect) in een haarloze C57BL/6J achtergond) van beide geslachten gebruikt, verdeeld over 5 groepen van ieder 14 dieren. De dieren zijn afkomstig uit de eigen fok.

Proefopzet en handelingen:
Voor dit experiment worden haarloze *Xpa* muizen met het CPD of 6-4PP specifieke fotolyase transgen (leeftijd 8 weken) gebruikt. Dieren leven vrij in hun kooi (1 muis/kooi; permanent geplaatst in een UV/wit licht belichtingsopstelling; kooiverrijking aanwezig) en worden dagelijks blootgesteld aan 2 minuten ultraviolet licht (80 J/m^2, Philips TL12 lampen), gevolgd door 3 uur wit licht (GE lightning polylux wit fluorescerende lampen). Wekelijks worden de muizen uitgebreid onderzocht; de aard, locatie, hoeveelheid en grootte van huidtumoren wordt genoteerd in een logboek. De verwachting is dat *Xpa* muizen na ongeveer 60 dagen de eerste tumoren ontwikkelen. De inductie van huidtumoren in fotolyase muizen wordt later verwacht, dan wel op hetzelfde moment, maar met een tragere progressie. Na 180 dagen (maximale looptijd van het experiment), of wanneer tumoren een doorsnee bereiken van meer dan 4 mm, of wanneer erytheemvorming optreedt, of wanneer op basis van de ongeriefscore en/of de Code of Practice "Dierproeven in het Kankeronderzoek" aanleiding onstaat voor euthanasie, wordt de muis gedood

d.m.v. cervicale dislocatie. De tumoren worden geïsoleerd voor histopathologisch onderzoek. Tevens wordt m.b.v. immunohistochemische technieken gekeken naar de aanwezigheid van bepaalde eiwitten zoals het tumorsuppressor eiwit p53.

Maximale cumulatieve mate van ongerief:
Gering/matig, > 30 dagen (groep 1)
Matig, > 30 dagen (groep 2-5)

Ethische afweging:
In de afweging worden door de commissie de volgende aspecten betrokken: het al dan niet aanwezig zijn van reële alternatieven, het belang van de doelstelling, het ongerief voor de dieren (als gevolg van zowel de experimentele handelingen, als huisvesting, eventuele genetische aanleg, duur van de proef, de toestand van het dier na afloop van de proef en alle andere factoren die het ongerief van de dieren kunnen beïnvloeden), alsmede eventuele andere ethische aspecten ten aanzien van het gebruik van dieren.
De commissie is ervan overtuigd dat voor het bereiken van de doelstelling van dit onderzoeksplan het gebruik van dieren onvermijdelijk is en dat die doelstelling niet te realiseren is met minder dieren en/of met minder ongerief voor de dieren.
Voorts is de commissie van oordeel dat het wetenschappelijk en maatschappelijk belang van de doelstelling van dit onderzoek opweegt tegen het voorgestelde gebruik van dieren en het met de huisvesting en experimenten gepaard gaande ongerief.
De commissie ziet geen reden te twijfelen aan de kwaliteit van de toetsing van de wetenschappelijke kwaliteit en de deugdelijke opzet van het onderzoek.

De commissie oordeelt derhalve dat de haar voorgelegde dierexperimenten ethisch toelaatbaar zijn.

Advies: Positief met ingang van 8 april 2009 voor de periode van 1 jaar.

De onderzoeker dient iedere verandering ten opzichte van hetgeen beschreven staat in het onderzoeksplan, eventuele eerdere correspondentie/adviezen en dit advies, alsmede onverwachte gebeurtenissen, onverwijld te melden aan de proefdierdeskundigen en de DEC.

Datum: 10 april 2009
Namens de DEC

Informatie: uw proefdierdeskundige

M. de Poel

stichting DEC Dier Experimenten Commissie

Consult

ADVIES DEC Nr. EUR 1901 (EMCnr. 139-09-16)

Vergunninghouder	*Erasmus MC*
Titel onderzoeksplan	*De rol van CPD en 6-4PP DNA beschadigingen in UV-geïnduceerde p53-patches*
Status	*Nieuw nog niet eerder uitgevoerd onderzoeksplan*
Ingediend door	*Prof.dr. G.T.J. van der Horst* g.vanderhorst@erasmusmc.nl
Afschrift aan	*onderzoeker, proefdierdeskundige, leden van de commissie*
Adviestraject	*Vergadering 7 oktober 2009 Bespreking OZP Advies*

Doel/vraagstelling:
Wat is de bijdrage van UV-geinduceerde DNA cyclobutaan pyrimidine dimeren (CPDs) en 6-4 fotoproducten (6-4PPs) aan het ontstaan van verbranding van de huid en het optreden van huidkanker.

Maatschappelijk en/of wetenschappelijk belang:
In de laatste decennia is de incidentie van huidkanker sterk gestegen. Inmiddels wordt ruim gewaarschuwd tegen onbeschermde blootstelling aan UV-licht, maar dat kan de gevolgen van blootstelling in het verleden, of van mensen die afhankelijk zijn van de zorg van anderen (kinderen, bejaarden) niet oplossen. Huidkanker tast de kwaliteit van leven aan en vormt een steeds hogere kostenpost voor de maatschappij. Onderzoek met de gegenereerde genetisch gemodificeerde muizen, zoals in dit protocol beschreven, levert fundamentele kennis op over het ontstaan van UV-geïnduceerde huidkanker. De verwachting is dat deze kennis in sterke mate bijdraagt aan de ontwikkeling van methoden voor therapie en preventie van huidkanker. Hierbij valt te denken aan het opruimen van DNA schade in aan de zon blootgestelde huid d.m.v. cremes/lotion met liposomen die het enzym fotolyase bevatten.
Voorts is het onderzoek van belang om de rol van DNA-repair mechanismen bij het al dan niet ontstaan van kanker (deze en andere vormen) in kaart te brengen.

Aangevraagde dieren en verantwoording daarvan: [*aantallen, M/V, diersoort, stam(men); verdeling over aantal groepen, groepsgrootte; herkomst*]
Er worden totaal 112 transgene fotolyase muizen (met of zonder *Csa* of *Xpc* DNA-herstel defect) in een haarloze C57BL/6J achtergond van beide geslachten gebruikt. De muizen zijn verdeeld over 8 groepen van ieder 14 dieren. De dieren zijn afkomstig uit de eigen fok.

Proefopzet en handelingen:
Voor dit experiment worden *Csa* en *Xpc* muizen met het CPD of 6-4PP specifieke fotolyase transgen (leeftijd 6-9 weken) gebruikt. Dieren leven vrij in hun kooi (1 muis/kooi; permanent geplaatst in een UV/wit licht belichtingsopstelling; kooiverrijking aanwezig) en worden dagelijks blootgesteld aan 8 (*Csa*) of 16 (*Xpc*) minuten ultraviolet licht (250 (*Csa*) of 500 (*Xpc*) J/m^2, Philips TL12 lampen), gevolgd door 3 uur wit licht (GE lichting polylux wit fluorescerende lampen). Dagelijks worden de muizen uitgebreid onderzocht: locatie, hoeveelheid en grootte van p53-patches wordt genoteerd in een logboek. De verwachting is dat *Csa* en *Xpc* muizen na ongeveer 25 dagen de p53-patches ontwikkelen. De inductie van deze patches in fotolyase muizen wordt later verwacht, dan wel op hetzelfde moment, maar met een tragere progressie. Na 25 dagen, of wanneer op basis van de ongriefscore en/of de Code of Practice "Dier-proeven in het Kankeronderzoek (Inspectie V&W) aanleiding onstaat voor euthanasie, wordt de muis gedood d.m.v. cervicale dislocatie. De huid wordt geïsoleerd voor histopathologisch onderzoek. Tevens wordt m.b.v. immunohistochemische technieken gekeken naar de aanwezigheid van bepaalde eiwitten zoals het tumorsuppressor eiwit p53. Tevens worden andere organen geïsoleerd (thymus, milt, lymfeklieren, etcetera) ten behoeve van analyse van parameters voor het effect van UV-bestraling en reparatiemechanismen op het immuunsysteem.
Maximale cumulatieve mate van ongerief:
Gering/ matig, > 30 dagen (onbestraald)

Matig, > 30 dagen (bestraald)

Ethische afweging:
In de afweging worden door de commissie de volgende aspecten betrokken: het al dan niet aanwezig zijn van reële alternatieven, het belang van de doelstelling, het ongerief voor de dieren (als gevolg van zowel de experimentele handelingen, als huisvesting, eventuele genetische aanleg, duur van de proef, de toestand van het dier na afloop van de proef en alle andere factoren die het ongerief van de dieren kunnen beïnvloeden), alsmede eventuele andere ethische aspecten ten aanzien van het gebruik van dieren.
De commissie is ervan overtuigd dat voor het bereiken van de doelstelling van dit onderzoeksplan het gebruik van dieren onvermijdelijk is en dat die doelstelling niet te realiseren is met minder dieren en/of met minder ongerief voor de dieren.
Voorts is de commissie van oordeel dat het wetenschappelijk en maatschappelijk belang van de doelstelling van dit onderzoek opweegt tegen het voorgestelde gebruik van dieren en het met de huisvesting en experimenten gepaard gaande ongerief.
De commissie ziet geen reden te twijfelen aan de kwaliteit van de toetsing van de wetenschappelijke kwaliteit en de deugdelijke opzet van het onderzoek.

De commissie oordeelt derhalve dat de haar voorgelegde dierexperimenten ethisch toelaatbaar zijn.

Advies: Positief met ingang van 7 oktober 2009 voor de periode van 1 jaar onder de volgende voorwaarde.

Voorwaarde:
Dit advies is pas van kracht na instemming met de reactie op onderstaande vragen en opmerkingen.

Vragen:
1. Welke methode wordt gebruikt om de p53 patch vast te stellen? Is het mogelijk om dit met het blote oog waar te nemen of wordt hier een andere methode voor gebruikt?
2. In de beantwoording van vraag 9 geeft u aan 2 dieren per groep toe te voegen vanwege eventuele uitval. Waardoor ontstaat deze uitval en waarop is het percentage van 15% gebaseerd?
3. Het ontstaan van p53 patches wordt ingeschat als matig ongerief. Kunt u aangeven hoe u tot deze inschatting bent gekomen?

Opmerkingen:
1. Bij vraag 9 geeft u aan dat 'kooiverrijking aanwezig ' is. De commissie gaat er vanuit dat dit een verschrijving is.

2. Gewichtsverlies is een van de criteria om de dieren uit proef te nemen, de commissie neemt aan dat het gewichtsverlies meegenomen wordt in de dagelijkse visuele inspectie van de dieren, omdat de dieren niet dagelijks worden gewogen.

De onderzoeker dient iedere verandering ten opzichte van hetgeen beschreven staat in het onderzoeksplan, dit advies, alsmede onverwachte gebeurtenissen, onverwijld te melden aan de proefdierdeskundigen en de DEC.

Datum:19 oktober 2009
Namens de DEC

M. de Poel

To my parents, Julieta and Buck Quayle, for inculcating in me curiosity, scientific spirit and principles. Above all, for their love, acceptance and support, always unconditional!
I love you so, so much!!!

ACKNOWLEDGMENTS

I have had the pleasure to work with some brilliant scientific minds during the development of this project, and I would like to start the acknowledgements by thanking them for dedicating their time and for all the knowledge they have given me.

First of all, I would like to thank Prof. Menck for welcoming me in his lab. Only eight years ago I started as an undergraduate who had never heard of “DNA Repair” and now leave as a PhD... Menck, your love for science is contagious. Thank you very much for the amazing opportunity, all the scientific knowledge and the guidance throughout this work! It has been a privilege to work with you.

To Prof. Jan Hoeijmakers, a truly amazing scientist and person! It has been an honor to be able to share scientific ideas with you! Thank you very much for giving me the opportunity to go to The Netherlands and develop this project after talking for just a couple of hours in that Scientific Meeting in Brazil just “a few” years ago…

A special acknowledgment to Prof. Bert van der Horst who openly received me in his lab in The Netherlands, and thought me so much about Science, photo and chronobiology, thank you very much for investing in me and in this project! It was a unique experience to work in your lab!

To Prof. Frank de Gruijl for welcoming me in his lab and teaching me so much on UV irradiation, skin structure, tumorigenesis and p53 patches in such a short time! Also special thanks to Heggert Rebel who took the time to walk me through a lot of techniques with such precision and willingness to help. It is hard to express how much of what I learned in this lab was fundamental for the development of this project.

In Rotterdam many people made this work possible. Special thanks to Yvonne Rijksen and Cíntia Bombardieri who were directly involved in some of the experiments and who finished things up when I had to return to Brazil! To Karen Basoalto, Pim and all mouse house technicians who were involved in mouse lineage establishment and maintenance.

To the Clock and Aging group and friends from the Genetics Department in Holland for welcoming me and making me feel at home in a foreign country: Antonio,

Karl, Win, Koos, Yanto, Sander, Magda, Renata, Gosia, Eugin, Maria, Inês, Ed, Fillipo, Roel, Monica, Alexandre, Herve and so many others. “Ik wil u bedanken voor uw vriendschap”!

To the close friends who really made Holland my home: Romana, Eva, Ric and Cíntia. Romana, you are an amazing person, an intelligent and dedicated scientist! From the beginning you have been a true friend! Thank you. Ric and Cíntia you were my “little Brazil” in Holland, true friends and amazing people. I am glad we spent that one year together!

To the lab friends who first welcomed me to Menck´s lab: Mê, Kero, Luis, Alice, Berra, Renatinha, Marinas, André, Mary, Ric, Raquel, Rodrigo, Dani, Apuã, Helots, Lu V, Lu A, Ste, Tati and Wa, what a group, what a family to enter! Thanks for teaching me so much!!!!

To the “new” (not so new) friends at Menck´s lab: Leo, Ale, Veri, Janu, Rosa, Lu G, Lele, Annabel, Eliza, Huma, Livia, Débora, Clarissa, Juliana, Ligia, Magna, Rachel, Vítor, Ali, Teiti, Nati, Rosana, Thiago and Susan. Thanks for welcoming me back from Holland and for such great times! I will miss you all!

Special thanks to Camila and Satoru, who have shared the responsibility of the mouse house with me for the last couple of years. Thank you for the devotion to the animals, patience, shared projects and friendship. Satoru, as my first student, you have taught me a lot, thank you for this great experience and for your scientific curiosity!

To all who were a part of the “I Winter Course on DNA damage responses: implications on aging and cancer”, what a journey…. Thanks for letting me be a part of it and may it be just the beginning!

To Silvia Massironi, PhD, for all the help in maintaining the mouse colony and all the constant support in how to establish new animal lineages and purify background and also for past and ongoing collaborative experiments. Thank you very much!

To Carlos Augusto da Silva for all the constant support in the mouse house in Brazil. Without you this work would not have been possible.

To Prof. Patrícia Gama, who showed me for the first time, as an undergraduate student, how to collect, fixate and mount tissues, stain sections and look at tissues under a microscope, thank you so very much! To Cruz Alberto Mendoza Rigonati and all the lab members for the indispensable help with samples processing.

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To Marco Antonio Stephano for taking me to the Butatan Institute, making sure I had a great first undergraduate internship, introducing me to the Science World.

To my great friends, Crau, Atila, Zá, Xans and Flávia, for all the great times! In particular, to Atila and Crau who have been closer the last couple of years and have seen this thesis through good and bad (and now, to the end)!

To the Wards and Youlins for so quickly showing me what family is all about. David and Chi, thanks for accepting me as I am and for being who you are. John and Nita, it feels like I´ve known you all my life. Thanks for opening up your home, your life and your hearts for me like that. I love you!

To my mom and dad, for reviewing this manuscript, over and over again. Special thanks to my mom for all the help with the pictures and graphics, too. And, of course, for simply being who you are and whom I love so much!

Imagine.

ABSTRACT

Quayle C. The specific role of CPDs and 6-4PP DNA photolesions in distinct local epithelial responses to UV light in DNA repair-deficient mice. 2013. 228 p. Ph. D thesis (Microbiology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

The ultraviolet (UV) component of sunlight is the most common environmental genotoxic factor, mainly inducing two photolesions in DNA: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). Photolyases are enzymes which use the energy of a photon to directly reconvert these dimers to monomers. Placental mammals do not have photolyases and count on the nucleotide excision repair (NER) pathway to remove photolesions. Deficiencies in genes involved in NER may lead to severe human syndromes, such as Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS), characterized by increased skin cancer incidence and progeria, respectively. Although the effects of UV on skin are widely known, the specific roles of each photolesion are not clear. Previous work with human fibroblasts demonstrated that, while in NER-proficient cells the removal of CPDs increases survival after UV irradiation, in NER-deficient cells both lesions play an important role in apoptosis induction. Furthermore, experiments with DNA repair-proficient mice suggested that CPDs are responsible for the majority of the deleterious effects seen after UV skin exposure. Therefore, the aim of this work is to investigate the specific roles of CPDs and 6-4PPs *in vivo*, in a system where the fast removal of photolesions by NER does not mask their function in the induction of local responses to UV irradiation. For that purpose, transgenic XPA and CSA knockout mice, expressing either CPD-photolyase or 6-4PP-photolyase, were UV exposed for 1, 25 or 36 consecutive days to a low UV dose, followed by photoreactivation. In XPA mice, ubiquitous CPD removal after acute UV exposure reduced suprabasal proliferation, partially preventing hyperplasia. Chronic UV irradiation induced significant hyperplasia, which was prevented by ubiquitous CPD removal, due to a partial reduction in basal proliferation. Furthermore, 6-4PP persistence in the genome induced significant melanin production, which was further increased by ubiquitous CPD removal. CPD removal from total epidermis prevented p53 overexpression in cluster cells, avoiding the beginning of a tumorigenic process. 6-4PP removal from basal keratinocytes partially prevented acute UV-induced hyperplasia, through basal proliferation reduction, but did not prevent chronic UV-induced hyperplasia or clustered p53 overexpression. UV-induced basal and suprabasal cell death were reduced, and skin pigmentation was prevented by 6-4PP removal from basal keratinocytes after UV exposure of XPA mice. In CSA mice, chronic UV exposure did not alter skin pigmentation but induced significant hyperplasia. CPD removal from basal keratinocytes partially prevented epidermal hyperplasia, due to a reduction in basal and suprabasal cell proliferation and an increase in basal cell death. 6-4PP removal from basal keratinocytes did not alter UV-induced hyperplasia, cell proliferation or apoptosis. These results indicate that not only the nature of the lesion, but also the total amount and location of genome damage, may play important roles in skin responses after acute and chronic UV exposure. CPDs seem to play a major role in cell proliferation, hyperplasia and tumorigenesis, whereas 6-4PPs are involved in melanogenesis and apoptosis.

Keywords: DNA photolesions. Ultraviolet radiation. DNA repair. Phtolyases. Skin. Pigmentation.

RESUMO

Quayle C. O papel específico das fotolesões de DNA CPDs e 6-4PPs em respostas epiteliais à irradiação ultravioleta em camundongos deficientes em reparo de DNA. 2013. 228 f. Tese (Doutorado em Microbiologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

O componente ultravioleta (UV) da luz solar é o fator ambiental genotóxico mais abundante, induzindo majoritariamente duas fotolesões no DNA: dímeros de pirimidina ciclobutano (CPDs) e pirimidina (6-4) pirimidona fotoprodutos (6-4PPs). Fotoliases são enzimas que utilizam a energia de um fóton para reconverter diretamente estes dímeros em monômeros. Mamíferos placentários não possuem fotoliases, contando com a via de reparo por excisão de nucleotídeos (NER) para remover fotolesões. Deficiências em genes desta via podem causar síndromes humanas severas, como Xeroderma Pigmentosum (XP) e Síndrome de Cockayne (CS), caracterizadas por alta incidência de câncer de pele e progéria, respectivamente. Os efeitos da radiação UV na pele são amplamente conhecidos, porém os papéis específicos de cada fotolesão não estão esclarecidos. Estudo com fibroblastos humanos demonstrou que, enquanto em células proficientes em NER apenas a remoção de CPDs aumenta sobrevivência após exposição à UV, em células deficientes em NER, ambas lesões são importantes na indução de apoptose. Experimentos *in vivo* sugerem que CPDs são responsáveis pela maioria dos efeitos deletérios da exposição da pele à UV. Assim, o objetivo deste trabalho é investigar os papéis específicos de cada fotolesão, *in vivo*, em um sistema onde sua rápida remoção por NER não mascare sua função na indução de respostas locais à irradiação UV. Para tanto, camundongos transgênicos *knock out* em XPA ou CSA, expressando CPD-fotoliase ou 6-4PP-fotoliase, foram expostos por 1, 25 ou 36 dias a baixas doses de UV, seguidas por fotorreativação. Em animais XPA, a remoção de CPD após exposição aguda à UV reduziu a proliferação suprabasal, parcialmente prevenindo hiperplasia; a redução parcial em proliferação basal e suprabasal após irradiação crônica preveniu hiperplasia. A persistência de 6-4PP aumentou a concentração de melanina na epiderme, efeito exacerbado pela remoção de CPDs. Superexpressão de p53 em clusters de células foi prevenida pela remoção de CPDs, evitando processo tumorigênico. Remoção de 6-4PPs de queratinócitos basais preveniu parcialmente hiperplasia aguda através da redução da proliferação basal, porém não preveniu indução de hiperplasia crônica ou a superexpressão de p53 em clusters de células após exposição à UV. A apoptose foi reduzida e a pigmentação da pele foi prevenida pela remoção de 6-4PP em queratinócitos basais em animais XPA. Em camundongos CSA, exposição crônica à UV não alterou a pigmentação da pele, mas induziu hiperplasia. A remoção de CPDs de queratinócitos basais preveniu parcialmente a indução de hiperplasia, devido à redução de proliferação basal e suprabasal e ao aumento de apoptose basal. A remoção de 6-4PP de queratinócitos basais não preveniu a indução de hiperplasia, proliferação ou morte celular. Estes resultados indicam que, não apenas a natureza da lesão, mas também, a quantidade total e localização de dano no DNA, podem desempenhar papéis importantes nas respostas da pele à exposição aguda e crônica à radiação UV. CPDs têm papel majoritário na proliferação celular, hiperplasia e tumorigênese enquanto 6-4PPs estão envolvidos em melanogênese e apoptose.

Palavras-chave: Fotolesões de DNA. Radiação ultravioleta. Reparo de DNA. Fotoliases. Pele. Pigmentação.

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LIST OF ABBREVIATIONS

Abbrev.	***Meaning***
αMSH	Alpha melanocyte stimulating hormone
βACT	Beta actin
6-4PP	Pyrimidine (6-4) pyrimidone photoproduct
8-HDF	8-hydroxy-5-deazaflavin
8OH-G	7,8-dihydro-8-oxoguanine
9-1-1	Complex RAD 9, RAD 1 and HUS 1
ACTH	Adrenocorticotropic hormone
AE	Acute exposure
APAF1	Apoptotic protease activating factor 1 (gene)
AT	Ataxia Telangiectasia
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and RAD3 related
ATRIP	ATR-interacting protein
B[α]P	Benzo[α]pyrene
BAX	BCL2 associated protein X
BCC	Basal cell carcinoma
BCL2	B-cell lymphoma 2
BER	Base Excision Repair
bFGF	Basic fibroblast growth factor
BLM	Bloom Syndrome
BRAF	v-raf murine sarcoma viral oncogene homolog B1 (gene)
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
CD14	Cluster of differentiation 14
Cdc25	Cell-division cycle protein 25
CE	Chronic exposure
cGMP	Cyclic guanosine monophosphate
CHK1	Checkpoint 1
CMM	Cutaneous malignant melanoma
CPD	Cyclobutane pyrimidine dimer
CS	Cockayne Syndrome
CSA	Cockayne Syndrome protein A
CSB	Cockayne Syndrome protein B
CSF2	Colony stimulating factor
DAG	Diacylglycerol
DCT	DOPAchrome tautomerase
DDB1	Damaged DNA binding complex 1
DDB2	Damaged DNA binding complex 2
DDR	DNA damage response
Dewar-PP	Dewar photoproduct

DHI	Dihydroxyindole
DHICA	Dihydroxyindole-2-carboxylic acid
DMBA	7,12-dimethylbenz[α]anthracene
DNA	Deoxyribonucleic acid
DNA-PK	DNA protein kinase
dNTP	Deoxynucleotide triphosphate
DOPA	L-3,4-dihidroxyphenylalanine
DSB	Double strand breaks
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELAM-1	Endothelial-leucocyte adhesion molecule
ERCC1	Excision repair cross-complementing rodent factor 1
ES	Embrionic stem (cells)
ET-1	Endothelin 1
ETOH	Etanol
FA	Fanconi Anemia
FAD	Flavin adenine dinucleotide
GG-NER	Global Genome Repair
HGPS	Hutchinson-Gilford Syndrome
hHR23B	Human RAD homolog 23 B
HR	Hairless
HUS 1	Hydroxyurea sensitive homolog to *S. pombae1*
ICAM-I	Intracellular adhesion molecule 1
IL	Interleukin
INF-ɣ	Interferon gama
IPD	Immediate pigment darkening
K14	Keratin 14 promoter
KO	Knockout
KIT1	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog 1
L*	Luminosity
Lig	Ligase
MA	Macrophage
mC	5-methylcytosine
MC	Mast cell
MED	Minimal erythema dose
MMR	Mismatch Repair
MTHF	5,10-methenyltetrahydrofolyl polyglutamate
NADH	Reduced form of nicotineamide-adenine-dinucleotide
NER	Nucleotide Excision Repair
NF-κβ	Nuclear factor kappa beta
NHEJ	Non-Homologous End Joining
NMSC	Non-melanoma skin cancer
OAG	Oleylacetylglycerol

PAF	Platelet activating factor
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PG	Prostaglandina
PGE_2	Prostaglandin E_2
PIC	Pre-incision complex
PKC	Protein kinase C
Pol δ	Polymerase delta
Pol κ	Polymerase kappa
Pol I	Polymerase 1
Pol V	Polymerase 5
Pol η	Polymerase eta
Pol ε	Polymerase epsilon
Pol ι	Polymerase iota
Pol Rev1	Polymerase Rev1
Pol ζ	Polymerase zeta
PPD	Persistent pigment darkening
PTCH	Protein patched homolog
RAD	RAS-related associated with diabetes
RAD 1	RAS-related associated with diabetes 1
RAD 9	RAS-related associated with diabetes 9
RAS	Rat sarcoma
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II
RNAse	Ribonuclease
ROS	Reactive oxygen species
RPA	replication protein A
RRS	Recovery of RNA synthesis
RT	Rothmund-Thomson Syndrome
RT	Room temperature
SCC	Squamous cell carcinoma
SILV	DHICA polymerase
SSB	Single strand breaks
ssDNA	Single-stranded DNA
TC-NER	Transcription Coupled Repair
TdT	Terminal deoxynucleotidyl transferase
TFIIH	Transcription factor II H
TGF-β	Transforming growth factor beta
Th	Helper T lymphocyte
TLS	Translesion synthesis
TNF-α	Tumor necrosis factor alpha
TOPBP1	Topoisomerase binding protein 1
Treg	Regulatory T cell
TTD	Trichothiodystrophy
TUNEL	TdT-mediated dUTP nick end labeling

TYR	Tyrosinase
TYRP I	DHICA oxidase
UCA	Uronic acid
UDS	Unscheduled DNA synthesis
UV	Ultravioleta
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
UvrA	Ultraviolet resistant protein A
UvrB	Ultraviolet resistant protein B
UvrC	Ultraviolet resistant protein C
WRN	Werner Syndrome
WT	Wild type
XP	Xeroderma Pigmentosum
XPA...XPG	Xeroderma Ppigmentosum complementation group protein A to G
XP-V	Xeroderma Pigmentosum Variant

LIST OF INITIALS

Initials	*Meaning*	
CEUA	Comissão de Ética no Uso de Animais	(Brazilian Animal Ethics Comittee)
EMC	Erasmus Medical Center	
DEC	Dier Experiment Comissie	(Dutch Animal Ethics Comittee)
ICB	Instituto de Ciências Biomédicas	(Biomedical Science Institute)
INCA	Instituto Nacional do Cancer	National Institute of Cancer
NIH	National Institute of Health	
USA	United States of America	
USP	Universidade de São Paulo	(University of São Paulo)
UTC	Universal Time Coordinated	

LIST OF SYMBOLS

Symbol	*Meaning*
A	Adenine
C	Cytosine
G	Guanine
e^-	Electron
E!	Energy
Py<>Py	Pyrimidine dimer
T<>T	Thymine dimer
T	Thymine
U	Uracil

SUMMARY

1 INTRODUCTION

1. 1 Structure and instability of the DNA molecule

Gregor Mendel is considered the father of Genetics. In 1866, in his work “Versuche über Plflanzenhybriden” (Experiments in plant hybridation), the Austrian monk was the first to demonstrate, with scientific experiments, that phenotypic characteristics are inherited through hereditary entities (1). In 1869 Friedrich Miescher isolated DNA for the first time (2). Almost a hundred years later, McCarty and Avery proved that the deoxyribonucleic acid (DNA) molecule is responsible for the transmission of inherited information (3). In 1949 Erwin Chargaff showed that DNA is composed of an equal percentage of the nucleotide bases guanine (G) and cytosine (C), as well as of adenine (A) and thymine (T) (4).

It was only in 1953 that the double-helix structure of the DNA molecule was discovered by James Watson and Francis Crick (5). This finding rendered them the Nobel Prize in 1962. Marshall, Casey and Nirenberg unraveled the universal genetic code in 1967 (6) and, two years later, Beckwith´s group isolated the first gene (7). In 1973, the first nucleotide sequencing method was developed by Sanger and co-workers, a two-dimentional chromatography technique (8). This laborious methodology has been thoroughly refined in the past forty years to what is now known as “third generation sequencing techniques”, which include several different methods capable of sequencing an entire bacterial genome in only one day, reviewed in (9).

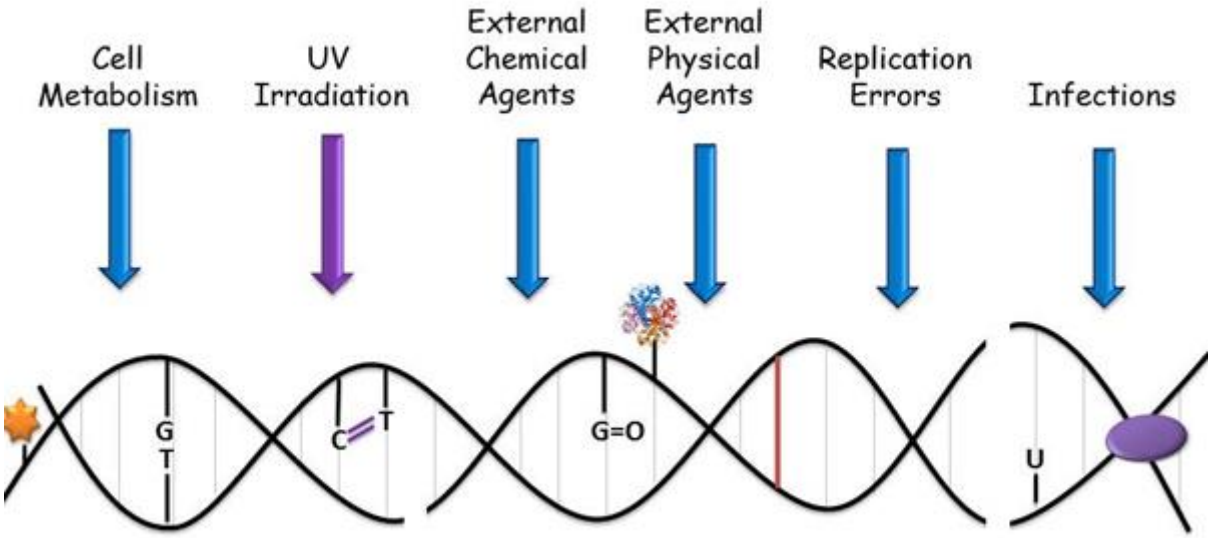
The chemical structure of DNA is well-known today. The double-helix is composed of repeated subunits, the nucleotides. Each nucleotide consists of one phosphate connected to a deoxyribose sugar, which is linked to a nucleotide base: A, C, G or T. On the same double-helix strand, each nucleotide is ligated to the next by covalent bonds in their phosphate groups. Each base is specifically paired to the opposite one in the complementary strand of the double-helix. On a canonic DNA double strand, A pairs with T and G pairs with C, via hydrogen bonds (10).

The DNA molecule contains all the genetic information of an organism and, therefore, the maintenance of its integrity is of the upmost importance to preserve cell functions and to guarantee the individual´s survival. Thus, one might expect the DNA molecule to be very stable and resistant to injuries. However, DNA is very unstable

and is constantly under attack from endogenous and exogenous chemical and physical agents which are capable of damaging its structure.

Different agents may cause several types of damages to DNA. Figure 1 presents some of the most common DNA damaging agents and DNA lesions.

Figure 1 - Common DNA damaging agents and DNA lesions



The most common endogenous and exogenous DNA damaging agents are presented in the upper part of the figure. Some of the most usual chemical and physical alterations found in DNA are represented in the DNA double-strand molecule (adapted from “I Winter course - DNA damage responses: implications in aging and cancer”).

The present work focuses on ultraviolet radiation (UV), the damages it causes in the DNA molecule and the cellular and tissue responses to those damages.

1.2 Ultraviolet radiation (UV)

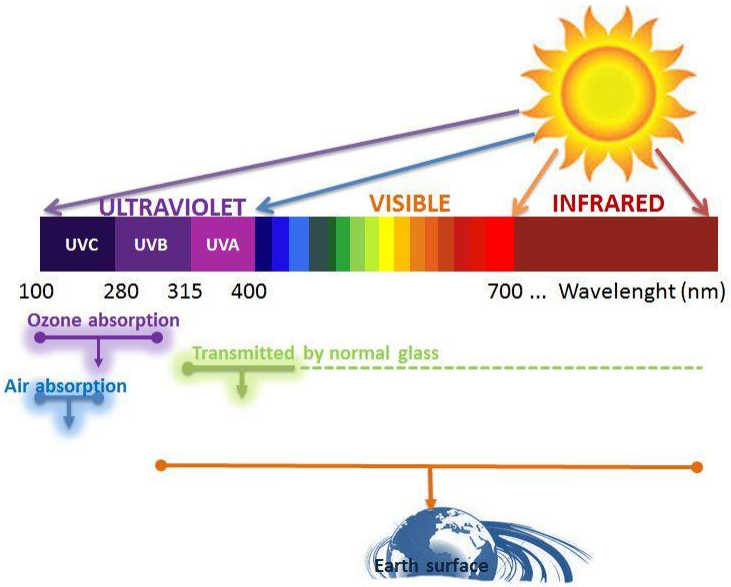
1.2.1 General characteristics of UV light

Sunlight is responsible for supplying natural energy to Earth´s biological system, enabling the photosynthetic process. However, the ultraviolet component of the sun has clear detrimental effects which correlate with skin cancer incidence, reviewed in (11).

UV light is comprised of wavelengths from 100 to 400 nm and is subdivided into three bands: ultraviolet A (UVA), ultraviolet B (UVB) and ultraviolet C (UVC).

The UVC band is composed of wavelengths from 100 to 280 nm and is totally blocked by the Earth´s ozone (O_3) layer and atmosphere [vaporized water, oxygen gas (O_2) and carbon dioxide and monoxide]. The UVB spectrum is comprised of wavelengths from 280 to 315 nm, 95 % of which is blocked by the Earth´s ozone layer. UVA consists of wavelengths from 315 to 400 nm and its totality reaches the planet´s surface (12). Figure 2 presents a scheme of the solar spectrum of light and the penetration of each wavelength through the Earth´s ozone layer and atmosphere.

Figure 2 - Schematic representation of the solar light spectrum and its penetration in Earth´s surface



Different ultraviolet bands have distinct penetration capacities in the Earth´s atmosphere: UVC (100-280 nm) is totally blocked by the ozone layer and atmosphere; 95 % of UVB (280-315 nm) is blocked by the ozone layer; and virtually the totality of UVA (315-400 nm) reaches the planet´s surface.

Four billion years ago the Earth´s atmosphere was anaerobic. The lack of O_2 allowed the totality of the UV spectrum to reach the planet´s surface. UV radiation was therefore a very strong selective agent for organisms, which had to adapt to this extreme condition. It is believed that this scenario was one of the biggest challenges for the terrestrial occupation by organisms. It is speculated that the rate of DNA damage in the ocean´s surface was a thousand times greater than today (13).

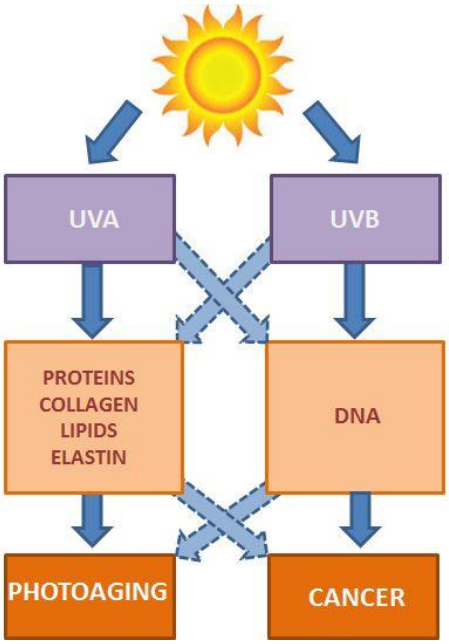
Almost a billion years later sunlight began to be used as an energy source for the photosynthetic process, which in turn began to fill the planet´s atmosphere with O_2. This modification of the Earth´s atmosphere allowed for the development of aerobic organisms, believed to be the main reason for the explosion of the Animal Phylum, about five hundred and forty million years ago, reviewed in (14).

Furthermore, UVC light reacted with the O_2 in the outer layers of the atmosphere, dissociating it´s molecules and permiting O_3 formation. This gas was trapped in layers between 10 and 50 Km from the surface and formed the ozone layer, about four hundred million years ago. As previously mentioned, the present concentration of ozone in the Earth´s atmosphere is largely responsible for blocking the totality of UVC rays and the majority of UVB light from reaching the Earth´s surface, reviewed in (15).

The percentage of UVA and UVB light which reaches the planet´s surface not only varies according to the latitude and longitude, but also with season, altitude and weather conditions (16).

Organic molecules are capable of absorbing different UV bands depending mainly on the characteristics of their chemical bonds. For instance, aromatic protein bonds and nucleic acids are very effective in absorbing UVB, whereas UVA is mainly absorbed by other chromophores, such as the reduced form of nicotineamide-adenine-dinucleotide (NADH), riboflavins and hemoglobin (17). Figure 3 represents the most common molecules which absorb UVA and UVB in the skin.

Figure 3 - Molecules which most commonly absorb UVA and UVB in skin



Because of their physical properties, UVA and UVB´s energy are better absorbed by different organic molecules: UVA is mainly absorbed by proteins, collagen, elastin and lipids, whereas UVB is mainly absorbed directly by DNA. However, as indicated by the doted arrows, UVB and UVA can also interact with other molecules.

The absorption of radiant energy may produce a photo-chemically reactive molecule, rendering molecules to be changed or damaged (11). UV light is considered the most genotoxic physical agent present in our environment today, reviewed in (11,18).

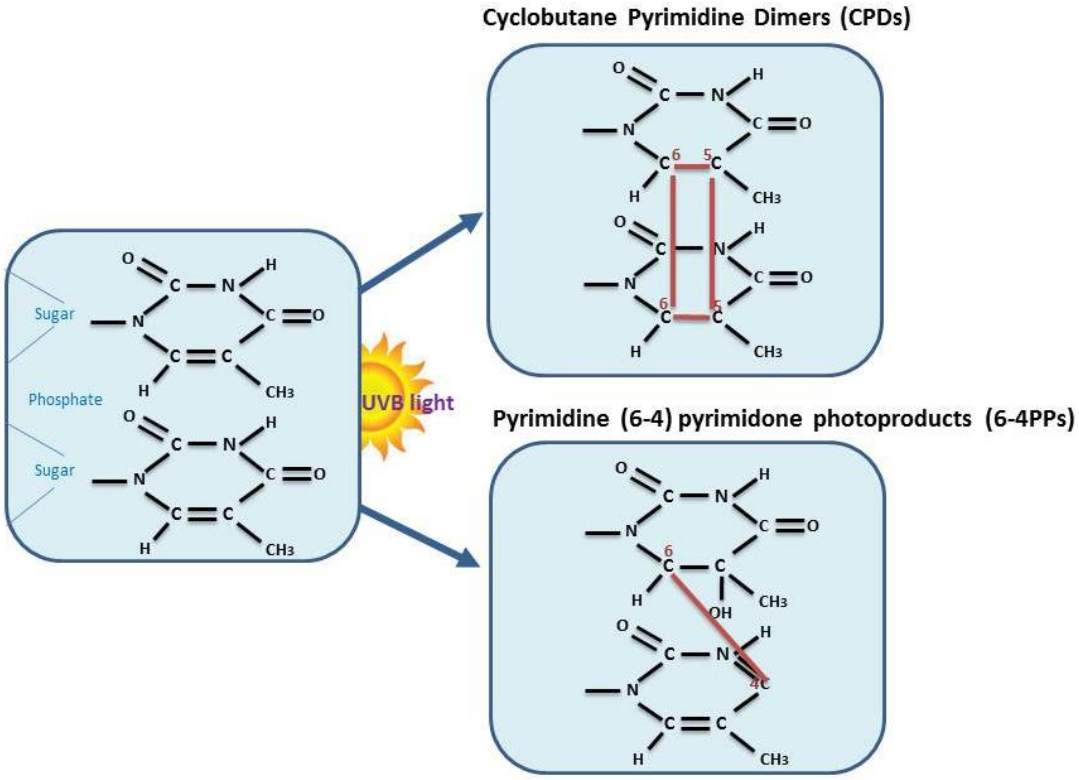
1.2.2 UV-induced photolesions in DNA

It has been known since 1877 that sunlight is capable of destroying bacteria (19). In 1885, it was proven that the germicide effect was caused by UV light from the sun (20). However, it was only in 1946 that the correlation between UV light and skin cancer was established (21).

When the photons of UV radiation are directly absorbed by the DNA molecule, two photolesions may be formed: the cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). The CPD lesions are characterized by two covalent bonds between carbons 5 and 6 of two adjacent pyrimidines in the same DNA strand, directly forming a cyclobutane ring. The 6-4PP lesions are formed due to one covalent bond between carbons 4 and 6 of two

adjacent pyrimidines in the same DNA strand, reviewed in (22). Figure 4 shows the chemical structure of each photolesion.

Figure 4 - Chemical structure of UV-induced photolesions

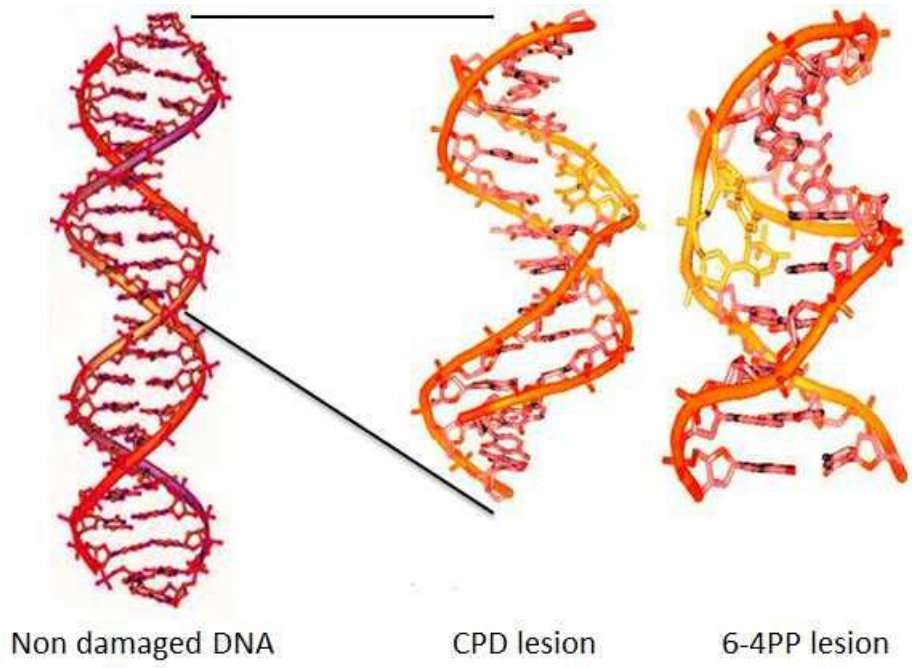


UV induces DNA photolesions through photon absorption, which leads to the formation of covalent bonds between two pyrimidines which are juxtaposed in tandem in the nucleotide DNA sequence. CPDs are characterized by the formation of two bonds between carbons 5 and 6 of the pyrimidines, whereas 6-4PPs are formed by one bond between carbons 4 and 6 of the adjacent bases.

CPD lesions can be formed in four different isoform configurations: *cis-syn*, *cis-anti*, *trans-syn* and *trans-anti*, the first being the most common in double-stranded B-form DNA (23).

CPD photolesions are three to five times more frequent than 6-4PPs, depending on the precise wavelength which reaches the DNA, nucleotide composition and chromatin structure of the DNA fragment (24). Furthermore, CPDs only cause a 30^{o} double-helix distortion (25), whereas 6-4PPs cause a significantly stronger 44^{o} distortion in the DNA double-helix (26), as illustrated in Figure 5.

Figure 5 - Representation of the double-helix distortion caused by CPDs and 6-4PPs



CPD formation causes a 30^{o} double-helix distortion whereas 6-4PP formation causes a more significant 44^{o} distortion. Adapted from Rastogi *et al*., 2011 (27).

In the core of a nucleosome, CPDs are formed regularly on an average of every 10.3 nucleotides, with a strong preference to form away from the histone surface (28). In linker DNA, as well as in naked DNA, CPD formation is nearly uniform, with no periodicity (29,30). Thus, it seems that DNA configuration is a deciding factor for CPD formation (31). In contrast, 6-4PPs are formed with no periodicity within the nucleosomes, but are formed six times more frequently in linker DNA (32). Other DNA-protein interactions, like those common in promoters, may also increase or decrease the DNA susceptibility to specific photolesion formation, i.e., the binding of a certain protein in a specific DNA region may be simultaneously inhibitory for 6-4PP formation and attractive for CPD formation, and vice-versa (33,34).

Furthermore, pyrimidine dimers have a different formation rate depending on the bases which form the photolesion and on the flanking bases 5´ and 3´ of the lesion. In naked UVC-irradiated plasmid, the relative yield of CPD formation is T<>T:C<>T:T<>C:C<>C (68:13:16:3) (35,36).

Even though CPDs and 6-4PPs are the most common photolesions induced in DNA after UV irradiation, they are not the only ones. 6-4PP lesions are formed through an oxetane or azetidine ring intermediate between carbons 5 and 6 of the 5´ pyrimidine and carbon 4 and the oxygen of the 3´pyrimidine. This very unstable ring

eventually breaks, transferring an amino or hydroxyl group to carbon 5 of the 5´pyrimidine, forming a stable bond between carbons 6 and 4 of the adjacent pyrimidines, reviewed in (37). Further stimulation of the 6-4PP lesion with a wavelength close to 315 nm (short UVA) may induce the formation of the 6-4PP valence isomer: Dewar photoproducts (Dewar-PP). Dewar-PP are formed by a slow electrocyclization reaction, generating the β-lactam ring characteristic of this type of DNA lesion (38).

For more information on photolesion structure, formation and distribution in DNA, please refer to the following reviews: Smerdon 1991 (39) and Pfeifer 1997 (22).

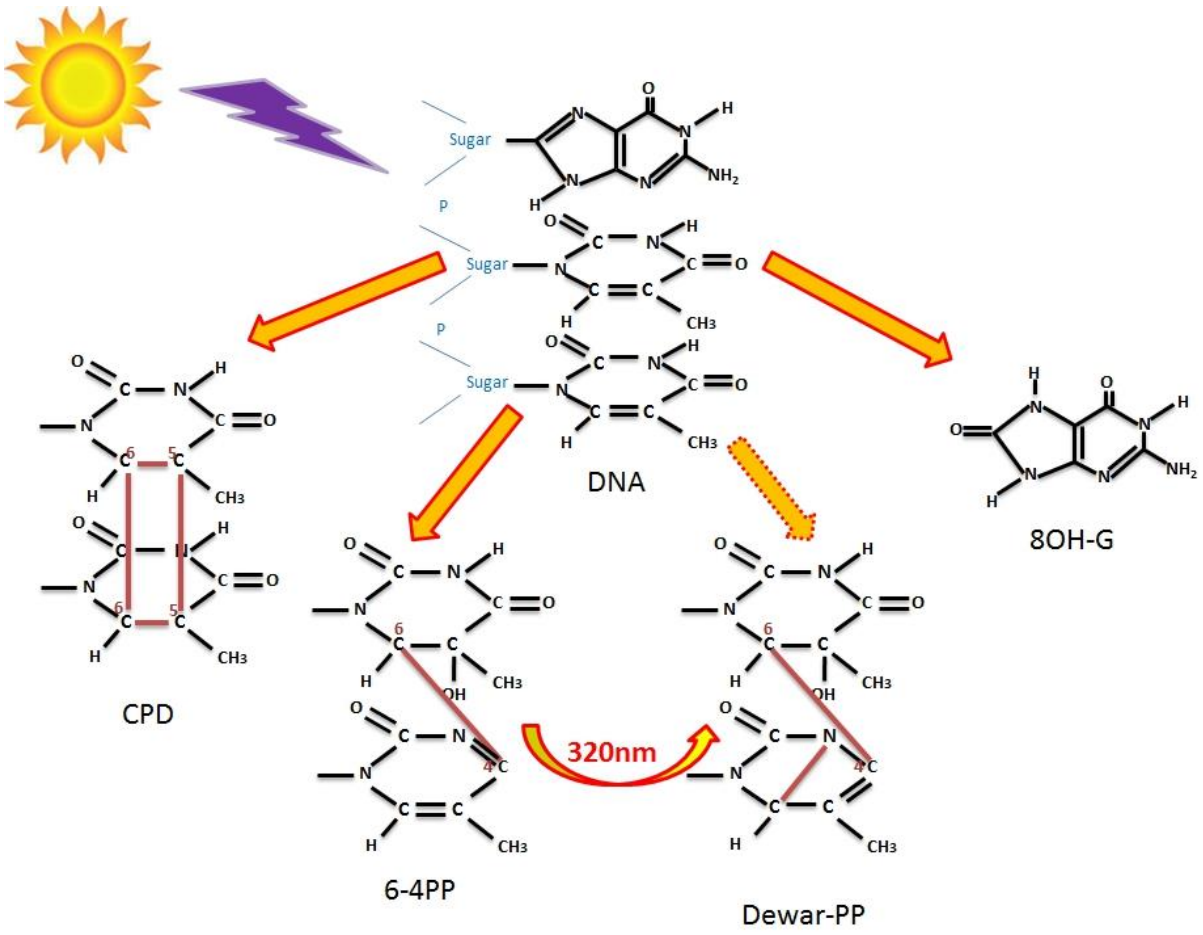
CPDs, 6-4PPs and Dewar-PP are the most common lesions induced by UVB in DNA. However, other DNA lesions can also be induced by UV light.

1.2.3 Other DNA lesions induced by UV radiation

The physical characteristics of each UV wavelength determine which organic molecules will absorb it best, reviewed in (40,41). As previously mentioned, nucleic acids and aromatic protein bonds are very effective in absorbing UVB, whereas UVA interacts mainly with chromophores (17). These specific properties directly correlate with the type of DNA damage caused by each UV wavelength.

It has been properly demonstrated in naked DNA that UVB produces a majority of CPDs, a significant amount of 6-4PP (which can be converted into the Dewar-PP isomer), and an almost undetectable quantity of oxidized lesions. UVA is also capable of directly inducing more CPDs than 6-4PPs, but at a lower frequency. On the other hand, UVA induces a significantly higher level of oxidized damage. Single strand breaks (SSBs) are virtually absent in this irradiation setup after both UVA and UVB exposures (42). Figure 6 represents the most common DNA lesions induced directly or indirectly by UVA and UVB cell irradiation.

Figure 6 - Schematic representation of the most common DNA lesions induced directly and indirectly by UVA and UVB irradiation



CPDs are the most common type of lesion induced by UVA and UVB cell irradiation, although at a lower frequency by UVA light. 6-4PPs are induced at a ~3 lower frequency than CPDs by both UVA and UVB, and may be converted to Dewar-PP by UVA irradiation. Oxidized damage, such as 8OH-G is generated at a relatively higher frequency by UVA than by UVB light. Strand breaks are generated at very low frequencies by both UVA and UVB irradiation.

Therefore, it can be concluded that UVA is capable of damaging DNA both directly, through photolesion formation, and indirectly. The indirect damages induced in DNA by UVA irradiation can be caused by two reactions, denominated type I or II. In the type I reaction, light is absorbed by endogenous chromophores, other than DNA, such as riboflavin, tryptophan or porphyrin, which interacts with the solvent or with the DNA molecule, damaging it. In the type II reaction, excited photosensitizers react with oxygen molecules, generating reactive oxygen species (ROS), which may in turn further damage DNA. Most of the damages caused in DNA through a type I or a type II reaction are oxidized lesions, such as 7,8-dihydro-8-oxoguanine (8OH-G) (43).

Alternatively, ROS can cause DNA strand breaks (44) or even interact with the nucleotide pools, producing oxidized nucleotides which can still be used in DNA transcription or replication (45,46).

Other types of DNA lesions may be formed after UV irradiation, such as 8,8-adenine dehydrodimer, pyrimidine hydrates, thymine glycol and spore photoproducts, among others. However, their formation rate is very low and/or their half-life is very short. Furthermore, some of them can only occur under very specific conditions, such as in an anhydrous environment. However, their occurrence is not part of the scope of this work and, therefore, will not be further mentioned. For a concise review please refer to chapter two of the book titled “DNA repair and Mutagenesis” (47).

Interestingly, the UV- signature mutations (C→T and CC→TT transitions) are the most common type of mutations seen in cells and tissues irradiated either with UVA or UVB light. This indicates that even though oxidized lesions may play an important part in cell responses to DNA damage, this type of lesion may not directly correlate with mutation induction after UV irradiation and its consequent genotoxicity (43,48).

1.2.4 UV-induced DNA mutations

DNA wavelength absorption peaks around 260 nm (UVC), where the highest yields of CPD and 6-4PP formation are found. This relative absorption is 10^2 times smaller for UVB wavelengths and 10^{-5} for UVA (49), which directly correlates with photolesion formation rates and, consequently, to mutation induction (48).

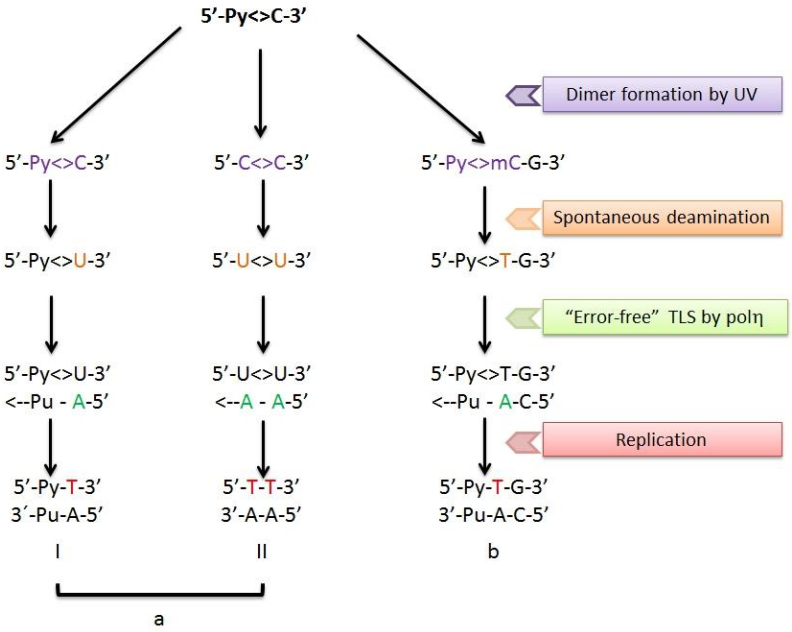
UV irradiation induces specific types of mutation at dipyrimidine sites: the most common is a base substitution of cytosine to thymine (C → T), and the relatively rare tandem base substitution of cytosines to thymines (CC → TT). These two types of mutation are known as UV-signature mutations (48,50,51).

One of the mechanisms through which UV-signature mutations occur is the cytosine deamination in the pyrimidine dimer, resulting in an uracil (U) base, followed by “error-free” translesion synthesis (TLS) of the bulky lesion by polymerase eta (pol η) during DNA replication. This TLS will insert an A opposite to the U, which, if not corrected, will result in a C → T conversion, fixating the mutation. In cases where the two pyrimidines in the dimer are C, both can undergo deamination, followed by the

TLS by Pol η, which may result in a CC → TT conversion, fixating the second type of UV-signature mutation in the DNA strand (52).

Alternatively, the deamination may occur in a methylated C (5-methylcytosine; mC), originating a T. In this case, TLS will probably add an A opposite to each T. In turn, just as in the previous model, after DNA replication this C → T conversion will be fixated as a mutation. This specific type of mutation, very common in methylated CpG islands, is known collectively as Solar UV-signature mutation (53), reviewed in (54). Figure 7 presents the aforementioned induction models for UV-signature mutations and Solar UV-signature mutations.

Figure 7 - Possible mechanisms of UV and Solar UV-signature mutations induction



Cytosine deamination of pyrimidine(s) leads to the formation of uracil(s) in the pyrimidine dimer; a) "error-free" translesion synthesis by Pol η inserts an adenine in front of each uracil. Alternatively, b) deamination of methylated cytosine leads to the presence of thymine bases in the pyrimidine dimer. Both mechanisms explain the formation and fixation of UV-signature and Solar UV-signature mutations, respectively (C → T and CC → TT). "<>" represent dimers. Adapted from Ikehata & Ono, 2011 (54).

However, TLS by Pol η alone cannot explain the UV-signature mutations, since the same mutational spectrum is seen in organisms which do not express Pol η or any homologue, such as *Escherichia coli* (*E. coli*) (55) (56). Furthermore, Xeroderma Pigmentosum Variant (XP-V) human cell lines, which fail to express a functional Pol η, present the UV-signature mutations and at a higher rate as well (57).

Therefore, at least part of the UV-signature mutations cannot be related to the Pol η activity. It is known that in *E. coli*, when polymerase 5 (Pol V) is absent, there is

a suppression of UV-induced mutagenesis (58). This enzyme is known as an “error-prone” TLS polymerase, usually inserting an A opposite to a pyrimidine dimer, which also explains the C → T and CC → TT conversions (59). It is possible that other TLS polymerases, such as polymerase Rev1 (Pol Rev1), polymerase kappa (Pol κ) and polymerase iota (Pol ι), may also play a similar role in UV-signature mutations induction, reviewed in (54).

For more detailed information on UV-photoproduct TLS, please refer to subsection 1.5 of the present work.

Furthermore, UV-signature mutations are induced mainly by CPDs (60). Even though it has been established that 6-4PP significantly contributes to the formation of UV-signature mutations (61,62), the mechanism by which these photoproducts lead to the induction of these mutations is unclear, probably involving the aforementioned “error-prone” polymerases: Pol Rev1, Pol κ and/or Pol ι (63,64).

Dewar-PP may also play an important role in UV-induced mutagenesis, since they are more stable than their isomer 6-4PP and cause a similar double helix distortion (65). However, the mechanism through which they may cause these mutations is not clear, but just like with 6-4PPs, it seems to involve “error-prone” polymerases (66).

In addition to the UV-signature mutations, the UV irradiation usually also leads to triplet mutations, especially in DNA repair-deficient backgrounds, such as alterations in the nucleotide excision repair (NER) pathway (67–69). Since CPDs are a poor substrate for NER, the lesions which are more probably involved in the induction of triplet mutations are the 6-4PPs and the Dewar-PPs. Therefore, the formation of the triplet mutations must involve “error-prone” polymerases. In the model proposed by Ikehata & Ono (54), the TLS polymerase would add a mismatched nucleotide 3´ to the dipyrimidinic site, consistent with the “error-prone” characteristic of some polymerases, such as Pol κ (63).

For more detailed information on UV-induced mutagenesis, please refer to the excellent review by Ikehata & Ono of 2011 (54).

1.3 DNA repair

Considering the relevance of genome integrity maintenance and the constant attack of endogenous and exogenous agents on DNA, it is no surprise that, during evolution, several DNA repair mechanisms have been selected in order to revert, correct, remove or adapt to the existence of such a wide variety of DNA lesions.

For instance, the Mismatch Repair (MMR) mechanism is capable of correcting wrongly paired DNA bases; Base Excision Repair (BER) deals with damaged DNA bases and SSBs; Translesion Synthesis (TLS) counts on specific DNA polymerases capable of replicating through DNA lesions; Homologous Recombination and Non-Homologous End Joining (NHEJ) deal with SSBs and, mostly, with double strand breaks (DSB) in the DNA, reviewed in (70).

Two DNA repair mechanisms are capable of dealing with the photolesions induced by UV irradiation (CPDs and 6-4PPs): photolyases and the NER pathway, which will be discussed in further detail in sections 1.3.1 and 1.3.2 of the present work, respectively.

1.3.1 Photolesion repair by photolyases

Photorepair of UV-induced DNA lesions was the first DNA repair mechanism to be discovered. In 1949, Albert Kelner was UV irradiating bacteria, searching for mutations which could lead to the production of novel antibiotics. Kelner was having trouble duplicating the mutation rates when he finally realized that the bacterial cultures which were exposed to white light after the UV exposure had lower mutation frequencies (71,72). At about the same time, Renato Dulbecco made similar discoveries working with bacteriophages and UV irradiation (73).

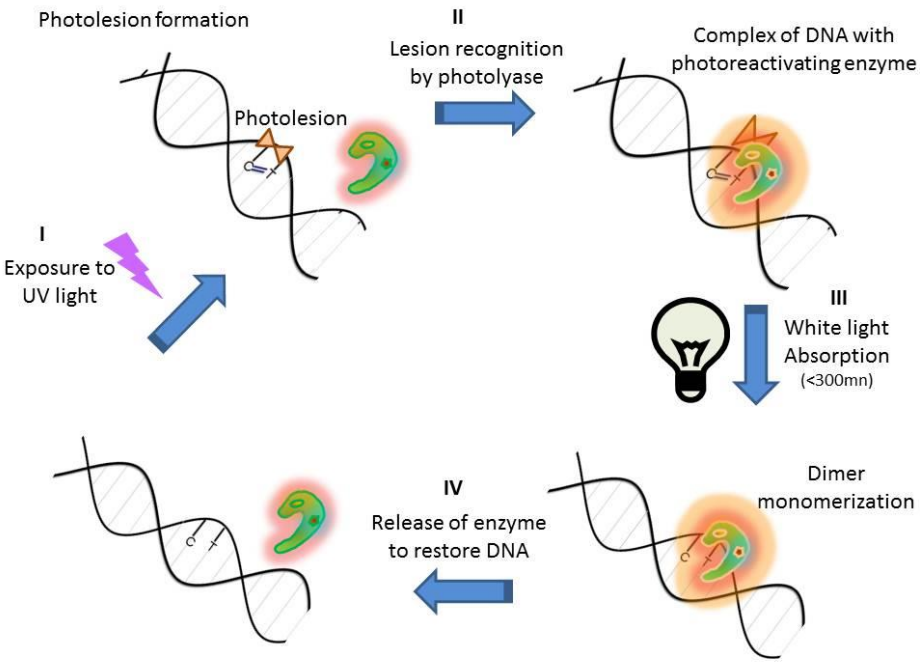
In Kelner´s second article about this subject, the process was named photoreactivation (72). Between 1958 and 1962, Claud Rupert demonstrated that the photoreactivation process actually depended on an enzyme, called photolyase (74–77). In his 1962 articles, Rupert also showed that the DNA-photolyase complex is formed in the dark and that the average turnover for lesion recovery is 1-2 minutes (76,77).

In the 80´s, the chromophores present in the photolyase enzyme were identified: all photolyases have a flavin adenine dinucleotide (FAD) as the first

chromophore. Photolyases may have an additional second chromophore, which varies from species to species: 5,10-methenyltetrahydrofolyl polyglutamate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF) (78–81). It was later proved that only FAD chromophore is essential for enzymatic activity in photolyases purified from several organisms, especially when FAD acts as a sensitizer and not the second chromophore (82–84).

Figure 8 presents a schematic representation of photolesion recognition by photolyase, lesion-enzyme complex formation in the dark and light-dependent lesion monomerization.

Figure 8 - Schematic representation of dimer photoreactivation

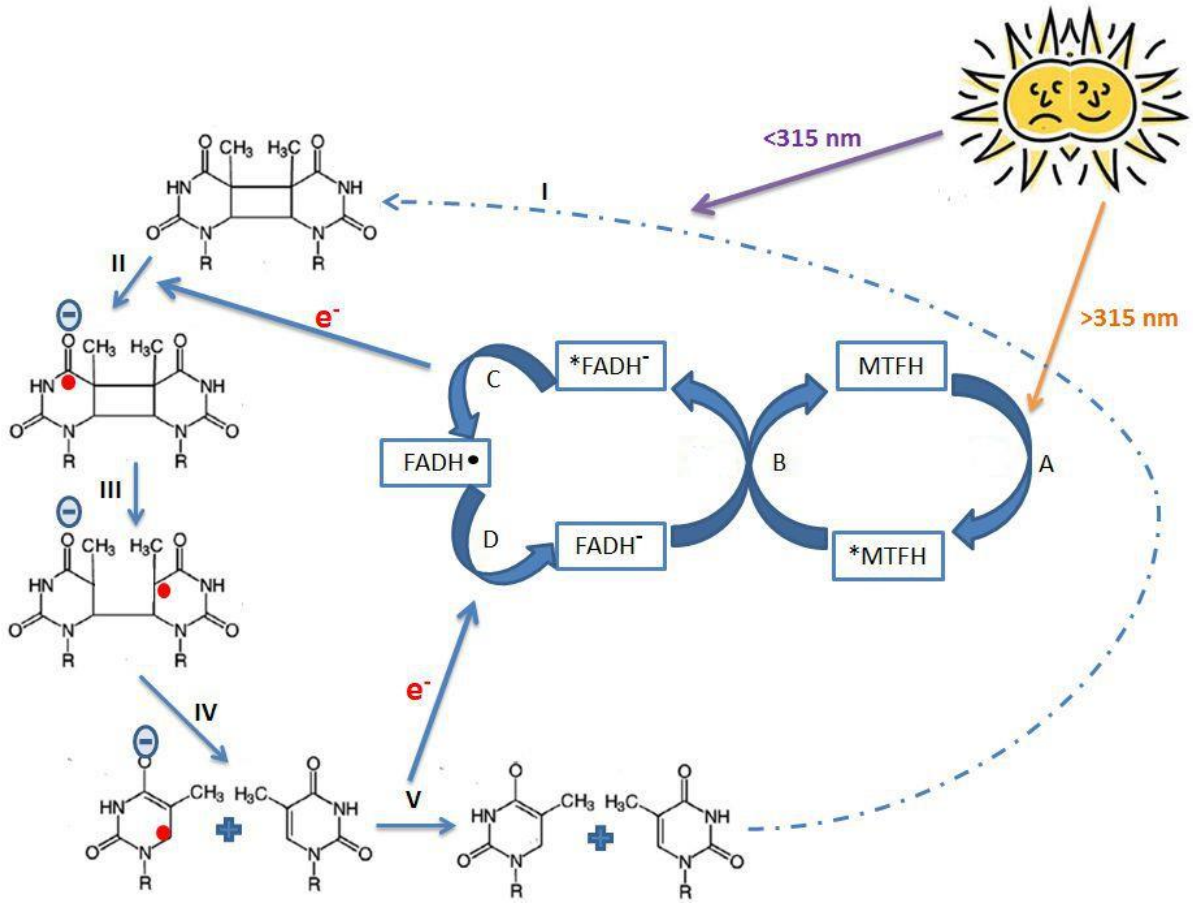


UV irradiation leads to the formation of pyrimidine dimer (I); in the dark, complex dimer-enzyme is formed (II); Photon absorption activates photolyase (III); and dimer is directly monomerized (IV); finally, the complex is undone and the enzyme is ready for another photoreactivatioin reaction.

Photolyases are capable of reconverting the pyrimidine dimers back to monomers through an electron transfer from the FAD chromophore to the lesion, breaking the extra covalent bonds which unite the dimer, allowing pyrimidine monomerization, followed by an electron transfer back to FAD. If FAD acts as a sensitizer, it will be directly excited by a photon. Alternatively, another chromophore (MTFH or 8-HDF) may first be sensitized by the photon and, in turn, excite FAD

which will transfer an electron to the dimer, as previously described. This reaction takes less than 1 ns (85) and is represented in Figure 9:

Figure 9 - Representation of chemical reactions involved in photoreactivation



A chromophore (MTFH) is sensitized by a photon (A) and transfers energy to the second chromophore (FAD) (B). A pyrimidine dimer (CPD in this case) is formed by UVB irradiation of DNA (I). FAD donates an electron (e^-) to the pyrimidine dimer (II), breaking the first (III) and then the second covalent bond which forms the cyclobutane dimer (IV); electron is transferred back to FAD, finalizing the photoreactivation process (V).

All previously mentioned studies on photoreactivation involved CPD repair by specific CPD-photolyase. It was not until 1993 that an enzyme capable of photoreactivating 6-4PPs was described in *Drosophila melanogaster* (86). CPD-photolyase and 6-4PP-photolyase are homologous, sharing an even higher amino acid sequence similarity then that between several CPD-photolyases (87).

There is strong evidence that 6-4PP-photolyase can also photoreactivate Dewar-PP, although with less affinity (88). This process begins with the Dewar-PP conversion into its 6-4PP isomer, followed by its photoreactivation, all of which occurs through 6-4PP-photolyase activity (89). CPD-photolyases (a) and 6-4PP-photolyases

(b) are very specific for their substrate: CPDs (a) and 6-4PP and Dewar-PP (b), with no cross-recognition having ever been described (87).

Both CPD-photolyases and 6-4PP-photolyases are part of the blue-light receptor family, together with the cryptochromes, which are involved in circadian rhythm control (87).

Blue-light receptor family genes are widely distributed throughout all kingdoms of life, including viruses, prokaryotes and eukaryotes (both plants and animals). However, placental mammals do not have photolyase encoding genes. One possible explanation for the absence of these genes is that placental mammals developed nocturnal habits during evolution, which may have released the evolutionary pressure for their presence (90).

For more detailed information on photolyase structure, function and distribution, please refer to the following works: the book titled “DNA Repair and Mutagenesis” (70) and Eker´s 2009 review (90).

There is no DNA repair process more efficient and effective than one involving only one enzyme, composed of one single polypeptide, and requiring only one step which does not include DNA cleavages or base substitution.

However, placental mammals cannot count on direct photoreversal of UV-induced lesions, and depend exclusively on the NER pathway to remove photolesions from their genomic DNA.

1.3.2 Photolesion repair by the Nucleotide Excision Repair (NER) pathway

The first evidences of the existence of a DNA repair pathway not involving photoreactivation came in 1958, when Hill discovered an *E. coli* strain particularly sensitive to UV irradiation (91), and in 1962, when Howard-Flanders and co-workers defined a genic *locus*, also in *E.coli*, which controlled photoproduct removal (92). In 1964, simultaneously and independently, Setlow & Carrier and Boyce & Howard-Flanders demonstrated that, in this lesion removal pathway, a small DNA fragment containing the lesion was excised (93,94). Only six months later, Pettijohn and Hanawalt showed that after the excision of the damaged DNA fragment there was a form of non-semiconservative DNA synthesis (95).

In the same year, Rasmussen & Painter demonstrated that DNA repair also occurs in mammalian cells (96). In the following decade several mechanisms of DNA

repair, including BER and MMR, were discovered, reviewed in (97). In 1985, Bohr and colleagues showed that lesions present in the transcribed strand of active genes are removed faster by a NER subpathway, the transcription coupled repair (TC-NER), than lesions elsewhere in the genome, which are removed by the global genome repair (GG-NER) (98).

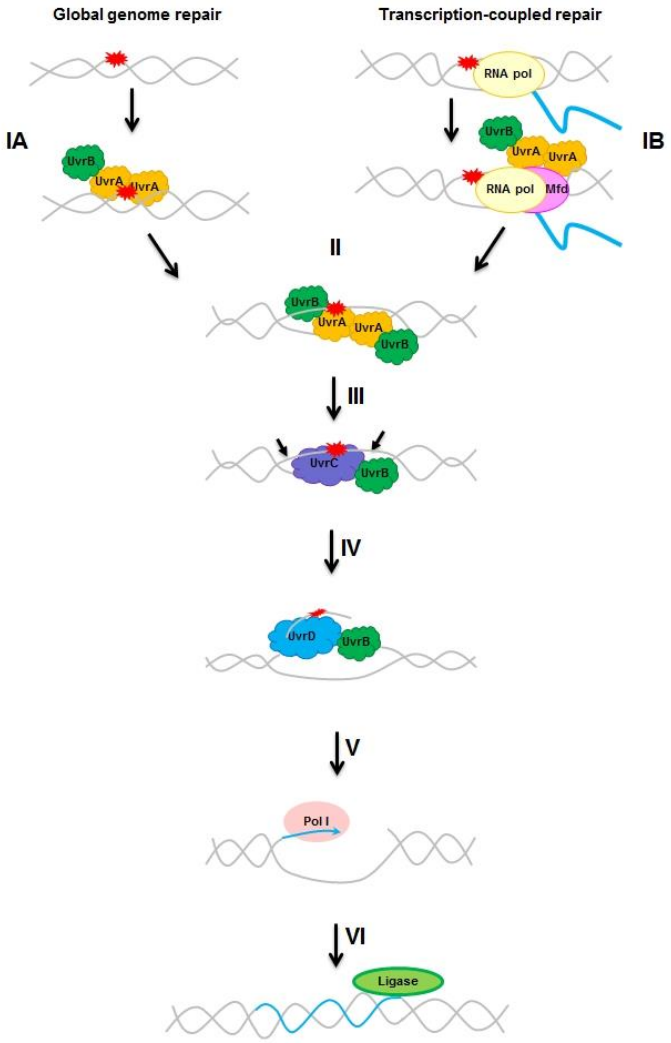
NER is a highly versatile pathway, capable of removing a wide variety of bulky lesions. Its substrates not only include UV-induced photoproducts (CPD, 6-4PP and Dewar-PP), but also oxidized damages and intrastrand crosslinks. Even though these lesions can be caused by several different endogenous and exogenous agents and have little to no chemical similarity, the recognition factors are able to identify the distortion they cause on the double-helix, despite of its origin (99).

The NER pathway is widely found in both prokaryotes and eukaryotes. In prokaryotes, only seven proteins are involved (four UV resistant proteins: UvrA, UvrB, UvrC and UvrD ; mutation frequency decline protein, Mfd; polymerase 1, Pol I; and a ligase, Lig) in the lesion removal, whereas in mammals this pathway requires the concerted action of over thirty proteins, reviewed in (99,100).

The first step in this lesion-removal system is (1) lesion recognition, followed by (2) region stabilization, (3) double-helix opening, (4) damaged fragment excision, (4) re-synthesis of the excised fragment, and (5) ligation to pre-existing contiguous DNA.

E. coli is the model organism in the study of prokaryotic NER. As schematized in Figure 10, the DNA lesion is either recognized by a stalled RNA polymerase (RNA Pol) with the aid of Mfd and $UvrA_2B$ in the TC-NER, or directly by the later protein trimer in the GG-NER. After lesion recognition, subpathways converge to the same pathway, a second UvrB is recruited and $UvrA_2$ leads to a DNA conformational change. The complex is released and only one UvrB stays at the lesion site. UvrC is recruited and forms the complex UvrBC. The fragment which contains the damage is excised about 5 nucleotides upstream and then on the eighth nucleotide downstream of the lesion by UvrC (101); UvrD aids in the removal of the excised fragment; Pol I synthesizes the DNA which is then ligated by a DNA ligase. Energy (adenosine triphosphate, ATP) is required for the NER pathway to be successful, reviewed in (99,100).

Figure 10 - Simplified model of nucleotide excision repair in *E. coli*



In the GG-NER subpathway, the lesion is recognized by the $UvrA_2B$ complex (IA). In the TC-NER subpathway, RNA Pol stalled by the lesion recognizes the damage with the aid of Mfd and the $UvrA_2B$ complex (IB). After the recognition step, subpathways converge into one pathway. $UvrA_2$ recruits a second UvrB and causes a DNA conformational change (II). The protein complex is then released, only one UvrB remains and UvrC is recruited to make incisions 5´ and a 3´ to the DNA lesion (III). UvrC is released and UvrD is recruited to aid in the removal of the damaged DNA fragment (IV). The gap is filled by Pol I (V) and the newly synthetized DNA is sealed by a DNA ligase (VI).

The proteins involved in the NER pathway are conserved inside prokaryotic and eukaryotic groups, but not between them, reviewed in (99). The eukaryotic system is significantly more complex.

In the eukaryotic GG-NER, the lesion is recognized by the complex xeroderma pigmentosum (XP) complementation group C (XPC) – human RAD 23 homolog B (hHR23B), potentially altering the double-helix structure at the damage site, allowing for the other factors to recognize and stabilize the damaged region (102). However, there is evidence that if the lesion severely distorts the DNA double-helix, XPC may not be required for its recognition (103). The UV-damaged DNA binding (UV-DDB)

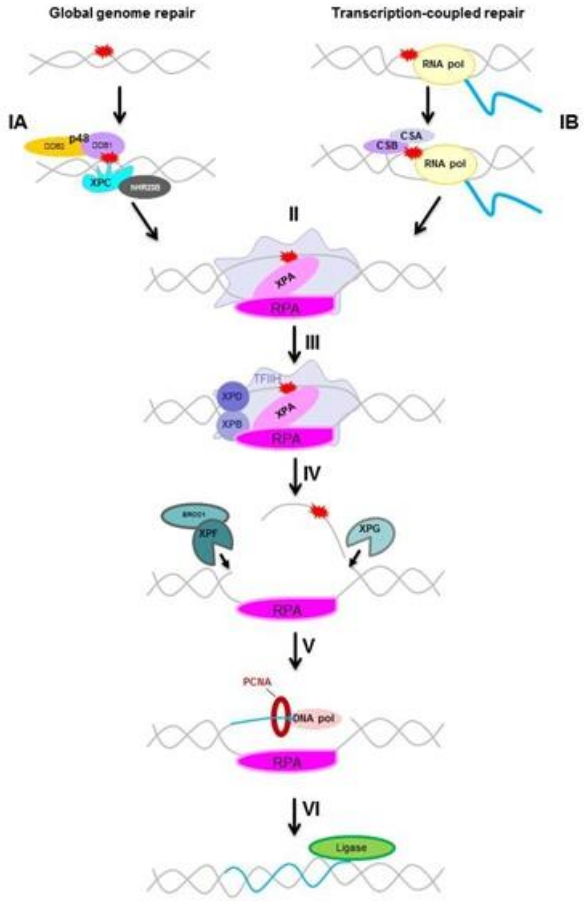
protein, also known as protein 48 (p48) or XPE, is composed of a dimer (DDB1-DDB2), and seems to aid XPC in the recognition of some specific types of damage, such as CPD (104). XPA also has affinity with damaged DNA and helps in lesion recognition and other NER factors recruitment (105).

Once XPA binds to the damage site, it recruits the transcription factor II H (TFIIH) complex (106). TFIIH is formed of nine subunits and has a basal role in transcription. Some of the TFIIH subunits required for NER are: XPB, XPD, the complex excision repair cross-complementing rodent factor 1 (ERCC1)-XPF (ERCC1-XPF) and XPG (107,108). After TFIIH is recruited, in the presence of XPG, XPC is released (106). XPB has a 5´-3´helicase activity, while XPD´s activity is 3´-5´ (109). While the helicases are opening the double-helix of the region containing the damage, the replication protein A (RPA), which has high affinity for single-stranded DNA (ssDNA), is recruited (110,111), forming a complex with XPA. The nucleases then take part, excising a 24-32 nucleotide fragment. ERCC1-XPF makes the incision 16-25 bonds 5´ of the damage (112) and then XPG cuts two to nine phosphodiester bonds 3´ of the lesion (113–115). This pre-incision complex (PIC) is then released and the gap is filled by a proliferating cell nuclear antigen (PCNA) dependent polymerase (probably polymerase delta, Pol δ, or polymerase epsilon, Pol ε) (116), with RPAs stabilizing the ssDNA (106). The gap is finally sealed by a DNA ligase, possibly Ligase I (117).

The previously described GG-NER subpathway is responsible for recognizing and removing lesions which can be situated anywhere in the genome. The other subpathway, TC-NER, differs only in the lesion recognition step and is responsible for lesion removal from the transcribed strands of active genes. In TC-NER, when RNA polymerase II (RNA Pol II) is stalled at a lesion site during transcription, it recruits the other NER factors, with the aid of Cockayne Syndrome proteins A and B (CSA and CSB). CS proteins seem to support the RNA Pol II complex allowing for its temporary removal from the lesion site (118,119). Both GG-NER and TC-NER require energy (ATP) in several of their steps.

Figure 11 presents a simplified model of the mammalian NER pathway. For more detailed information about the prokaryotic and eukaryotic NER pathways, please refer to the reviews by Wood (120) and Batty (99).

Figure 11 - Simplified model of the mammalian nucleotide excision repair (NER) pathway



In the GG-NER subpathway, the lesion is recognized by the XPC-hHR23B complex, with the aid of the p48 protein (IA). In the TC-NER subpathway, RNA Pol II stalled by the lesion recognizes the lesion site (IB). After the recognition step, both subpathways converge into one pathway. XPA and RPA are recruited and stabilize the lesion site (II). TFIIH complex (XPB, XPD, ERCC1-XPF, XPG) is recruited and DNA double-helix is unwinded by helicases XPB and XPD (III). ERCC1-XPF excises 5´ of the lesion and then XPG excises 3´ (IV). Proteins are released and the gap is filled by a PCNA dependent DNA polymerase (V) and the newly synthetized DNA is sealed by a DNA ligase (VI). Adapted from Quayle *et al*., 2011 (121).

While CPD-photolyase and 6-4PP-photolyase are able to specifically remove CPDs and 6-4PPs at the same rate (122), the NER pathway removes 6-4PPs much faster than CPDs. The totality of 6-4PPs is removed within 3 hours, whereas only 60 % of CPDs is removed in 24 hours, even for low UVC doses (10-15 J/m^2) (123,124). This difference is probably related to the different DNA distortion caused by each photolesion (27) and to the fact that XPC and XPA recognize lesions in DNA by the distortion they cause on the double-helix.

Therefore, when considering GG-NER, the more distortive the DNA lesion is, the quicker it will be recognized and removed. In this model, both types of lesion

present in the transcribed strand of active genes must be removed by TC-NER with the same efficiency, reviewed in (22,106).

Furthermore, photolesion removal by TC-NER is significantly quicker than by GG-NER: in eight hours, 80 % of CPD lesions are removed from the transcribed strands of active genes, while less than 30 % is removed from the rest of the genome (125). This difference is probably related to the more efficient surveillance exercised by TC-NER, which counts on RNA Pol II stalling as a recruiting signal for the NER proteins, and also to lesion accessibility (22).

As previously mentioned, p48 seems to aid XPC in CPD recognition (104). However, rodents, such as mice, lack this protein and are virtually unable to remove CPD lesions through GG-NER (126). Therefore, these lesions must accumulate throughout their genome, except in active genes.

When photolesions are not repaired by either photolyases nor by the NER pathway, there is a mechanism which helps cells to deal with their presence, avoiding the elicitation of other cellular responses, such as senescence and apoptosis (viewed in more detail in section 1.5 of the present work): the translesion DNA synthesis.

1.4 Translesion synthesis (TLS) of UV-induced photoproducts

Replicative DNA polymerases are not able to accommodate most damaged DNA in their active site (including CPDs, 6-4PPs and Dewar-PPs), promoting a physical blockage to strand elongation, reviewed in (127). Therefore, the presence of photolesions during cell replication may lead to replication fork collapse which, in turn, may culminate in cell death (reviewed in section 1.5 of the present work).

There is, however, a mechanism which helps cells to cope with the presence of DNA damage during replication: DNA translesion synthesis (TLS). TLS was first observed in 1968 by Rupp and Howard-Flanders, when they identified gaps after DNA synthesis in *E. coli* strains defective for DNA repair (128). The idea of TLS was already circulating in the 1970´s, but it was only in 1996 that the first TLS polymerase was identified (129), reviewed in (127).

Nowadays, several TLS polymerases are known. Some of them are “error-free” for specific types of DNA lesions, while others are considered to be “error-

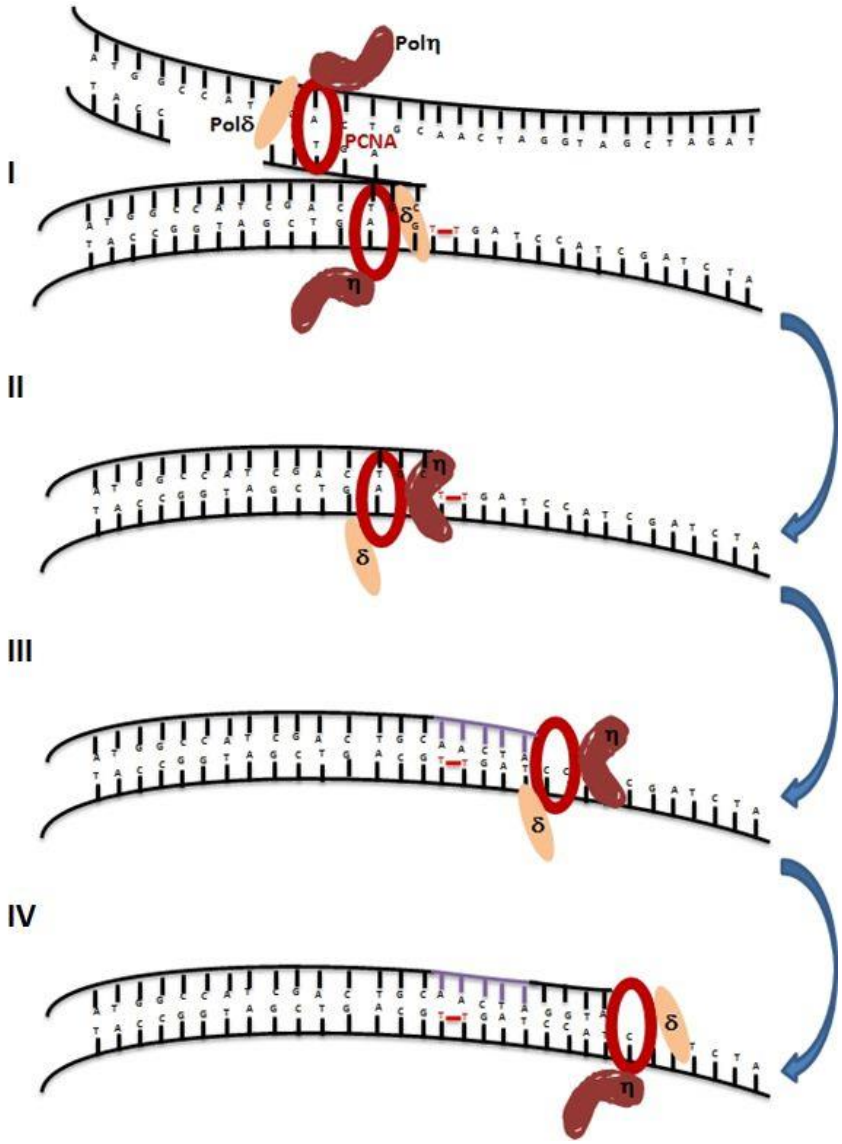
prone". TLS polymerases are found in all domains of life, which suggests they play a very important role in genome maintenance, reviewed in (130).

The general principle by which TLS polymerases are capable of replicating through DNA damage is that after the replicative polymerase (Pol ε or Pol δ) is stalled at the DNA damage site, PCNA suffers a series of modifications (ubiquitilation, sumoylation and/or phosphorylation), reducing its affinity with that polymerase and increasing the affinity for a specific TLS polymerase, which is then recruited to the site of the lesion. In combination with PCNA, this TLS polymerase will replicate the damaged site and, possibly, extend the patch for a few nucleotides before the replicative polymerase resumes the DNA duplication process. Alternatively, depending on the type of lesion and on the TLS polymerase involved, a second TLS polymerase, or even the replicative polymerase, may make the patch extension, reviewed in (127).

The present work focuses on Pol η, which is capable of transposing *cys-syn* T<>T CPD lesions with accuracy. Pol η is part of the Y polymerase family and was first identified in yeast in 1999 (131) and, later in the same year, in humans (132,133).

The active site of Pol η is particularly large and can accommodate both bases of the pyrimidine dimer. It also stabilizes the T<>T, the most common type of CPD, so that two As can be paired with two Ts. To ensure that replication is accurate after Pol η is released and a replicative polymerase resumes the DNA synthesis, it also adds three nucleotides after the lesion while still stabilizing the dimer, guaranteeing that there is no DNA distortion after the translesion patch (134,135). Figure 12 presents a simplified model for the translesion of T<>T by Pol η, a process considered "error-free" (~2 % errors), reviewed in (135).

Figure 12 - Simplified translesion mechanism of Pol η of T<>T lesion



The replicative polymerase is stalled at the presence of T<>T dimers (I). It is substituted by the TLS "error-free" Pol η (II), which is capable of adding two As pairing the two Ts and three more bases before the replication fork switches back to the replicative polymerase.

When the photolesion in question is 6-4PP or Dewar-PP, or when Pol η is not present, it seems that other TLS polymerases take place, in a more "error-prone" fashion. There is strong evidence suggesting that the polymerases involved in this process are Pol κ or Pol ι, in combination with polymerase zeta (Pol ζ) (63,64,136,137). It has recently been shown that Pol ζ is not only involved in "error-prone" TLS but also in "error-free" (< 2 % errors) 6-4PP TLS, independently of Pol κ or Pol ι (138).

For more detailed information about the structure and action mechanism of TLS polymerases, including Pol η, please refer to the following reviews: Livneh, 2010 (139) and Sale, 2012 (127).

1.5 Cellular responses to UV-induced photoproducts

The main regulators of cellular responses to DNA damage are ataxia telangiectasia mutated (ATM), ATM and RAD3 related (ATR) and DNA protein kinase (DNA-PK). It is known that ATM and DNA-PK generally respond to DSB, whereas ATR is mainly activated by the presence of ssDNA, reviewed in (27).

ATR is the principal regulator of the cell responses against UV-induced DNA damage, mostly through RPA signaling in ssDNA regions at stalled replication forks. This DNA structure also recruits the ATR-interacting protein (ATRIP) and the 9-1-1 complex [RAS-related associated with diabetes (RAD) 9 (RAD 9), RAD 1 and hydroxyurea sensitive homolog to *S. pombae* 1 (HUS 1)] which in turn recruits topoisomerase binding protein 1 (TOPBP1). This protein then activates ATR through ATRIP, reviewed in (140).

Activated ATR is capable of phosphorylating a series of effector signals which may lead to specific cell cycle checkpoint activation, recruitment of DNA repair systems, senescence induction, different types of cell death (such as apoptosis or necrosis), or autophagy. It is possible that several of these responses are elicited at the same time or in sequence. For instance, the presence of DNA damage may induce checkpoint activation followed by the recruitment of a DNA repair pathway; if the damage is not satisfactorily removed, the cell may undergo apoptosis, reviewed in (27).

One of the main targets of ATR is checkpoint 1 (CHK1). Phosphorylated CHK1 phosphorylates cell division cycle protein 25 (CDC25), whose consequent degradation will lead to G1/S and/or intra-S phase checkpoint activation, preventing cells to enter mitosis, reviewed in (27).

Another ATR target is the transcription factor protein 53 (p53), a central regulator of DNA damage response. P53 controls a series of UV-responses, from checkpoint activation, to DNA repair proteins recruitment, and even apoptosis and cell proliferation. This broad number of roles that p53 plays in cell responses to UV irradiation is due to a high diversity of targets. For instance, p53 interacts with the

CDK inhibitor protein 21 (p21), which arrests cells in the G1/S checkpoint, reviewed in (27).

Checkpoint activation is particularly important since it allows cells time to recruit DNA repair proteins to the DNA damage site, preventing the cell to attempt to replicate with damaged DNA. Replication of damaged DNA may lead to mutation, or cell death induction, reviewed in (141).

The UV-induced mutation pathways have been previously explained in section 1.2.4 of the present work. It has also been previously explained how dipyrimidine lesions can cause transcription fork arrest (142) (section 1.4), which in mammal cells is usually repaired by TC-NER (section 1.3.2). By the same principle, it is also possible for photolesions to stall replication fork (subject of the previous section).

Considering that photolesions are capable of stalling transcription forks, they may be able to alter the transcription pattern of a given cell, with severe consequences, depending on the genes involved (143,144).

However, if the photolesion stalls a replication fork, and it is not repaired, it may lead to fork collapse, which can cause DSBs. This type of DNA break can not only lead to genomic instability, chromosome rearrangements and mutations, but it is also a very strong apoptotic signal (145).

UV irradiation can induce cell death through several mechanisms, including necrosis when the injury is too severe for the cell to deal with. Most commonly, when lesions are not efficiently removed, apoptosis is induced in a p53/p21/B-cell lymphoma 2 (BCL2) associated protein X (BAX) dependent manner, reviewed in (146).

1.6 NER deficiency related disorders

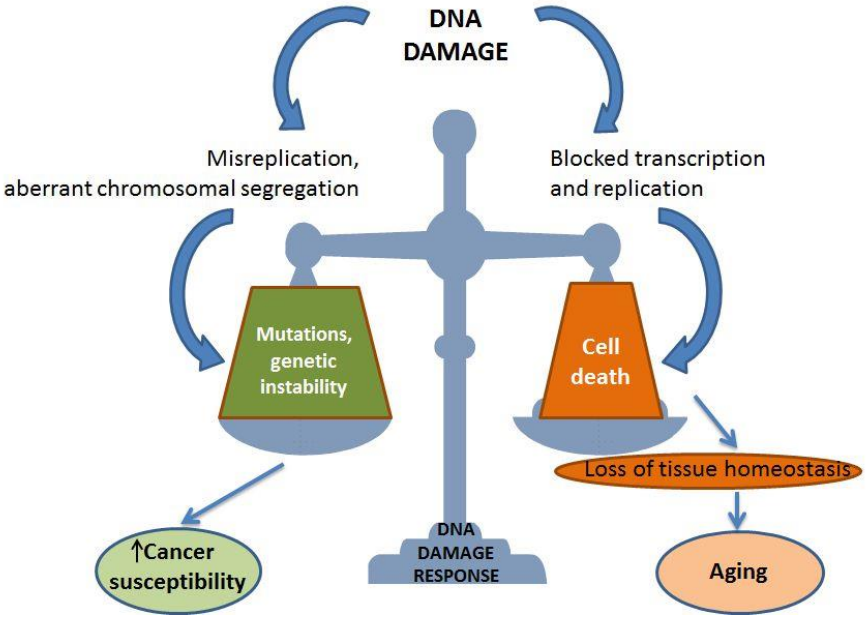
If the fact that DNA Repair is spread throughout all kingdoms of life is not enough proof of the importance of these mechanisms, the existence of several disorders related to different deficiencies in DNA Repair should provide enough evidence. These include Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS), Trichothiodystrophy (TTD), Werner Syndrome (WRN), Bloom Syndrome (BLM), Rothmund-Thompson Syndrome (RT), Fanconi Anemia (FA), Ataxia Telangiectasia (AT) and Hutchinson-Gilford Syndrome (HGPS).

All of the previously mentioned conditions may, at least in some cases, manifest themselves with progeria. The term “progeria” comes from the Greek “pro” (before) and “géras” (old age) and means “segmented premature aging”. The term “segmented” is added to emphasize that not all tissues from the organisms necessarily age at the same speed, meaning that, in some diseases, only a few organs may be affected.

Even though since the late 1950´s the theories from Failla and Szillaard suggested that normal aging was probably related to genomic mutations, the intimate connection between DNA repair deficiencies and aging was not well understood for a long time, reviewed in (147–149). It was only in the late 1990´s that Hoeijmakers´ group was able to establish this link, analyzing data from several mouse models with DNA repair deficiencies and progeria.

Comparing the deficiencies and phenotypes of NER deficient animals Hoeijmakers´ group observed that TC-NER deficient animals tended to have increased cell death and progeria, as did CS and TTD patients. On the other hand, GG-NER deficient mice tended to accumulate genetic mutations and have enhanced cancer susceptibility, as observed in most XP patients. With these observations, they were able to conclude that the link between DNA repair and aging was the DNA damage response (DDR): cell death culminated with loss of tissue homeostasis and consequent aging, whereas low cell death rate and consequence genetic mutation accumulation led to an enhanced cancer susceptibility, reviewed in (141,150,151). Figure 13 represents the delicate balance between cancer and aging.

Figure 13 - The delicate balance between aging and cancer



DDR regulates the cell´s response against DNA damage: if the lesion is too severe, it may lead to cell death and consequent loss of tissue homeostasis (right side of the scale); on the other hand, if lesions are not removed and the cell does not die, it may accumulate damages that can induce mutations, which will increase cancer susceptibility (left side of the scale).

The present work will focus on two NER deficiency related diseases, investigating the correlation between their cancer susceptibility and UV light: XP and CS.

1.6.1 Xeroderma Pigmentosum (XP)

Xeroderma pigmentosum (XP) is a rare autosomic recessive disorder which affects ~1:200.000 people. It is characterized by photosensitivity, actinic skin, increased cancer risk (>1000) in sun-exposed areas (skin, mucous membranes and eyes) and, in some patients, neurologic degeneration. Children are normal at birth and the external symptoms increase with sun exposure: freckles start to appear and eventually the skin starts to show poikiloderma (areas of hyperpigmentation, hypopigmentation, atrophy and/or telangiectasias). The first skin cancer usually appears between 9 and 10 years of age, almost 60 years before the average occurrence in the normal population (152), reviewed in (153,154).

XP was first described by Moriz Kaposi in 1874, who worked with four patients (155). In 1883, Albert Neisser reported two XP siblings with progressive neurological

degeneration (156). Today it is known that about 25 % of XP patients present this specific symptom (152).

In 1964, Gartler showed that XP cells presented hypersensitivity to UV (157), which was correlated with DNA repair deficiencies four years later by Cleaver (158). In 1969, it was demonstrated that photoproducts were not removed by XP cells and that they lacked an efficient NER pathway (159–162).

By 1999, all genes involved in XP development had been identified and isolated, reviewed in (154). Cell fusion experiments showed that there are seven XP complementation groups (A-G) (163–167) and a variant form (XP-V), which is NER-proficient but defective for the polymerase H (*POL H*) gene (Pol η) (168).

For more detailed information about XP clinical features, etiology and molecular aspects, please refer to the reviews of Lehmann (153) and DiGiovanna (154), or to Ahmad & Hanaoka and Balajee´s books (169,170).

Several mouse models with different XP deficiencies have been created (171). In the present work, two XP mouse models were used: XPA and XPC. Detailed information about these two lineages are given in the next subsections of the present work.

1.6.1.1 XPA mouse model

XPA patients present a very severe phenotype, since deficiency in this protein completely abolishes NER activity. These subjects present high cancer incidence in sun exposed areas and, in some cases, neurodegeneration.

XPA mice were generated substituting exons 3 and 4 of this gene with a resistance cassette to neomycin in embryonic stem (ES) cells. These animals are knockout (KO) for XPA. 50 % of XPA embryos die after the 13th day post coitum. However, the embryos which come to term develop normally, with a normal lifespan (172).

When UVB irradiated (310 J/m^2), these animals presented a very similar response to that of XPA patients exposed to sunlight: erythema and hyperplasia, solar keratosis and skin tumors (squamous cell carcinomas, SCC), in a higher intensity and incidence than the heterozygous littermates. Eye abnormalities were also observed (172).

XPA mice are also sensitive to other DNA damaging agents, such as 7,12-dimethylbenz[*α*]anthracene (DMBA) and benzo[*α*]pyrene (B[*α*]P). Furthermore, with old age these animals present a higher incidence of internal cancer when compared to wild type (WT) animals, reviewed in (170).

1.6.1.2 XPC mouse model

XPC patients may have a mild phenotype, since the absence of this protein only disrupts a subpathway of NER, the GG-NER. In this case, lesions present on the transcribed strand of active genes are still efficiently removed. These patients present an intermediary increase in cancer incidence in sun exposed areas and no neurodegeneration (173).

XPC mice were generated through inactivation of exon 10 via insertion of a resistance cassette to neomycin by homologour recombination in ES cells. KO animals were born at a mendelian rate and present a normal lifespan (173).

Furthermore, animals do not present a lower minimal erythema dose (MED) compared to WT mice, unlike XPA mice (174). However, just like XPC patients, XPC mice present an increased cancer predisposition on UV exposed areas (175). This indicates that damage in the transcribed strand of active genes may be the trigger for erythema and edema but not for cancer predisposition.

1.6.2 Cockayne Syndrome (CS)

Cockayne Syndrome (CS) is a rare autosomal recessive disorder, with an incidence of ~1:200.000 births. CS patients are characterized by mild photosensitivity, with no increase in cancer incidence, and progressive ataxia, neurodegeneration and progeria, which results in a reduced lifespan: an average of 12 years, reviewed in (176,177).

CS was first described in 1936 by the pediatrician Edward Cockayne (178). The patient´s apparent sensitivity to UV light was proven with UV irradiation of cultured cells in 1977 (179). The comprehension that CS patients have a defective TC-NER came from the observation that their cells present a normal level of unscheduled DNA synthesis (UDS) (180), but a very slow recovery of RNA synthesis (RRS) (181) after UV irradiation .

Deficiencies in several genes can lead to a CS phenotype: *CSA*, *CSB*, *XPB*, *XPD* and *XPG*. Mutations in *CSA* or *CSB* hinder TC-NER. Mutations in *XPB*, *XPD* or *XPG* can affect the whole NER pathway. Therefore, it is not surprising that, depending on the specific protein motif which is affected, patients with mutations in any of these three genes can present different phenotypes: XP, CS, TTD or a combination (XP/CS, XP/TTD or CS/TTD). Considering that these proteins also seem to have other roles in the cell might help to explain this heterogeneity. For instance, CSB also seems to be involved in DNA repair of oxidized damage and chromatin remodeling; XPB and XPD are helicases, part of the TFIIH transcription complex; and XPG is a nuclease, also part of TFIIH.

Most UV Signature mutations found in skin tumors in mice and humans are C → T conversions in non-transcribed strands. Interestingly, in CSB patients and CSB mice, these transitions are mainly found in the transcribed strands of active genes. Furthermore, unlike CSB patients, CSB mice present an increase in cancer susceptibility, which may be due to the lack of CPD removal by GG-NER (182). Contradictory data suggests that, specifically in keratinocytes, DDB-2 is expressed in sufficient levels to ensure CPD removal and prevent carcinogenesis (183). However, enhanced DDB-2 expression increases mice resistance to UV-induced carcinogenesis (184).

For more detailed information on CS, please refer to the reviews of Friedberg (176) and Hanawalt (177).

Several mice models with the same mutations as CS patients have been created. However, in the present work, only CSA animals have been used and will be described in detail.

1.6.2.1 CSA mouse model

CSA patients present mild photosensitivity, with no increase in cancer predisposition, but with several progeroid characteristics, including progressive neurodegeneration (176,177).

CSA mice were generated with the insertion of a hygromycine resistance gene in exon 2 of the *Csa* gene in ES cells. Animals were born at a mendelian rate (185).

Like CSA patients, CSA mice also show mild photosensitivity but, unlike the patients, present increased predisposition to skin cancer after UV irradiation and

almost no progeroid characteristics, with the exception of age-related retinopathy. CSA mice have a normal lifespan (185).

This observed difference in the phenotypes of mice and men may have a correlation with the fact that rodents lack the p48 protein and therefore have no GG-NER of CPDs.

1.7 UV radiation and the skin

The skin is the largest organ of the human body and it functions as an interface between internal and external environments. Because of this characteristic, the skin is under constant attack from genotoxic exogenous agents, such as UV light. Therefore, the last part of this introduction will focus on skin responses to UV light.

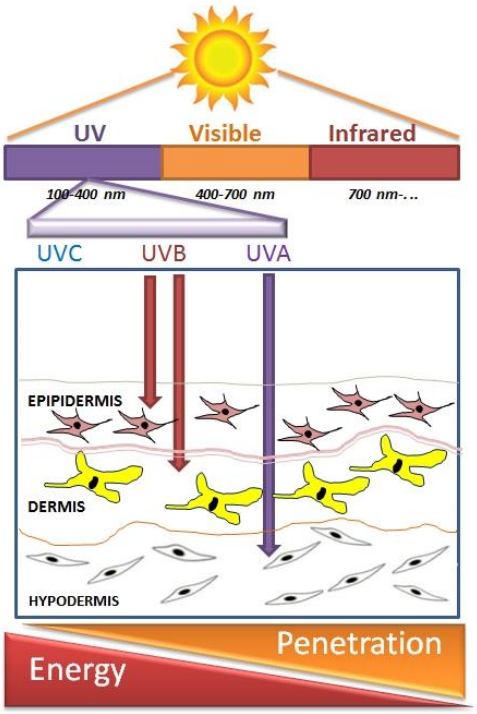
1.7.1 UV penetration in the skin

Electromagnetic radiation, such as UV light, is capable of transporting energy (E!) without carrying particles. It obeys the same physical laws as visible light, suffering diffraction, reflection, polarization and interference. The electromagnetic waves loose E! while propagating in any vehicle. When withstanding the interface between two environments (such as the skin/air surface), part of the light´s E! will be reflected and part transmitted to the second environment (in this case, the skin), where it may be absorbed. How far the light will go in a given vehicle depends not only on its E!, but mainly on the characteristics of the environment, reviewed in (186).

For instance, the UVB wavelength (280-315 nm) is strongly absorbed by nucleic acids and the protein´s aromatic amino acids, both present in high levels in the epidermis. Therefore, UVB light is totally absorbed by this external skin layer, virtually never reaching the dermis. On the other hand, UVA wavelengths are not efficiently absorbed by the structures present in the epidermis and go straight through. In the dermis, UVA photons are scattered several times by collagen bundles, before being absorbed by other chromophores, such as NADH, riboflavins and hemoglobin, reviewed in (17).

Figure 14 represents the UV light spectrum and the penetration capacity of each UV wavelength in the skin. To be noted: the penetration spectrum of human and mouse skin is similar.

Figure 14 - UV light spectrum and the penetration of each wavelength in the skin



UVC light is totally blocked by the ozone layer and the atmosphere. The great majority of UVB is absorbed by the epidermis whereas almost all of UVA is absorbed in the dermis. The greater the energy of the wavelength, the lower its penetration capacity in the skin. These properties apply to human and mouse skin.

To better understand the interaction between UV light and the skin, it is very important to comprehend the skin structure.

1.7.2 Skin structure

The epithelial tissue serves as interface between the environment and the inside of our bodies. Therefore, its integrity is fundamental not only to conserve the integrity of higher organisms, but also to preserve essential body functions, such as temperature regulation and substance absorption and excretion.

The epithelial tissue is constituted on its outside layer by the epidermis, originated from the ectoderm, and more internally by the dermis and the hypodermis, both originated from the mesoderm.

The hypodermis is mainly composed of adipocytes, but also by fibroblasts, blood vessels and nerves. It is basically responsible for thermoregulation, nutrient hoarding and protection against mechanical trauma.

The dermis is comprised of fibroblasts, which secrete collagen and elastin, and by blood and lymphatic vessels, muscle bundles and nerves. The dermis is the conective tissue responsible for the structural and functional support of the skin, also ensuring epidermal nutrition and oxygenation.

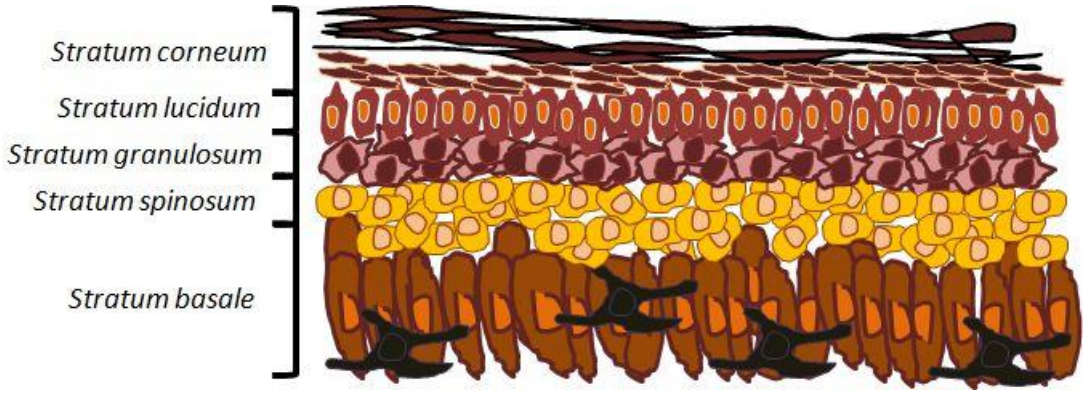
The epidermis is formed by keratinocytes, melanocytes, and Langerhans´ and Merkel´s cells. Keratinocytes are the cells responsible for synthetizing keratins, the proteins of the intermediate filament, which are characterized as being resistant and impermeable. Only the keratinocytes of the epidermal basal layer, which are the cells in contact with the basement membrane (just above the dermis), are capable of proliferating. Once these cells divide, they start migrating to the outer layer of the epidermis. During this period, cells change the type and amount of keratin they produce and accumulate, which leads to the loss of cell function and cell flattening, culminating in cell death on the stratum corneum.

Proliferating keratinocytes in contact with the basement membrane form the *stratum basale* or germinative. These keratinocytes present an elongated morphology and low keratin levels. In the *stratum spinosum*, cells are rich in desmosomes, the keratin content starts to increase and they become more oval-like. The keratinocytes in the *stratum granulosum* present lozenge morphology and are rich in filaggrin granules. In the *stratum lucidum*, cells no longer present a nucleus and accumulate more keratin. Finally, in the *stratum corneum*, cells are no longer metabolically active, are very compact and extremely rich in keratin.

Melanocytes are mainly found in the deeper epidermal layers. These cells are responsible for producing melanin (eumelanin and pheomelanin). Each individual presents a different level of each type of melanin, which is responsible for characterizing the different skin tones. Melanin is synthesized and exported to keratinocytes in melanosomes. Melanin production can be stimulated by UV light, which will be discussed in detail in the next subsections.

Figure 15 represents the epidermal layers formed as keratinocytes migrate to the epithelial surface, loosing nuclear and metabolic activities.

Figure 15 - Epidermal layers formed through keratinocyte migration and differentiation



Only the keratinocytes in contact with the basement membrane (*stratum basale*) are capable of proliferating. These cells then start a journey of migration and differentiation towards the outer layer of the epithelium, loosing nuclear and metabolic activities and accumulating keratin. Cells in the *stratum spinosum* and *granulosum* still present a nucleus, which is lost in the *stratum lucidum*. In the *stratum corneum*, cells are no longer metabolically active. Melatinocytes are represented as dark cells in the *stratum basale*.

For more detailed information about skin anatomy and physiology please refer to Bolognia´s book, chapter 1 in particular (187).

Mouse skin structure is fundamentally the same as human skin. However, each *stratum* is constituted of fewer layers and the intermediate ones are very hard to observe. In total, the mouse epithelium presents an average of 2-3 layers (188). Human epidermis is almost ten times thicker: 50 µm for human epidermis (187) compaired witht 6 µm for mouse epidermis (189). This characteristic makes mouse skin more sensitive to UV light. Still, despite differences in the intensity of the effects observed in the skin of these two animals, the acute and chronic responses to UV light are essentially the same.

The epidermis, in direct contact with the environment, is highly exposed to a series of harmful physical and chemical agents and its most important function is to protect complex organisms from injuries caused by exogenous factors, such as UV light.

Factors such as skin phototype, body region and age influence skin sensitivity to UV and determine the intensity of acute and chronic responses. For instance, fair-skinned people, as well as children and the elderly present a higher sensitivity to UV. The same is true for areas with a more delicate and thinner skin, such as the face and neck, reviewed in (190).

1.7.3 Acute effects of UV light in the skin

Acute skin responses to UV light usually peak around 24 hours after UV irradiation and can be observed up to 72 hours after exposure. They include, but are not restricted to, apoptosis, inflammation and hyperplasia (epidermal thickening). These responses are UV dose dependent and elicited by UVA and, with greater intensity, UVB (191).

These responses seem to be p53-related: p53 levels peak approximately 12 hours after UV exposure. This transcription factor is activated by the presence of DNA damage and leads to cell cycle checkpoint activation, followed by an arrest in cell cycle progression in the G1 phase. Alternatively, when injuries are too severe to be repaired, p53 may elicit a programed cell death response via p21/BAX/BCL2 response.

All of the aforementioned acute responses are also observed in p53 KO animals, but with a 12-24 hour delay (192). Therefore, evidence suggests that p53 is not essential but presents a stimulatory effect in acute skin responses to UV light (192).

Immediate responses to UV light also include vitamin D synthesis, inflammation, erythema (skin redness) and edema (skin swelling).

1.7.3.1 <u>Sunburn cells</u>

Sunburn cells are apoptotic cells characterized by pyknotic nucleus and shrunken cytoplasm. The UV-induced cell death is usually observed from 6 to 72 hours, peaking 36 hours after exposure. The dying cells start to migrate to the outer epidermal layers, eventually co-localizing with the hyperkeratosis areas. There is a UV-dose effect in the induction of sunburn cells and high levels of cell death can be observed as macroscopic peeling (192).

Interestingly, after UV irradiation, despite the high number of dying cells, what is observed is epidermal thickening and not shrinkage. This phenomenon is observed because of a secondary response which starts approximately 12 hours after UV irradiation, when p53 levels decrease: cell proliferation (193).

1.7.3.2 Hyperplasia

Hyperplasia is an increase of epidermal thickness due to the rise of the total number of keratinocytes, leading to an augmentation in the number of epidermal layers. In hyperplastic skin tissue, it is common to observe an enlargement of cell size.

The hyperplastic response seems to be independent of genetic background and is pigment-independent. This reaction is considered the most effective skin photoprotection defense mechanism in individuals who tan poorly (190). The epidermal thickening physically blocks UV light, diminishing the total amount of rays which can reach deeper epithelial layers, protecting the proliferative cells from its damaging effects.

The observed hyperplasia is the result of the combination of two cell responses: cell death of the previously existing keratinocytes, and cell proliferation in the basal epidermal layer (192). The hyperplastic response starts 12 hours after exposure, when p53 levels start to decrease. It peaks in 48 hours, and is observed up to 72 hours after UV irradiation (192). This response seems to be dependent on the epidermal growth factor (EGF), since epithelium lacking its receptor (EGFR) does not present hyperplasia after UV exposure (194,195). Typically, the reversion of the hyperplastic response starts 96 hours after the proliferative stimulus has been removed (193).

1.7.3.3 Erythema and edema

Erythema (skin redness) and edema (skin swelling) walk hand-in-hand and are caused initially by infrared and UVA rays, which reach the dermis and cause vasodilation and consequent increase of blood content in the skin. This response starts only a few minutes after UV exposure and fades within 4 hours, unless there has been enough damage to start an inflammatory response which will sustain vasodilation. Edema and erythema induction is UV-dose dependent and may even lead to blistering, when the damage is too severe, reviewed in (190).

1.7.3.4 Inflammation

UV-induced inflammation starts a few hours after UV exposure and peaks 6-24 hours after exposure (196) and includes, as a consequence of lipid peroxidation and DNA damage, increased production of prostaglandins (PG), tumor necrosis factor alpha (TNF-α), nuclear factor kappa beta (NF-κβ), histamine, kinins and inflammatory cytokines (interleukins – IL - 1α, 1β and 6), reviewed in (197,198).

Together, these molecules regulate the expression of adhesion molecules (intracellular adhesion molecule 1, ICAM-I, and endothelial-leucocyte adhesion molecule, ELAM-1) in vascular endothelium and keratinocytes, recruiting mononuclear cells and neutrophils. As a consequence, vasodilation, lymph infiltration and inflammation can be observed. The inflammatory process increases the formation of ROS and there is evidence that this may aid in the launching of the tumorigenic process through the increase of DNA damage levels, reviewed in (197,198).

1.7.3.5 Vitamin D production

Vitamin D is crucial for the maintenance of endogenous calcium levels and skeleton mineralization. The vitamin D precursor (pre-vitamin D or colecalciferol) is formed in the epithelium with the aid of UV-irradiation. The active form of this vitamin, calcitriol, is then produced in the kidneys and liver.

It is well known that UV light exposure can lead to several types of skin cancer. However, recent works present evidence that vitamin D production may aid not only in skeletal health, but also in skin cancer prevention and regression. Therefore, even though low UV exposition is sufficient for a satisfactory calcitriol production (10-15 min exposure, 2-3 times/week), extreme care has to be taken when advising people to “stay out of the sun”, since this seems to be another delicate balance, reviewed in (199).

1.7.3.6 Pigmentation

There are three types of UV-induced pigmentation: the immediate pigment darkening (IPD), the persistent pigment darkening (PPD) and the late tanning

response. IPD starts 1-2 minutes after UV irradiation, lasting no more than a few minutes. PPD is frequently confused with tanning, but consists of a phenomenon similar to IPD, starting a few minutes after UV exposure and lasting up to a few days. Both IPD and PPD are also known as Meirowsky phenomena and are caused by photoxidation of melanin and its precursors. IPD pigment is more grayish, whereas PPD pigment is brownish; none of them seem to have photo-protective properties, reviewed in (198,200). The IPD and the PPD capacities are independent of the tanning ability of the individual, even though higher melanin content in the skin increases the IPD and PPD responses.

Late skin pigmentation (or tanning) starts 2-3 hours after UV irradiation and peaks in 3 weeks. This late response correlates with melanin production by melanocytes and its distribution to surrounding keratinocytes and will be discussed in more detail in the next sections of the present work.

1.7.4 Chronic effects of UV light in the skin

Chronic effects comprise delayed local and systemic responses to UV light which can be observed from 2 days up to several months or years after UV exposure. These chronic responses include photoaging, immunosuppression, persistent hyperplasia, delayed pigmentation and tumorigenesis. Hyperplasia and pigmentation are considered photoprotective/photoadaptive responses and are mostly related to the overexpression of genes related to cell cycle control, DNA damage response or apoptosis, such as *p53*, *GADD45* and *BAX* (201,202).

1.7.4.1 **Photoaging**

Aging is the progressive decline of function and reserve capacity of all organs in the body, due to the loss of tissue homeostasis. Skin photoaging is the superposition of intrinsic aging and environmental factors, such as chronic UV exposure. Photoaged skin is characterized by several macro and microscopic changes, including variation in epidermal thickness and pigmentation, dermal elastosis, alterations in the collagen composition and levels, inflammatory infiltrates, vessel ectasia, and wrinkling, reviewed in (203).

Several types of local damages and responses contribute to the development of skin photoaging, such as genomic and mitochondrial DNA damage, protein oxidation, telomere shortening, UV-signaling pathways and loss of tissue homeostasis, reviewed in (203).

Prevention of skin photoaging can be achieved by limiting sun exposure and regular use of sunscreens that protect against both UVA and UVB. Other products which antagonize the UV-signaling pathway, such as tretinoin and antioxidants, or that aid in the reduction of metalloproteinase levels and in collagen reconstitution, may help in the treatment of this condition. A better comprehension of the photoaging process, as well as the development of preventive and palliative methods, aids not only in improving skin appearance of middle-aged people and beyond, but also helps to reduce the burden of skin cancer, reviewed in (203).

1.7.4.2 Immunosuppression

In 1974, Kripke published the first evidence that UV light has an impact in the immunologic system (204), which helped to explain a series of previous data and successful light treatments against psoriasis and tuberculosis (205). A clear sign UV-induced immunosuppression is the enhanced susceptibility to several types of infection after UVB exposure (206–208).

Immunological responses usually arise from a series of signals which compose an intricate network. In photo-immunosuppression, one of the main signals seems to be DNA damage in cells present in the epithelium, including those from the immune system, such as Langerhans cells (LCs). In fact, removal of CPDs, but not 6-4PPs, is capable of preventing UV-induced immunosuppression. Interestingly, lesion removal from only keratinocytes did not produce the same results (209).

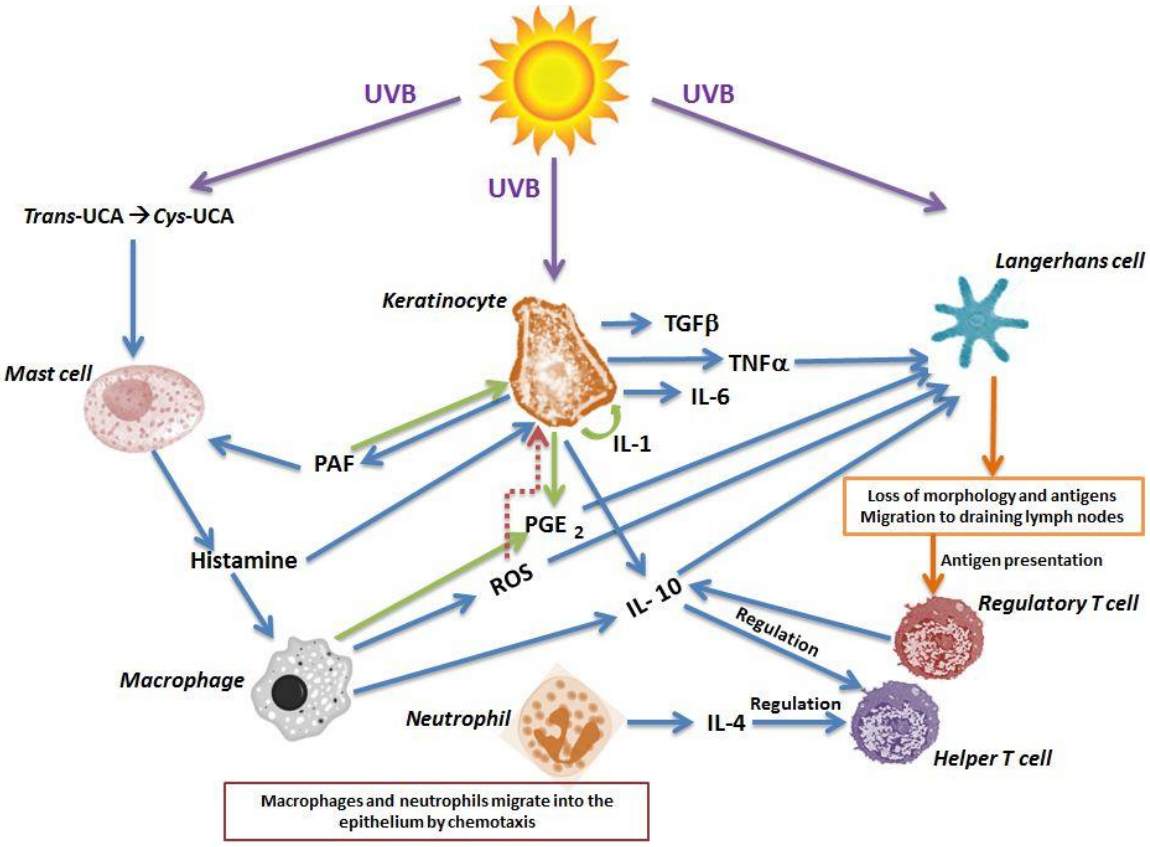
An increase in local immunosuppression is obtained by TC-NER blockage (CSB animals) (210), but an increase in systemic immunosuppression is only achieved with the simultaneous inactivation of TC-NER and GG-NER (XPA animals) (211).

The exact pathway through which UVB induces immunosuppression is still not clear and may vary depending on the wavelength in question, exposure duration, previous exposures, skin area and local pigmentation.

The presence of photoproducts and oxidized damages in DNA cause keratinocytes to secrete TNF-α, interleukin (IL) 10 (IL-10), IL-6, IL-1, transforming growth factor beta (TGF-β) and the platelet activating factor (PAF). TNF-α and IL-10 prevent LCs to differentiate into dendritic cells and stimulate their migration to draining lymph nodes.

UV converts *trans*-uronic acid (UCA) to *cys*-UCA, which induces mast cells (MCs) to release histamine. Histamine, IL-1 and PAF promote PGE_2 secretion by keratinocytes. Macrophages (Mas) and neutrophils migrate into the epithelium by chemotaxis 72 hours after injury. Histamine induces MAs to secrete IL-10 and produce ROS. ROS increase the production of several of the aforementioned signaling molecules, probably through DNA damage, reviewed in (212). Interferon gama (INF-γ), IL-4, IL-10 and IL-12 regulate the levels of helper T lymphocytes (Ths) (213). Reduced levels of Ths and increased levels of regulatory T lymphocyte (Treg) are major markers of UV-induced immunosuppression, reviewed in (214,215). Figure 16 presents a simplified version of the known pathways which regulate UV-induced immunosuppression.

Figure 16 - Pathways which regulate UV-induced immunosuppression



DNA damage causes keratinocytes to secrete TNF-α, IL-10, IL-6, IL-1, TGF-β and PAF. TNF-α and IL-10 prevent Langerhans cells to differentiate and stimulate their migration to draining lymph nodes. UV converts *trans*-UCA to *cys*-UCA, which induces mast cells to release histamine. Histamine, IL-1 and PAF promote PGE_2 secretion by keratinocytes. Macrophages and neutrophils migrate into the epithelium by chemotaxis. Histamine induces macrophages to secrete IL-10 and produce ROS. ROS increase production of signaling molecules, which increase levels of regulatory T cells. IL-4 and IL-10 also regulate the levels of helper T lymphocytes.

UVB-induced immunosuppression can be prevented with low UVA exposure (216), probably through the induction of INF-ɣ and IL-12 (217). IL-12 seems to increase GG-NER, thus reducing the levels of remaining photodamage in DNA (218).

It is important to highlight that UV-induced immunosuppression has an important role in tumor development since the immune system surveillance may detect and combat early tumors. For instance, the absence of IL-10 prevent Treg-induced immunosuppression, reducing tumor induction in mice (219).

1.7.4.3 Persistent hyperplasia and dysplasia

Hyperplasia is not only an acute response against UV-induced skin damage. As one of the most important epidermal responses against this type of injury, hyperplasia persists for as long as the damaging agent is present (220). If tissue homeostasis is preserved, the hyperplastic response starts to be reversed 96 hours after the stimulus has been removed (193).

However, when tissue homeostasis is lost, hyperplasia becomes persistent. Histologically, there is no difference between acute and chronic hyperplasia, but in persistent hyperplasia signs of loss of tissue homeostasis are usually seen, such as dysplasia. Dysplasia is characterized by the loss of tissue morphology, usually presenting alteration of cell differentiation patterns in the epidermis.

It is important to emphasize that an acute stimulus, if strong enough, can also lead to persistent hyperplasia and dysplasia.

1.7.4.4 Delayed skin pigmentation (tanning)

Two types of melanin pigmentation may be observed in the skin: a) constitutive, which is the genetically determined skin color observed in different individuals; and b) induced, which is a reversible increase in tanning as a response to specific stimulatory agents, such as UV radiation, reviewed in (190).

According to Fitzpatrick (221), human skin can be classified in six different phototypes (I – VI). Phototype I is the most sensitive, with individuals presenting white skin and a great tendency to sunburn and no capacity to tan. As the scale progresses sun sensitivity decreases: the tendency to sunburn disappears and the ability to tan increases. Phototypes V and VI are the least sensitive, with individuals presenting brown and black skin, respectively, reviewed in (198,222).

Tanning capacity is genetically determined and depends on the ability of melanocytes to produce melanin, the total amount of melanin formed within each melanosome, and melanosome distribution. Fair-skinned individuals present clustered small melanosomes (0.6 – 0.7 μm) which are only partially melanized; while dark-skinned individuals present bigger melanosomes (1 μm) which are heavily melanized and more evenly dispersed. Therefore, fair-skinned individuals (skin photoypes I and II) are less efficient in producing visible pigment than dark-skinned

individuals (phototypes V and VI), who have an epithelium more efficient in absorbing light, reviewed in (198).

Tanning becomes visible 3-5 days after UV exposure. There is an increase in melanin production, related to the elevation of melanocyte activity and number, augmentation of melanocyte dendrites elongation and branching, and to an increase in melanosome quantity and size. After melanin is produced, melanosomes are exported to keratinocytes where they are diffusely distributed, but group above the nucleus forming a cap, reviewed in (198).

Curiously, UVA-induced melanin tends to stay in melanosomes located in the basal epidermal layer, whereas UVB-induced melanin is distributed to the upper keratinocyte layers, reviewed in (198). There is further evidence that the tanning mechanisms differ between UVA and UVB stimulation. In cell culture, UVB-induced melanogenesis requires the presence not only of melanocytes, but also of keratinocytes, suggesting that signaling molecules, such as endothelin 1 (ET-1) and IL-1α, may be required to elicit this process. In UVA-induced melanin production, keratinocytes are not required but oxygen is, which suggests that ROS formation may be required in this process, reviewed in (223).

Furthermore, it has been shown that UVA-induced pigmentation requires the presence of melanin pigment and depends on the production of oxidized damage in melanocytes. ROS can damage DNA (base oxidation and SSB), lipids, proteins and other molecules, initiating a signaling pathway which will culminate in melanogenesis. On the other hand, UVB-induced melanogenesis is pigment-independent and relies on direct DNA damage (6-4PPs and CPDs), reviewed in (224). Despite their different mechanisms of melanogenesis induction, UVA and UVB exposures have the same general outcome: increase of melanin production. Interestingly, UVA and UVB have an additive property for both erythema and pigment induction, reviewed in (223)

The aforementioned changes in melanocytes and melanosomes are regulated by a series of direct and indirect effects of UV on melanocytes. UV damages keratinocytes´ DNA, which leads to the expression of many citokynes, such as ET-1, granulocyte-macrophage colony-stimulating factor (GMCSF), basic fibroblast growth factor (bFGF) and stem cell factor (SCF), which are recognized by melanocytes and regulate melanogenesis. Furthermore, DNA damage in keratinocytes also leads to an increase in p53 levels, provoking higher POMC expression and, consequently, of its derivatives: alpha melanocyte stimulating hormone (αMSH), adrenocorticotropic

hormone (ACTH) and beta endorphin. αMSH is recognized by its receptor in melanocytes, MC1R, which stimulates adenylate cyclase (AC). Cyclic adenosine monophosphate (cAMP) is increased by AC, consequently raising protein kinase A (PKA) levels. PKA phosphorylates the cAMP response element-binding protein (CREB), raising microphthalmia transcription factor (MITF) expression. In turn, MITF induces the expression of a series of proteins related to the melanogenic pathway, such as tyrosinase (TYR), L-3,4-dihidroxyphenylalanine (DOPA) chrome tautomerase (DCT) and dihydroxyindole-2-carboxylic acid (DHICA) oxidase (TYRP1). Alternatively, a series of other pathways may lead to an increase in melanogenesis, in a MITF dependent or independent manner, reviewed in (222).

Evidence suggests that not only the presence of DNA damage but, more importantly, its repair is responsible for iniciating the aforementioned melanogenic pathway. Nucleotide fragments produced during NER are capable of inducing melanogenesis. For instance, the addition of small damaged DNA fragments *in vitro* and *in vivo* was proved capable of stimulating melanin production (225,226).

Several investigations confirm the central role of p53 in melanogenesis, mostly through the increase of POMC expression (227,228). Curiously, POMC null mice can still produce normal melanin levels, indicating that this is not the only pathway involved in melanogenesis induction (229). Furthermore, after UV exposure, keratinocytes secrete ET-1, colony stimulating factor 2 (CSF2), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog 1 (KIT1), PGE_2 and NO. These factors are recognized by melanocytes and/or their precursors (melanoblasts), inducing cell proliferation and differentiation; dendricity increase; melanogenesis; and melanosome formation, transport and density, reviewed in (198,223,230–232).

Melanogenesis inhibition is achieved through the secretion of a series of factors by keratinocytes, such as IL-1α, TNFα, interferons and bFGF. A fine tuning between all these molecules is responsible for melanogenesis control, reviewed in (223).

Interestingly, αMSH may also play an important role in preventing UV-induced apoptosis, aiding in the removal of photolesions from keratinocytes and melanocytes, in a NER-dependent manner (233).

After melanogenesis is stimulated, three pathways control the production of eumelanins and pheomelanin, reviewed in (234).

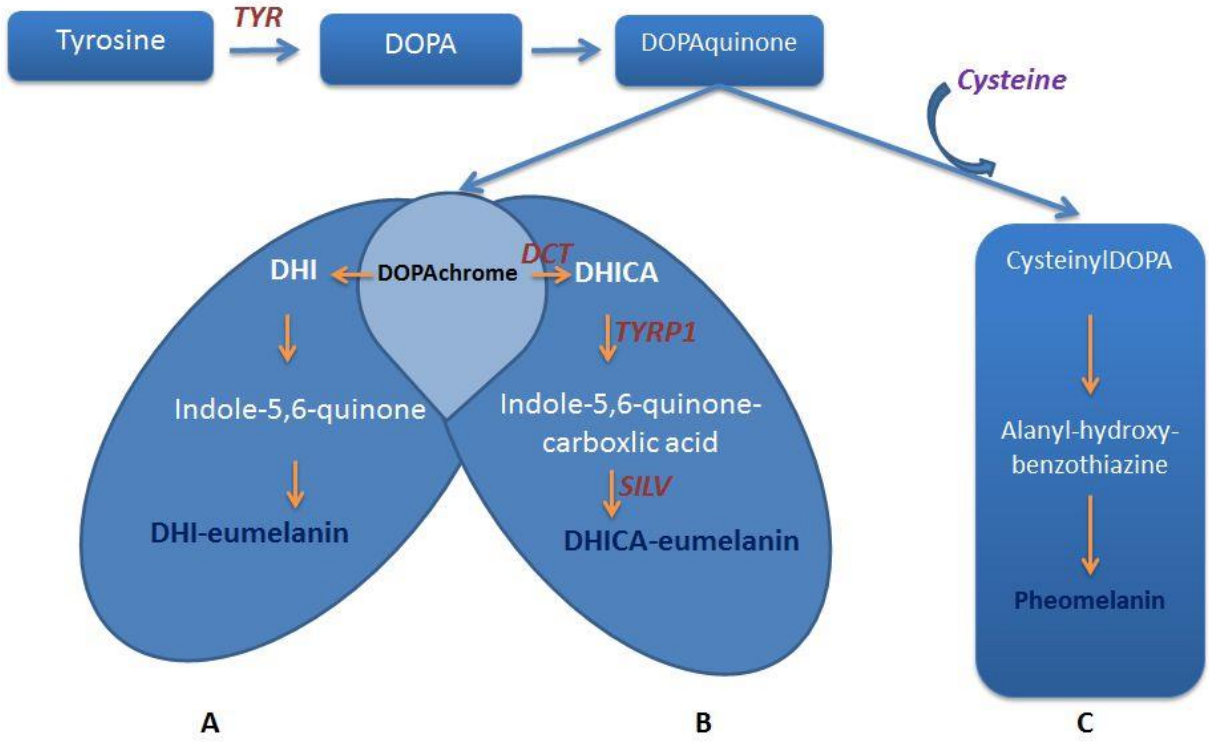
Dihydroxyindole-eumelanin (DHI-eumelanin) is produced by the Raper-Mason pathway. TYR converts tyrosine to its dihydroxylated form, DOPA. The subsequent reactions are spontaneous: DOPA oxidazes to DOPAquinone, which then cyclizes to DOPAchrome. DOPAchrome decarboxylates to DHI, which oxidazes to indole-5,6-quinone. Polymerization of these quinone and indole-quinone intermediates originates the dark black pigment known as DHI-eumelanin.

In an alternative pathway, when the enzyme DCT is present, it converts DOPAchrome to a carboxylated intermediate, DHICA, which is then oxidized by TYRP1 into indole-5,6-quinone-carboxylic acid. When this last form is further oxidized, it can polymerase, with the aid of DHICA polymerase (SILV), and form a dark brownish pigment: DHICA-eumelanin.

A third pathway is responsible for forming pheomelanin, a light brown pigment. DOPAquinone is converted to cysteinylDOPA with the addiction of the amino acid cysteine. CysteinylDOPA is converted to alanyl-hydroxy-benzothiazine, which polymerases, forming pheomelanin.

These pathways are represented in Figure 17.

Figure 17 - Melanogenesis pathways for the production of eumelanin and pheomelanin



Tyr is the limiting enzyme which converts tyrosine in DOPA, which is spontaneously converted to DOPAquinone and then to DOPAchrome. Through the Raper-Mason pathway, DOPAchrome is spontaneously converted to DHI and then to indole-5,6-quinone. The polymerization of these quinone and indole-quinone intermediate factors forms the darker form of melanin: DHI-eumelanin. On a secondary pathway, DCT converts DOPAchrome to DHICA that is then converted by TYRP1 to indole-5,6-quinone-carboxylic acid. Polymerization of these intermediate factors with the aid of Silv leads to the formation of the brownish form of eumelanin: DHICA-eumelanin. Alternatively, cysteine may be spontaneously added to DOPAquinone forming cysteinylDOPA, which is spontaneously converted to alanyl-hydroxy-benzothiazine, which polymerases into pheomelanin.

The exact mechanism for the activation of different melanogenesis pathways is not well understood. TYR levels determine total melanin production. DCT levels seem to define what type of eumelanin is formed; and environmental factors, such as cysteine availability, lead to pheomelanin production. It is believed that the two additional pathways to the Raper-Mason pathway have evolved to aid in the elimination of the intermediates generated in melanin production which are very toxic to cells. The same reason could explain why this pigment is produced inside specific vesicles, the melanosomes, which have detoxing enzymes and prevent ROS from damaging other cell structures, reviewed in (234).

Furthermore, it is also known that UV irradiation leads to increased levels of Tyr and reduced levels of DCT, TYRP1 and SILV, culminating in an increased DHI-eumelanin production, the darkest of all melanins. In fact, DHI-eumelanin is the most

efficient in protecting skin against UV light side damaging effects, followed by DIHCA-eumelanin and pheomelanin, respectively, reviewed in (234).

Contrary to popular belief, tanning has only a moderate effect in protecting the skin from the injuries caused by UV irradiation: a deep tan only increases protection by a factor of two or three. It is even argued that melanin might not have been evolutionarily selected to protect organisms from the damaging effects of UV exposition, but for camouflage and temperature maintenance, reviewed in (190). Nevertheless, fair-skinned individuals are over 10 times more sensitive to the effects of UV light than those that are dark-skinned, which puts that hypothesis in question, reviewed in (198).

However, in addition to blocking the passage of UV light, and thus protecting epithelial tissue from direct UV damage, melanin can also act as a chromophore which absorbs UV light and produces ROS, increasing cellular and indirect tissue damage. Until recently it was believed that the presence of pheomelanin was deleterious for the organism because of its low UV-blockage capacity and increased ROS production (224). However, recent evidence suggests that the higher photosensitive of skin phototype I individuals is not related to their higher pheomelanin/eumelanin ratios, but to non-pigmentary roles of MC1R, which is mutated in red hair subjects. Both eumelanin and melanin have been shown to efficiently protect cells from UV rays (235).

Melanocytes are resistant to UV-induced photodamage because of their high melanin content and are particularly resistant to UV-induced, p53 mediated, apoptosis, probably due to enhanced BCL2 (BAX suppressor) levels and upregulation of the growth arrest and DNA damage-inducible protein alpha (GADD45a), an apoptosis inhibitor, reviewed in (236). However, melanocytes have been described as having lower DNA repair capacities for oxidized damage and photolesions, which may be due to the presence of melanin (237).

1.7.4.5 Skin tumorigenesis

Skin cancer is the most common form of cancer in Brazil and in the world, constituting 25 % of all registered malignant tumors, in men and women (INCA). The incidence has been escalating due to an expansion of recreational exposure, such as sun tanning and outdoor sports; sunbed tanning (238); absent or incorrect use of

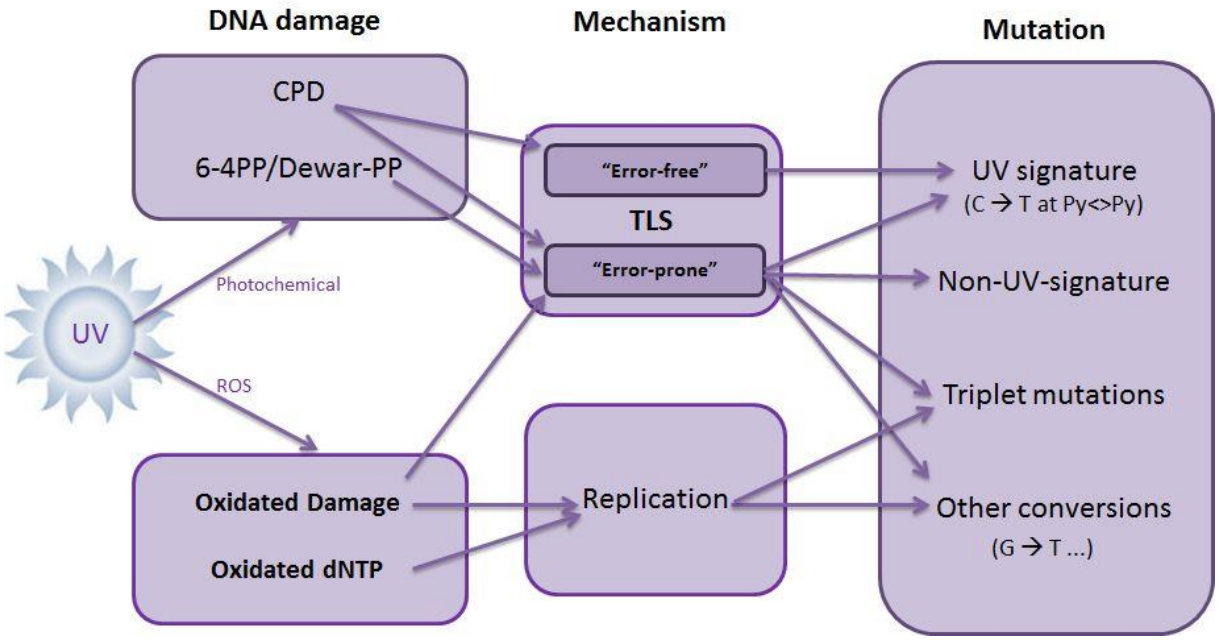
sunscreens (or use of ineffective sunscreens) (239); and to a progressive reduction of the ozone layer in certain areas, reviewed in (240).

Exposure to UV light is one of the biggest risk factors for the development of skin cancer. As explained in detail in subsection 1.2, UV light causes two types of photolesions on DNA, CPDs and 6-4PPs, in addition to indirect oxidized damage. If these lesions are not successfully removed, they can lead to mutations, which can culminate in cancer induction.

There is a set of mutations found in virtually all UV-induced skin tumors, called UV-signature mutations: C → T and CC → TT conversions. As shown in Figure 7, they are formed through deamination of pyrimidines in a photodimer followed by "error-free" TLS. Triplet mutations are also commonly found in these tumors.

Figure 18 summarizes the mechanisms through which UV-signature mutations and other UV-induced mutations may be formed in DNA.

Figure 18 - Induction mechanisms of UV- signature mutations and other UV-induced mutations



UV irradiation can damage the genomic DNA, generating CPDs, 6-4PPs and oxidized damages. TLS of CPD by "error-free" Pol η may lead to UV-signature mutations (C → T and CC → TT conversions). TLS of CPDs, 6-4PPs and oxidized damages by "error-prone" polymerases may lead to the formation of UV-signature mutations or other mutations, such as G → T and T → G conversions. Replication (without TLS) of oxidized damages or the use of damaged nucleotides during replication can lead to the formation of non UV-signature mutations. Adapted from Ikehata & Ono, 2011 (54).

Carcinogenesis is a multistep process that demands a sum of mutations in proto-oncogenes and tumor suppressor genes and/or in genes which regulate their expression. Two of the most commonly mutated genes found in UV-induced skin cancer are p53 and rat sarcoma (Ras), reviewed in (241).

During the carcinogenic process, cells have to suffer enough mutations to be able to resist cell death signals, sustain proliferative signals, deregulate cell energetics, evade growth suppression, enable replicative immortality, induce angiogenesis, avoid destruction from the immunologic system, and activate invasion and metastasis mechanisms, reviewed in (242).

A series of factors influences photocarcinogenesis: UV dose and specific wavelength, repetitive exposure, skin area exposed, age of the individual, skin type, use of sunscreens and diet, reviewed in (241).

Three types of skin cancers correlate with UV exposure: basal cell carcinoma (BCC), SCC, which are collectively referred to as non-melanoma skin cancer (NMSC), and cutaneous malignant melanoma (CMM), reviewed in (241). On average, BCC comprises 75 % of all skin cancers, followed by SCC with 21 % and CMM with 4 %.

Fair-skinned individuals (phototypes I and II), people with light-colored eyes and hair, with a tendency to sunburn and not to tan, immunosuppressed subjects, and those with a family history are considered high-risk groups for skin cancer (243).

BCC is the most frequent cancer in Caucasians, although rarely found in Orientals and Afro-Americans. It is a solid malignant skin cancer caused by the transformation of basal keratinocytes and presents a slow progression and low metastatic rates. BCC afflicts more men than women and its onset usually occurs around 69 years of age. It is more common on the head and neck and in inhabitants of the tropics. In the United States of America (USA), 750.000 new cases of BCC are diagnosed every year, reviewed in (240).

BCC is associated with intermittent UVB exposures, followed by intense sunburns, especially in childhood. Specific risk factors include mutations that lead to alterations in skin pigmentation and in vitamin D production. Mutations in p53 and in protein patched homolog (PTCH) are commonly found in BCC, reviewed in (240).

SCC is a solid malignant tumor, formed by transformed keratinocytes (not from the basal epidermal layer), with low metastatic rates and mortality. It is the second most common skin tumor in Caucasians, with a higher incidence in males. Although

UVB light is considered the most important cause of SCC, it is also commonly caused by chemical (arsenic and benzantracen) and biological agents (human papillomavirus, HPV). In the USA, approximately 200.000 new SCC cases are diagnosed every year, (244). The average age of SCC incidence is 68 years for women and 72 for men (245).

Risk factors include cumulative UVB exposition, inhabiting lower latitudes, immunosuppression, fair-skinned, people with light-colored eyes and hair, tendency to sunburn, and family history. The presence of pre-malignant lesions, the actinic keratosis, is an indicator of future development of SCC. Mutations in p53 are the most common in SCC, together with several chromosomal alterations (244), reviewed in (240).

CMM is a solid, malignant tumor, composed of transformed melanocytes, usually occurring in the epidermal-dermal junction. It comprises only 4 % of the totality of skin tumors, but it is their most lethal form, due to the high metastatic rate. The total incidence is 10-15/100.000 habitants in Europe, 45/100.000 habitants in Australia and it is estimated to inflict around 2.5-7.4/100.000 habitants in Brazil. CMM is very rare in children, presenting its higher frequency in the elderly, with an average incidence at 50 years of age. CMM incidence is a little higher in females (246).

The presence of nevi is a risk factor, especially atypical and familial nevi. Mutations in rat sarcoma (*RAS*), cluster of differentiation 14 (*CD14*), protein 16 (*p16*), apoptotic protease activating factor 1 (*APAF1*) and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) are commonly found in CMM (246).

CMM occurs in sun-exposed areas, and it is believed that intermittent UV exposure, with sunburns in early childhood, severely increases the risk of later development. There is evidence suggesting that UVA plays an important role in melanoma induction, probably due to direct absorption by melanin (247), reviewed in (248). However, CMM has not been successfully developed in mice or other placental mammals solely with UVA exposures. Therefore, UVB exposition is still considered the factor that is mainly responsible for CMM development, reviewed in (240).

Table 1 presents the estimated incidence of NMSC and CMM in Brazil in 2010 and 2012, as estimated by the National Institute of Cancer (*Instituto Nacional de Câncer, INCA).*

Table 1 - Estimation of skin cancer occurrence in Brazil between 2010 and 2012

Gender	*Type of skin cancer/ year*	*2010*	*2012*
Men	CMM	2.960	3.170
	NMSC	53.410	62.680
Women	CMM	2.970	3.060
	NMSC	60.440	71.490
Total	CMM	5.930	6.230
	NMSC	113.850	134.170

Skin cancer incidence predictions have increased from 2010 to 2012 in Brazil. Cutaneous malignant melanoma (CMM) affects more men than women, while the reverse is true for non-melanoma skin cancer (NMSC). Data obtained from INCA.

1.8 Final Considerations

The scientific community has long accepted the intimate relationship between UV exposure, photolesion formation in DNA, mutation induction (Signature UV-induced mutations) and the development of skin tumors. However, the specific role played by each photolesion, CPDs and 6-4PPs, in cell and tissue responses to UV irradiation, such as erythema, edema, pigmentation, hyperplasia and tumorigenesis, is still not clear.

Previous works have explored this question, but employing mice proficient in DNA repair mechanisms, which may have masked the role of 6-4PPs in skin responses to UV irradiation due to fast removal of these lesions by NER. In these studies, only the removal of CPDs was capable of preventing UV-induced local and systemic responses (209,249,250).

Furthermore, an *in vitro* research revealed that, in XPA cells, the removal of CPDs as well as the removal of 6-4PPs reduced apoptosis (251). Four years later, another study using adenoviral vectors for the delivery of photolyase transgene in several DNA repair-deficient fibroblasts demonstrated that the removal of both photolesions was able to reduce UV-induced apoptosis in XPA, XPD and XPG cells. Interestingly, in WT, XPV and CSA cell lines only the removal of CPDs had an impact in cell death after UV irradiation (122). These results suggest that depending on the DNA repair status of the organism, each photolesion may play different roles.

Therefore, it becomes of interest to study the specific role of each UV-induced photolesion in skin responses to UV exposure in a system where the fast removal of

6-4PP lesions by NER does not mask its role in hyperplasia, pigmentation, cell proliferation, cell death and tumorigenesis induction. This knowledge will not only increase the base knowledge of these processes, but may also aid in the development of prevention and treatment strategies for the unwanted UV side-effects, especially for subjects with photosensitivity, such as Xeroderma Pigmentosum patients.

2 OBJECTIVES

The main goal of the present work is to investigate the specific role played by each photolesion, CPD and 6-4PP, in skin responses after acute and chronic UVB irradiation. For that purpose, local epithelium alterations after UV exposure (UV lamp: 54 % UVB + 46 % UVA) of DNA repair-deficient mouse models carrying specific photolyases were evaluated: erythema, edema, pigmentation, hyperplasia, cell proliferation, apoptosis and tumorigenesis.

The specific objectives of this work are:

-Establish the desired mouse models through the backcross of two pre-existing transgenic mice lineages: one lineage that is KO in specific Nucleotide Excision Repair proteins (CSA, XPA or XPC), with one lineage that expresses a direct photolesion repair enzyme (CPD-photolyase or 6-4PP-photolyase). Further backcross these animals with a hairless lineage (SKH-1) to facilitate the UV irradiation process;

-Evaluate acute responses of these animals´ epidermises to UV irradiation: erythema, edema, hyperplasia, cell proliferation and apoptosis;

-Evaluate epithelial responses of the transgenic mouse lineages to chronic UV irradiation: erythema, edema, pigmentation, hyperplasia, cell proliferation, apoptosis and p53 overexpression.

3 MATERIAL AND METHODS

3.1 Mouse lineage establishment and maintenance

3.1.1 Mouse lineage establishment

Experiments were performed using transgenic animals expressing either CPD-photolyase or 6-4PP-photolyase. Depending on the mouse lineage, these genes can be under the control of two different promoters: chicken beta actin (βACT) with cytomegalovirus (CMV) enhancer or human keratin 14 (K14). The βACT promoter is responsible for expressing genes in all cells of a given organism (252). On the other hand, genes under the control of the K14 promoter are only expressed in keratinocytes (253). Photolyase constructs are described in detail elsewhere (249).

Mice lineages were established by backcrossing previously created transgenic lineages. βACT-CPD-photolyase/XPA/HR lineage was established through crossing the βACT-CPD-photolyase (249) and XPA (172) with the hairless (HR) SKH-1 mouse lineage [Erasmus Medical Center (EMC), Rotterdam - The Netherlands, inbreed strain, originally obtained from Charles River´s Laboratory, Wilmington/MA – USA]. K14-6-4PP-photolyase/XPA/HR lineage was established by crossing the following lineages: K14-6-4PP-photolyase (209), XPA and SKH-1. K14-CPD-photolyase/XPA/HR lineage was obtained backcrossing the K-14-CPD-photolyase (209) with the XPA and HR lineages. K14-CPD-photolyase/CSA mouse lineage was obtained through the crossing of K14-CPD-photolyase and CSA animals (185), K14-6-4PP-photolyase/CSA mice were obtained by backcrossing K14-6-4PP-photolyase and CSA animals. The mice lineages K14-CPD-photolyase/XPC and K14-6-4PP-photolyase/XPC were obtained by crossing K14-CPD-photolyase and K14-6-4PP-photolyase with the previously established XPC lineage (173), respectively. All animals were backcrossed for at least five generations before experiments were performed.

In the present work, chronic UV exposure experiments with βACT-CPD-photolyase/XPA/HR, K14-6-4PP-photolyase/XPA/HR, K14-CPD-photolyase/CSA and K14-6-4PP-photolyase/CSA mouse lineages are presented. Acute UV exposure experiments were performed with βACT-CPD-photolyase/XPA/HR and K14-6-4PP-photolyase/XPA/HR animal lineages. Acute and chronic UV exposure experiments

with XPC lineages, as well as acute UV exposure experiments with CSA mice, have not yet been performed due to lack of available animals, but will be conducted in the near future.

Animals were kept in an isolated environment (microisolator cages) in a 12/12 h day/night cycle, under a constant 21 ^{o}C temperature, with food and water available *ad libitum*.

This study was carried out in strict accordance with the recommendations of the "Dutch Experiments on Animals Act", which served as a basis for the implementation of "Guidelines on the protection of experimental animals" proposed by the Council of Europe, Directive 86/609/EC. All experiments were performed with the Erasmus Medical Center Animal Ethics Committee (*Dier Experimenten Comissie, DEC*) approval, under the protocol numbers 139-09-10 (EUR1758) and 139-09-16 (EMC1901). Approval from the Brazilian Animal Ethics Committee (*Comissão de Ética no Uso de Animais - CEUA*), from the Institute of Biomedical Sciences (*Instituto de Ciências Biomédicas, ICB*) of the University of São Paulo (*Universidade de São Paulo, USP*), was obtained under the following protocols: CEUA #059, page 46, book 2 (19/06/12) and CEUA #103, page 103, book 2 (23/05/12). No animal suffering was observed during the course of the experiments, according to the criteria suggested by the European Federation of Laboratory Animal Science Association (FELASA). Final procedures were performed under anesthesia.

3.1.2 Mice genotyping

Mice lineages establishment and maintenance were performed with the constant aid of genomic DNA genotyping.

Genomic DNA was obtained from the animals´ tails as follows. A 0.2-0.5 cm fragment of the tip of the tail was isolated and immediately frozen at -20 ^{o}C. Tissue was digested in 0.5 ml of Lysis Buffer [100 mM tris(hydroxymethyl)aminomethan (Tris) [Sigma, 154563 (Sigma-Aldrich, Saint-Louis, M.O., USA)] pH 8; 5 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma, E6758); 0.2 % sodium dodecyl sulphate (SDS) (Sigma, L4390); 200 mM sodium chloride (NaCl) (Sigma, S7652)] with 0.4 mg/ml Proteinase K [Bioline, BIO-37039 (Bioline, London, United Kingdom)] and 0.4 mg/ml ribonuclease (RNAse) [(Invitrogen, 12091-039 (Invitrogen, Carlsbad, CA, USA)], overnight at 55 ^{o}C. The following day, 0.5 ml of fenol:chloroform:isoamyl

acid (25:24:1) (Invitrogen, 15593-031) was added and the tubes inverted for 2 min. The samples were centrifuged at 14000 rpm (RCF: 92 RAD/mm) at 4 ^{o}C for 10 min. Upper phase was transferred into new tubes containing 0.5 ml of isopropanol [Merck, 100993 (Merck & Co, Whitehouse Station, N.J., USA)]. Tubes were inverted and re-centrifuged. Supernatant was discarded, pellets were washed with ethanol (ETOH) (Merck, 108543) and dried at room temperature (RT). Precipitates were then resuspended in 50-200 µl MilliQ water and stored at 4 ^{o}C.

Polymerase chain reactions (PCRs) were performed with GenetBio´s Taq Polymerase (with magnesium chloride in the buffer) [GenetBio, G-1000-1 (GenetBio, Yuseong-gu, Daejeon, Korea)] and a pool of A, T, C and G deoxynucleotide triphosphates (dNTPs) from Sigma (Sigma, DNTP100). Each reaction was performed with 100 pg – 1 ng/µl of DNA and the following extension primers and reaction protocols.

Table 2 presents extension primers used in genotyping PCRs.

Table 2 - List of extension primers used in genotyping PCRs

Gene	*Primer*	
	Name	*Sequence*[1]
CPD-Photolyase	CPD P1	TGA GAC TCA TCT CCC AGG AC
	CPD P2	CAC CAA TGC CAT GTG TTT GC
6-4PP-Photolyase	6-4PP P1	GCA CGA TTC AGC AAG CAA GG
	6-4PP P2	CGG TAC CTC TAC CTA TTT GAG TT
HR	HR P1	GCGTTACTGCAGCTAGCTTG
	HR P2	TGTAGCCTGTGGTCGCATAG
	HR P3	CTCCTGTTTGCTTGGTCATC
XPA	XPA-PGK	GGCCACTTGTGTAGCGCCAA
	XP26 155	GTGTCAGGCATAAGATCTATGACAA
	XP47 156	AGGCAAGCACCTGCAGCTGT
CSA	CSA 6	TCCTGGGGCTGGAGTTAAAC
	CSA 7	AAAGGCAAGATTTTTCTGCA
	CSA PGK3	TAGGGGAGGAGTAGAAGGTG
XPC	XPC 1	ATTGCGTGCATACCTTGCAC
	XPC 2	TATCTCCTCAAACCCTGCTC
	XPC 3	CGCATCGCCTTCTATCGCCT

[1] All primers are presented in a 5´ → 3´ sequence

Table 3 presents the PCR mixes used in each genotyping PCR.

Table 3 - PCR mixes used in each genotyping PCR

Gene	*Primers (pM)*	*dNTP (pM)*	*Buffer 10x[1] (ul)*	*Taq Polymerase (U)*	*H_20 MilliQ (ul)*
Photolyases & HR	0.25	0.25	2.25	0.5	qsp 20
XPA	0.8	0.2	3.75	0.625	qsp 25
CSA	0.66	0.2	3.25	2.5	qsp 25
XPC	0.132	0.2	3.25	2.5	qsp 25

[1] With $MgCl_2$

Table 4 presents the termocycles applied in the genotyping PCRs.

Table 4 - Termocycles applied in the genotyping PCRs

Gene	*Steps*												
	1		*2*		*3*		*4*		*5*	*6*		*7*	
	T[1] (°C)	t[2] (s)	T (°C)	t (s)	T (°C)	t (s)	T (°C)	t (s)	# of repetitions of steps 2 to 4	T (°C)	t (s)	T (°C)	t (s)
Photolyases & HR	95	60	95	30	58	30	72	30	30	72	600	4	?
XPA	95	120	95	30	62	30	72	60	35	72	300	4	?
CSA	95	300	95	60	62	60	72	60	40	72	600	4	?
XPC	95	120	94	30	62	30	72	60	35	72	300	4	?

[1] T = Temperature; [2] t = time.

Table 5 presents the expected band sizes for genotyping PCR amplification products ran on 2 % agarose (Sigma, A9539) gel in 0.5x TBE buffer [5,4g Tris; 2,75 g boric acid (Sigma, B7901); 1 mM EDTA pH 8; qsp 1 L H_20] at 160 V.

Table 5 - Expected band sized for genotyping PCR amplification products

Gene	*Allele band*	
	WT	*KO*
Photolyases	no band	300 bp[1]
HR	~ 400 bp	~ 250 bp
XPA	300 bp	200 bp
CSA	230 bp	140 bp
XPC	~ 250 bp	~ 150 bp

[1] bp = base pairs

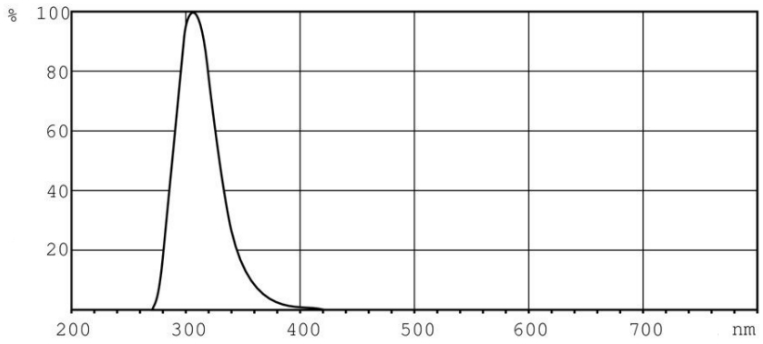
3.2 UV irradiation and photoreactivation of mouse skin

3.2.1 Minimal erythema dose (MED) definition

To determine the MED for our XPA/HR animals in our setup, 3 groups of 3 animals each were UV-irradiated with distinct UV doses: 20, 40 and 60 J/m^2, since literature suggests that 1 MED for these animals is equivalent to 40 J/m^2 (254). Twenty-four hours after exposure they were macroscopically scored for erythema induction, wounds and peeling. 1 MED was considered as the lowest dose tested which was capable of inducing erythema, without causing wounds or peeling. Animals were observed for 7 additional days to make sure the selected UV dose did not cause any delayed responses.

Irradiation was performed with 2 UV Philips bulbs TL12-40 W (54 % UVB – 280-315 nm -, 46 % UVA – 316-400 nm) (Philips, Amsterdam, The Netherlands), preheated for 15 min, at a distance of 128 cm from the cage base. Quantification of the irradiation dose was performed with a UV dosimeter [Waldman, 585100 (Hebert Waldman GmbH & Co, Villingen-Schwenningen, Germany)] positioned inside the cage at a ~5 cm height to simulate the UV dose which reached the mouse dorsum. Figure 19 presents the wavelength spectrum of the TL12-40 W Philips light.

Figure 19 - Wavelength spectrum of the TL12-40 W Philips light



TL12-40 W Philips lamp has a peak at 310 nm and emits 54 % of UVB light (200-315 nm) and 46 % of UVA (316-400nm). Source: Philips website (255).

Literature indicates that the MED for CSB mice is the same as that for XPA animals (254); and that CSA and CSB animals present the same general phenotype (185,256). However, hairless animals tend to have a MED which is half that of furred

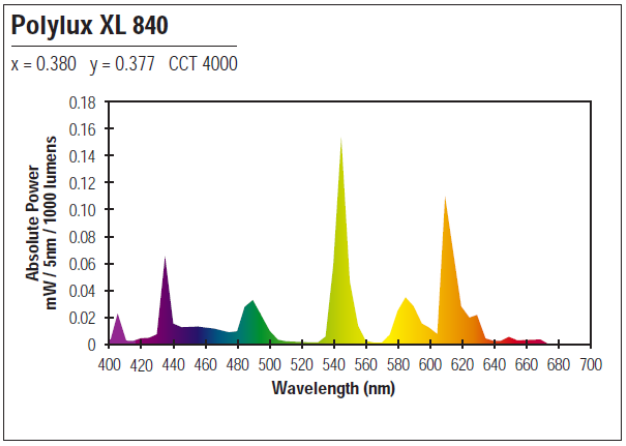
animals (257,258). Therefore, once the MED for XPA/HR mice was determined, the MED definition for CSA animals was performed with groups of 3 individuals irradiated with 40, 60, 80 and 100 J/m^2. Irradiations, UV dose assessment and MED definition were performed as previously described for XPA/HR animals.

3.2.2 Acute UV irradiation

Mice were irradiated at 10 *ante-meridian* (a.m.) (Coordinated Universal Time, UTC +1 h, Rotterdam – The Netherlands) with 1.5 MED (60 J/m^2) with 2 UV bulbs, followed by 3 hours of photoreactivation with 4 white Polylux bulbs [GE, XL F36 W/840 (General Electric-GE, Fairfield, Connecticut, USA)]. Thirty-nine hours after the single UV exposure, animals received an intraperitonial injection of 5 mg of 5-bromo-2-deoxyuridine (BrdU) (Sigma, 59-14-3). After 1 h animals were anesthetized with xylazine [Bayer, 18694 (Bayer, Leverkusen, North Rhine-Westphalia, Germany)] and ketamine (Bayer, 0856-2013-01) (110 mg Ketamine + 11 mg Xylazine/Kg of body weight), followed by cervical dislocation.

Figure 20 presents the wavelength spectrum of the Polylux XL F36 W/840 light.

Figure 20 - Wavelength spectrum of Polylux F36 W/840 white light.



Polylux F36 W/840 bulbs emit white light (<400 nm and >700 nm). Source: GE website: (259).

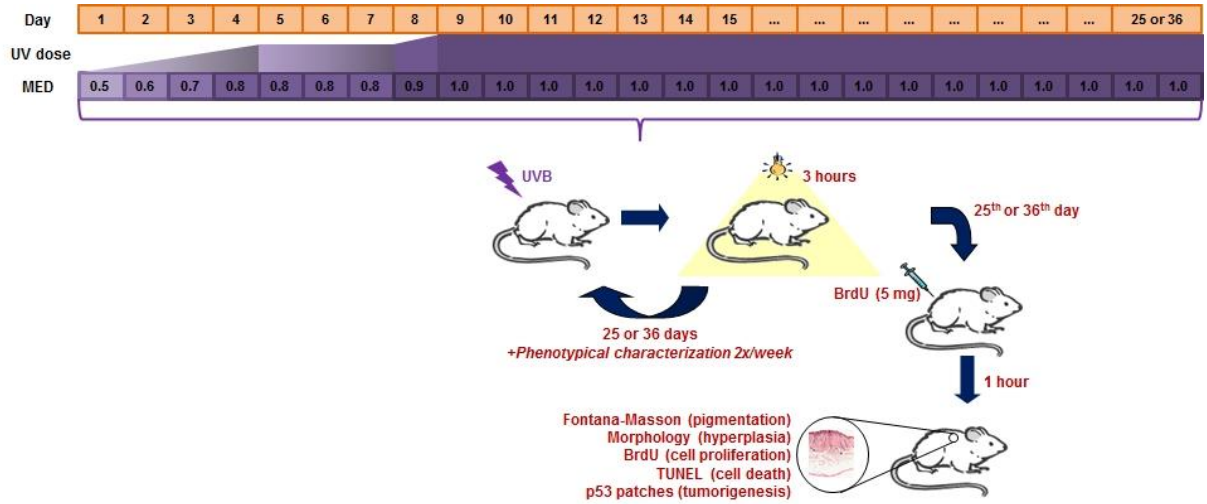
Two Acute Exposure (AE) experiments were performed: AE1 with β-ACT-CPD-photolyase/XPA/HR mouse model; and AE2 with K14-6-4PP-photolyase/XPA/HR animals. Each experiment had a sample of 3-7 animals and the

UV irradiated and non-UV irradiated control groups. Animals were 6-9 weeks old and a similar number of males and females were used in each group.

3.2.3 Chronic UV irradiation

Mice were irradiated daily at 10 a.m. for 25 or 36 consecutive days starting at 0.5 MED and increasing 0.1 MED/day until 0.8 MED was reached and maintained for 4 days. 0.1 MED was then increased each day until 1 MED (40 J/m^2) was reached and maintained until the end of the experiment, as illustrated in Figure 21. Immediately after each UV irradiation, animals were photoreactivated for 3 hours, as previously described. According to two different exposure protocols, on the 25th or 36th day, two hours into the photoreactivation period, mice received an intraperitonial injection of 5 mg of BrdU. After 1 h animals were anesthetized and sacrificed as described in section 3.2.2.

Figure 21 – Schematic representation of chronic UV irradiation process



Animals were irradiated with UVB followed by 3 hours of photoreactivation with white light for 25 or 36 consecutive days. The first irradiation was performed with 0.5 MED and each following day the dose was increased in 0.1 MED until 0.8 MED was achieved (day 4) and sustained for 4 days (day 7). The UV dose was again increased in 0.1 MED each day until 1 MED was reached and sustained until the end of the experiment (day 25 or 36). After the last irradiation, one hour before the end of the photoreactivation, animals were inoculated with BrdU (5 mg). After an 1 hour pulse animals were sacrificed and skin samples were collected for future analysis of morphology, pigmentation, cell proliferation, apoptosis and p53 patch formation.

Using the β-ACT-CPD-photolyase/XPA/HR mouse model, two Chronic Exposure (CE) experiments were performed with an endpoint of 25 days (CE1 and CE2) and one with an endpoint of 36 days (CE3). Group samples were of 5-7

animals/group in CE1, 3-5 animals/group in CE2, and 6 animals/group in CE3. Results are presented as an average of the two 25 day exposure experiments (CE1 and CE2) in comparison to the 36 consecutive day exposure experiments (CE3), unless otherwise stated.

Applying the K14-6-4PP-photolyase/XPA/HR mouse model, two experiments were performed with an endpoint of 25 days (CE4 and CE5) and one with an endpoint of 36 days (CE6). Group samples were of 5-7 animals/group in CE4, 3-5 animals/group in CE5, and 6 animals/group in CE6 experiment. Results are presented as an average of the two 25 day exposure experiments (CE4 and CE5) in comparison to the 36 consecutive day exposure experiments (CE6), unless otherwise stated.

Using the K14-CPD-photolyase/CSA and K14-6-4PP-photolyase/CSA mouse models, one experiment with an endpoint of 25 days was performed (CE7). Each group had 3-6 animals. Animals utilized in CE7 were not hairless. Therefore, one day prior to the beginning of the experiment, animals were anesthetized, as previously described, and plunked. When fur started to grow during the experiment, animals were shaven with the aid of a razor [Gilette Sensor Excel for women (Gilette, Boston, M.A., USA)] and shaving cream (Gilette Series foam mousse for men, Gilette).

In all experiments, animals were 6-9 weeks of age and a similar amount of males and females were used in every group. Each experiment, in addition to the UV-irradiated test group, also had two control groups: an UV irradiated control group which received the exact same treatment, but whose animals expressed no photolyase (XPA/HR or CSA animals); and a non UV irradiated control group which was only photoreactived. Table 2 presents a summary of all Chronic and Acute Exposure experiments.

Table 6 - Summary of performed Acute and Chronic Exposure experiments

Experiment	UVB		Window between last exposure and end of experiment	Test group genotype	Number of animals per group								
					Test group			Non UV irradiated control			UV irradiated control		
	Dose	# exposures			♀	♂	Total	♀	♂	Total	♀	♂	Total
AE1 [1]	1.5 MED	1	40 h	β-ACT-CPD-photolyase/XPA/HR	1	3	4	4	3	7	3	5	8
AE2 [1]	1.5 MED	1	40 h	K14-6-4PP-photolyase/XPA/HR	1	2	3	4	3	7	3	5	8
CE1 [2]	1 MED	25	3 h (Pr*)	β-ACT-CPD-photolyase/XPA/HR	1	4	5	4	3	7	3	4	7
CE2 [3]	1 MED	25	3 h (Pr)	β-ACT-CPD-photolyase/XPA/HR	2	1	3	3	2	5	2	1	3
CE3 [4]	1 MED	36	3 h (Pr)	β-ACT-CPD-photolyase/XPA/HR	3	3	6	3	3	6	2	4	6
CE4 [2]	1 MED	25	3 h (Pr)	K14-6-4PP-photolyase/XPA/HR	5	2	7	4	3	7	3	4	7
CE5 [3]	1 MED	25	3 h (Pr)	K14-6-4PP-photolyase/XPA/HR	1	2	3	3	2	5	2	1	3
CE6 [4]	1 MED	36	3 h (Pr)	K14-6-4PP-photolyase/XPA/HR	4	2	6	3	3	6	2	4	6
	1 MED	25	3 h (Pr)	K14-CPD-photolyase/CSA	3	2	5						
CE7 [5]	1 MED	25	3 h (Pr)	K14-6-4PP-photolyase/CSA	2	1	3	2	4	6	3	3	6
	3 MED	25	3 h (Pr)	K14-CPD-photolyase/CSA	3	3	6						

[1]–AE 1 e AE 2 experiments were performed at the same time and, therefore, shared the same animals for the non UV irradiated and the UV irradiated control groups.
[2]–CE1 and CE4 experiments were performed at the same time and, therefore, shared the same animals for the non UV irradiated and the UV irradiated control groups.
[3]–CE2 and CE5 experiments were performed at the same time and, therefore, shared the same animals for the non UV irradiated and the UV irradiated control groups.
[4]–CE3 and CE6 experiments were performed at the same time and, therefore, shared the same animals for the non UV irradiated and the UV irradiated control groups.
[5]–CE7:all test groups are part of the same experiment and, therefore, shared the same animals for the non UV irradiated and the UV irradiated control groups.
* –"Pr" stands for "photoreactivation".

3.3 Macroscopic analysis: erythema, pigmentation and hyperplasia

In the Acute Exposure experiments, 24 hours after UV irradiation and immediately prior to sacrifice, erythema and edema levels were observed and classified. During the Chronic Exposure experiments, twice a week, the levels of erythema, pigmentation and hyperplasia were macroscopically observed and classified. In the Chronic Experiments, on the last experimental day, photographs were taken and skin pigmentation was directly quantified with the aid of a chromometer [Minolta, CR-200 (Minolta, Osaka, Japan)], which evaluated the luminosity (L*) of the skin. L* represents the capacity of the analyzed surface to reflect white light. Lower L* indicates a darker surface. L* values were obtained from irradiated (dorsal) and non-irradiated (ventral) areas, and three individual measurements of each area were collected for data normalization. Skin pigmentation was not quantified for CE1 and CE4 due to lack of appropriate equipment.

3.4 Tissue collection and fixation for immunohistochemistry

At the end of the experiment, dorsal skin samples of 1 cm^2 were collected and fixated in 1.48 % formaldehyde (Sigma, 252549) in phosphate-buffered saline (PBS) [Lonza, 17-516F (Lonza, Basel, Switzerland)] overnight at 4 ^{o}C. After fixation, a series of ETOH dehydration at room temperature (RT) with 1 h immersion in each

solution took place: PBS, ETOH 50 %; ETOH 70 %; ETOH 80 %; ETOH 90 %; 2 x ETOH 100 %. After dehydration, samples were submerged 2 times for 1 h in xylene (Merck, 108661) and 2 times in melted paraffin (Merck, 107158) at 60 ^{o}C for 1 and 4 h, respectively. Paraffin blocks were then mounted and kept at RT until 5 µm sections were cut in a microtome [Micron, HM335E (Micron, Boise, I.D., USA)] and placed on positively polarized slides [Super Frost Plus Menzel-Gläser, J1800AMNZ (Menzel-Gläser, Braunschweig, Brunswick, Germany)] with ETOH 10 % at 50 ^{o}C, until total fluid evaporation. Sections were fixated on the slide at 37 ^{o}C overnight and then stored at 4 ^{o}C until staining.

3.5 Melanin quantification

Skin sections were deparaffinized through sequential immersion for 2 min in the following solutions: 2 x ETOH 100 %; ETOH 95 %; ETOH 70 %; ETOH 50 %. Melanin quantification was performed using a Fontana-Masson staining kit [EasyPath, EP-11-20024 (EasyPath, Brazil)]. Staining was performed according to manufacturer´s instructions. Briefly, slides were air-dried, followed by immersion in Reagent A (ammoniacal silver) for 35 min at 56 ^{o}C. Samples were then washed in water (H_2O) for 3 min at RT and incubated in Reagent B (gold chloride) for 15 s at RT. After a quick wash in H_2O, slides were incubated with Reagent C (thiossulfate) for 3 min, followed by a quick wash in tap water (H_2O). Finally, samples were counterstained with Reagent D (eosin) for 1 min, and dehydrated through sequential 5 s immersion in: ETOH 50 %; ETOH 70 %; ETOH 95 %; 2 x ETOH 100 %, followed by fixation through 2 x 2 min immersion in xylene. Slides were then mounted with Entellan (Merck, 1866) and cover slides (Menzel-Gläser, BB022060A1).

Quantification of melanin was performed in 5 fields per cut, in 3 sections, for a total of 15 analyses per animal sample, with a 400 x magnification. Pictures of each field were taken using a Zeiss optical microscope and Zeiss program AxioVision Release 4.8.2 [Zeiss, 06-2010 (Carl-Zeiss AG, Oberkochen, Germany)]. Melanin quantification was performed blindly through picture analysis. Each field was classified on an arbitrary scale from 0 to 8, indicating crescent melanin concentrations.

This staining was only performed for experiments with XPA/HR animals since no difference was observed in skin luminosity in CSA mice. Due to limited time, the

Fontana-Masson coloration was only performed for CE1 and CE4 slides. In the near future, slides from the CE2, CE3, CE5 and CE6 will also be analyzed.

3.6 Epidermal hyperplasia quantification

Skin sections were deparaffinized, quickly immersed in H_2O and transferred to hematoxylin (Merck, 1051740500) for 5 min. Slides were washed for 10 min under constant indirect tap H_2O flow, and transferred to eosin (Merck, 1098441000) for 1 min. Tissues were then dehydrated, fixated and mounted, as previously described. The whole protocol was performed at RT.

Hyperplasia was then quantified, with the aid of an optical microscope (Zeiss, Axiovert 200) in a 1000 x magnification with immersion oil (Zeiss, 518F), taking 3 diameter measurements on 3 fields of 3 slices of each sample, for a total of 27 data per sample. Pictures and measurements were taken with the aid of the Zeiss program AxioVision Release 4.8.2 (Zeiss, 06-2010). Measurements were taken from the dermis/epidermis junction to the outermost epidermal layer, excluding peelings.

3.7 Cell proliferation assay

For detection of proliferating cells in the epidermis, slides were deparaffinized, treated for 30 min at RT with 50 % methanol (METOH) (Merck, 1060351000) 1 % hydrogen peroxide (H_2O_2) (30 %) (Merck, 822287), washed for 2 x 5 min in PBS and incubated for 30 min in 0.6 mg/ml pepsine (Merck, 107185) in 100 mM hydrochloric acid (HCl) (Sigma, 7647-01-0) at 37 ^{o}C. Slides were then washed for 5 min in PBS and incubated at 56 ^{o}C for 20 min with 1 M HCl, followed by incubation 2 x 5 min with 100 mM sodium borate anhydrous in PBS pH 8.5 (Sigma, 1330-43-4) at RT. After pH neutralization, samples were washed for 3 x 5 min in PBS. Slides were incubated for 10 min RT in blocking solution [5 % rabbit serum (local production) in 1 % PBS/bovine serum albumin (BSA) (Sigma, A9647)], followed by treatment overnight at 4 ^{o}C with anti-BrdU antibody [Roche, 11 170 376 001 (Roche, Indianapolis, I.N., USA)]) diluted 1:50 in blocking solution. Slides were washed for 3 x 5 min in PBS and incubated for 1 h at RT in secondary antibody [Dako, P0161 (Dako, Denmark)] diluted 1:50 in 1 % PBS/BSA. After 3 x 5 min washing in PBS, substrate reaction was performed with 3,3'-Diaminobenzidina (DAB) (Dako, K3467) at RT, until positive

nuclei were visible. Slides were abundantly washed with PBS, counterstained for 1 min with hematoxylin and washed for 10 min in an indirect flow of tap H_2O. After dehydration and 2 x 2 min xylene immersions, slides were mounted with Entellan and cover slides.

Quantification of positive and total nuclei per area was performed in 3 fields per cut, in 3 cuts, for a total of 9 analyses per sample, with a 1000 x magnification with the aid of a Zeiss optical microscope and the AxioVision Zeiss program. Basal epidermis was defined as the first cell layer, in contact with the basement membrane. All the other superior epidermal layers, excluding peeling, were considered as part of the suprabasal epidermis. Basal data is presented as the percentage of stained basal cells in reference to the total number of cells in the basal layer. Suprabasal data is presented as the number of marked cells in the suprabasal layer per 100 cells present in the basal layer.

3.8 TUNEL assay

After deparaffinization slides were incubated for 5 min in 3 % H_2O_2 (37 %) in PBS at RT, followed by 2 x 5 min washes in PBS. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was then performed according to the manufacturer's instructions [Millipore, S7111 (Millipore, Billerica, M.A., USA)]. Briefly, slides were incubated for 10 min at RT with equilibration buffer followed by 1 h incubation with TdT solution at 37 ^{o}C. Samples were then transferred to stop buffer for 10 min at RT. Slides were washed for 3 x 1 min in PBS and incubated for 30 min at RT with anti-digoxigenin conjugate. After 2 x 2 and 1 x 5 min washing with PBS, substrate reaction was performed with DAB at RT until positive nuclei were visible. Slides were abundantly washed with H_2O and counterstained for 10 min at RT with methyl green solution (Sigma, M8884). After abundant washing in tap H_2O, slides were dehydrated and incubated 2 x 2 min in xylene. Slides were mounted with Entellan and cover slides.

Separate analysis was performed for basal and suprabasal epidermis, applying the same procedures previously explained for cell proliferation quantification.

This staining was performed for the following experiments: AE1, AE2, CE1, CE4 and CE7. In the near future, slides from the CE2, CE3, CE4 and CE5 will also be analyzed.

3.9 Detection of p53 overexpression

For the p53 analysis, 3 x 2 cm skin sections were collected and the epidermis was isolated by overnight floating in 200 μg/ml termolysine (Sigma, P1512) in PBS with 2 mM sodium chloride (Sigma, 7647-14-5) at 4 ^{o}C, followed by separation from the dermis with the aid of a polystyrene tube. Tissue fixation was performed in 1.48 % formaldehyde for 10 min, followed by 3 x 5 min washing in PBS. Samples were boiled at 110 ^{o}C for 5 min in 10 mM sodium citrate dehydrate (Sigma, W302600). After cooling down to RT, sheets were washed for 1 x 5 min in PBS at RT, followed by internal peroxidase blockage for 20 min under incubation in 1.5 % H_2O_2 in MTOH at RT. Samples were washed for 3 x 5 min in washing solution [PBS/0.5 % Tween 20 (Merck, 9480-OP)] at RT and incubated for 10 min with blocking solution [5 % rabbit serum (local production)/0.2 % BSA in PBS] at RT, followed by overnight incubation with anti-p53 antibody [Novocastra, CM5, NCL-p53-CM5p (Novocastra, New Castle, United Kingdom)] diluted to 1:500 in blocking solution at 4 ^{o}C. Epidermis was transferred to washing solution and then incubated with secondary antibody goat anti-rabbit biotin [Vector Laboratories, BA-1000 (Burlingame, C.A., USA)] diluted to 1:300 in PBS/0.1 % BSA/0.1 % saponin (Merck, 558255) in PBS. Samples were incubated with streptavidin-ABC-peroxidase [Amersham, RPN1051V (Amersham, Buckinghamshire, England)] diluted 1:100 in PBS for 45 min at RT, and washed for 3 x 5 min in PBS. Substrate reaction was performed with DAB until positive nuclei were visible. Samples were washed for 3 x 5 min in PBS. Epidermis was then mounted on slides with the aid of Kaiser´s mounting medium (Merck, 1092420100) and cover slides.

Quantification of groups of cells overexpressing p53 was performed with a 1000 x magnification with the aid of a Zeiss optical microscope. The entire area of the epidermis (2 x 3 cm) was analyzed. Groups of 10 or more consecutive positive nuclei were considered patches, as described elsewhere (260).

This staining was only performed with epidermal sheets from experiments CE1 and CE4 since the primary antibody used is no longer manufactured and no appropriate substitute could be found.

3.10 Data analysis

Column data was analyzed with one-way Anova followed by Bonferroni´s Multiple Comparison test. Paired columns data was analyzed by Two-way Anova followed by Bonferroni´s Multiple Comparision test. P value <0.05 was considered significant (*), p value <0.025 was considered very significant (**), and p value <0.001 was considered extremely significant (***). Data analysis was “blind” and performed with the aid of Prism 5.0 program (GraphPad Software, La Jolla, C.A., USA).

4 RESULTS

4.1 Mouse lineage establishment and maintenance

In the present work, six mouse lineages were successfully established: βACT-CPD-photolyase/XPA/HR, K14-6-4PP-photolyase/XPA/HR, K14-CPD-photolyase/CSA, K14-6-4PP-photolyase/CSA, K14-CPD-photolyase/XPC and K14-6-4PP-photolyase/XPC, through the backcrossing of previously existing lineages. In all lineages, mice were born at mendelian rate and presented normal life span. Mice lineages establishment and maintenance were performed with the constant aid of genomic DNA genotyping, as described elsewhere (209,249,250).

Acute and Chronic Exposure experiments with βACT-CPD-photolyase/XPA/HR, K14-6-4PP-photolyase/XPA/HR, K14-CPD-photolyase/CSA and K14-6-4PP-photolyase/CSA mouse lineages are presented. Acute and Chronic UV Exposure experiments with XPC lineages, as well as acute UV exposure experiments with CSA mice, have not yet been performed due to lack of available animals in sufficient number, but will be conducted in the near future.

4.2 Minimal erythema dose (MED) definition

To confirm the 1 MED for XPA/HR mice indicated in the literature (40 J/m^2) (254) for the animals applied in the present experiment, with the specific UV setup used, groups of 3 animals were exposed to 20, 40 and 60 J/m^2. Twenty four hours after UV exposure they were scored for erythema induction, wounds and peeling. 1 MED was considered as the lowest dose tested which was capable of inducing erythema, without causing wounds or peeling. Based on macroscopic observations, 40 J/m^2 was defined as 1 MED for XPA/HR mice. No delayed UV effects were observed up to 7 days after animals were exposed to this UV dose.

Literature indicates that the MED for CSB mice is the same as that for XPA animals (254). CSA and CSB animals present the same general phenotype (185,256). However, hairless animals tend to have a MED which is half that of furred animals (257,258). Therefore, once the MED for XPA/HR mice was determined, the MED definition for CSA animals was performed with groups of 3 individuals irradiated with 40, 60, 80 and 100 J/m^2. Twenty-four hours after UV exposure, based on the

occurrence of edema and erythema, 1 MED was defined as 40 J/m^2. Animals´ skin responses were followed for 7 additional days and no delayed responses were observed.

4.3 CPD ubiquitous removal in a XPA background

The experiments described in the present section (AE1, CE1, CE2 and CE3), employed XPA/HR mice transgenically expressing a CPD-photolyase under the control of a βACT promoter. Therefore, CPDs were ubiquitously removed during photoreactivation. In addition to the test group, all experiments were performed with UV irradiated and non UV irradiated control groups.

4.3.1 Acute UV exposure

In the experiment Acute Exposure 1 (AE1), animals were irradiated with 1.5 MED of UV, followed by photoreactivation for 3 h. Animals were observed 24 h and 40 h after irradiation. Samples were collected for analysis 40 h after UV exposure. Table 7 summarizes AE1 animal groups.

Table 7 - Acute Exposure 1 (AE1) experimental groups

Experiment	*Group*	*Mice genotype*	*# animals*	*UV dose*	*Experiment duration*	*Remaining photolesions in the genome*
AE1	A	βACT-photolyase/XPA/HR and XPA/HR	7	0 MED	40 hours	Ø
	B	βACT-photolyase/XPA/HR	4	1.5 MED		6-4PP
	C	XPA/HR	8	1.5 MED		CPD & 6-4PP

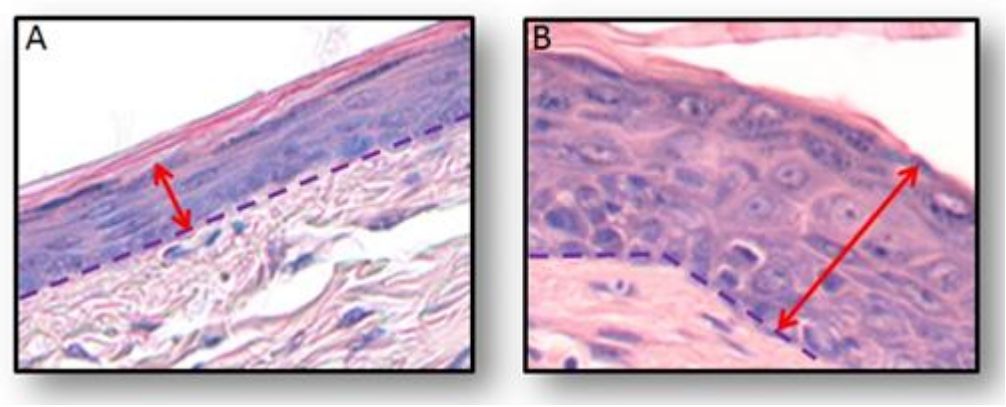
4.3.1.1 <u>Macroscopic observations</u>

Macroscopic observation of mice 24 h after UV irradiation showed that all exposed animals presented erythema and edema. However, in animals where CPD lesions were removed, these skin responses were less intense than in animals where both photolesions persisted. Forty hours after irradiation, all animals presented similar levels of erythema and edema, which were less severe than at 24 h, data not shown.

4.3.1.2 Skin hyperplasia and dysplasia

Dysplasia was observed in all hyperplastic tissues. Hyperplasia was defined as a significant increase in epidermal thickness as measured in skin sections stained with hematoxylin and eosin (H/E). Figure 22 illustrates how epidermal thickness was quantified.

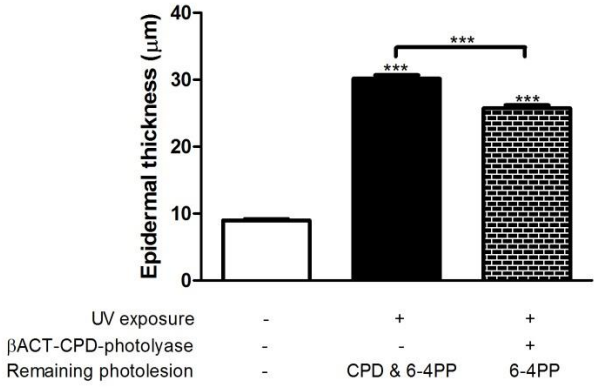
Figure 22 - Representation of epidermal thickness quantification



Epidermal thickness was quantified through perpendicular measurements of the tissue extension between basement membrane and *stratum corneum*, excluding keratin deposits in sections stained with H/E. A) normal epidermis of XPA/HR mice with no thickening and B) severe epidermal thickening 40 h after 1.5 MED of UV. Observe the increase in epidermal layers and cell size in B. Purple dotted line indicates the encounter between epidermis and dermis (basement membrane); red arrows represent how epidermal thickness is measured. Magnifcation: 400x.

Epidermal thickness quantification showed that acute UV exposure induced significant hyperplasia (30.1 μm) in comparison with normal epidermal thickness (8.9 μm). Animals where CPDs were ubiquitously removed presented an intermediate epidermal thickness (25.7 μm), which was statistically different from both control groups, as shown in Figure 23.

Figure 23 - Acute UV exposure and epidermal hyperplasia in βACT-photolyase/XPA/HR mice

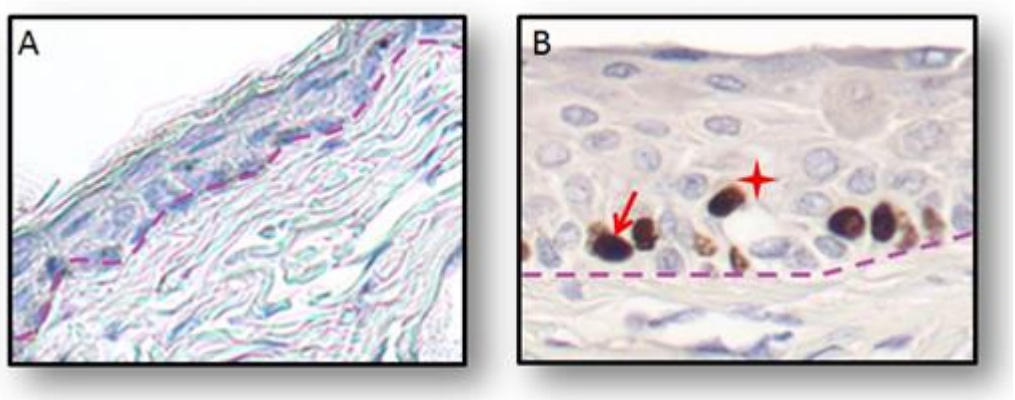


Acute UV irradiation induced significant hyperplasia in XPA/HR animals. CDP removal was capable of partially preventing epidermal thickening (One-way Anova: $p<0.0001$). * indicates p value<0.05; ** indicates p value<0.025; and *** indicates p value<0.001.

4.3.1.3 Cell proliferation

Cell proliferation was detected by the identification of cells labeled with BrdU, which characterize cells in S-phase. Figure 24 exemplifies BrdU incorporation in basal and suprabasal epidermis.

Figure 24 - Representation of BrdU incorporation in basal and suprabasal epidermis



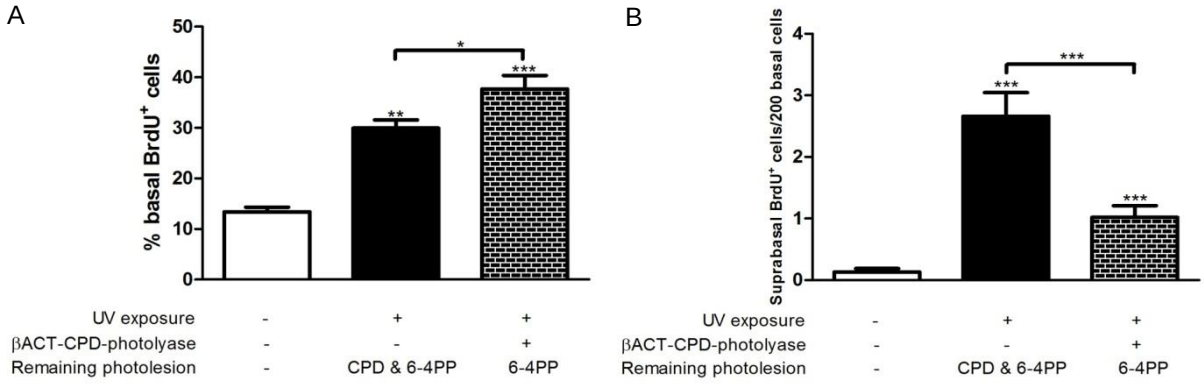
BrdU is analogous to thymine and is incorporated in DNA during S-phase cell cycle. Brown nuclei indicate BrdU incorporation. A) negative control and B) BrdU incorporation observed in a XPA/HR hyperplastic mouse epidermis 40 h after 1.5 MED of UV. The purple dotted line indicates the division of dermis and epidermis. The red arrow points to a basal proliferative cell and the red star indicates a dividing suprabasal cell. Magnification: 400x.

Normal basal epidermal proliferation (13.3 %) was significantly increased by acute UV irradiation of XPA/HR mice (29.9 %). CPD removal did not prevent UV-induced BrdU incorporation in this epidermal layer. On the contrary, there was a

small increase in basal cell proliferation when this photolesion was removed (37.6 %), as shown in Figure 25A.

Constitutive suprabasal cell proliferation (0.2 suprabasal $BrdU^+$ cells/100 basal cells) was increased by UV exposure (6.3 suprabasal $BrdU^+$ cells/100 basal cells). Intermediate levels of suprabasal cell proliferation were found in epidermis where only 6-4PP lesions persisted (3.5 suprabasal $BrdU^+$ cells/100 basal cells), which was significantly different from both control groups, as presented in Figure 25B.

Figure 25 - Acute UV exposure and epidermal proliferation in βACT-photolyase/XPA/HR mice

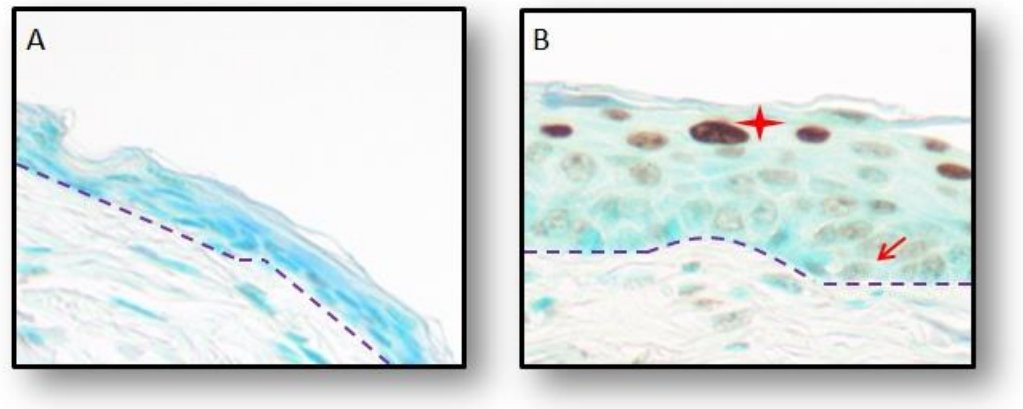


A) Acute UV irradiation induced significant basal epidermal cell proliferation, which was further increased by CPD removal in XPA/HR mice (One-way Anova: $p<0.0001$). B) Suprabasal cell proliferation was induced by UV exposure, which was partially prevented by CPD removal (One-way Anova: $p<0.0001$).

4.3.1.4 Cell death

Cell death in the epidermis was detected by TUNEL staining, as illustrated in Figure 26.

Figure 26 - Representation of epidermal TUNEL staining

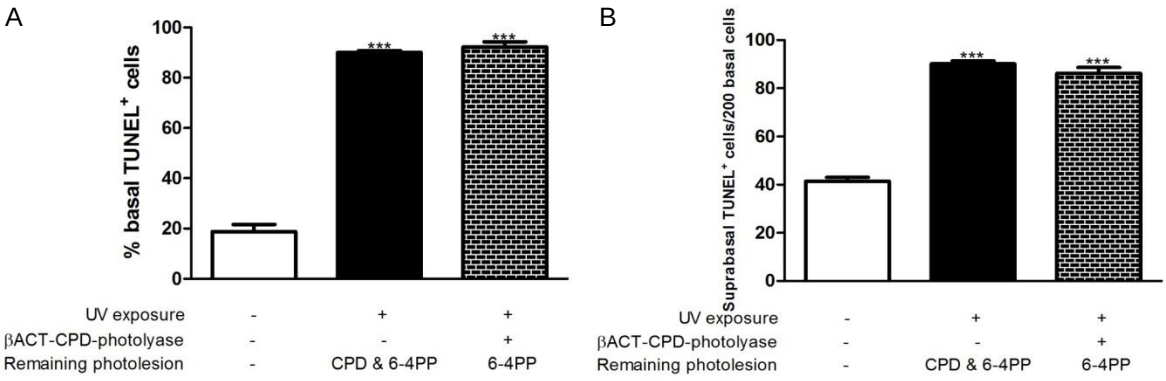


TUNEL staining labels 3´-OH free DNA extremity. Brown nuclei indicate apoptotic cells. A) negative control and B) TUNEL labeling in hyperplastic XPA/HR mouse epidermis 40 h after 1.5 MED of UV. The purple dotted line indicates the location of the basement membrane. The red arrow points to a basal apoptotic cell and the red star indicates a suprabasal apoptotic cell. Magnification: 400x.

Quantification of TUNEL positive cells the basal epidermal layer showed that acute UV irradiation significantly increased apoptosis (91.4 %) as compared to normal apoptotic levels (18.7 %). CPD removal did not reduce UV-induced basal cell death (92.2 %), as presented in Figure 27A.

Analysis of apoptosis in suprabasal epidermis demonstrated that UV irradiation also induced cell death in this layer (180.2 suprabasal $TUNEL^+$ cells/100 basal cells), compared with constitutive apoptotic levels (82.8 suprabasal $TUNEL^+$ cells/100 basal cells). There was no significant reduction of UV-induced suprabasal apoptotic levels when CPD photolesions were ubiquitous removed (172.4 suprabasal $TUNEL^+$ cells/100 basal cells), as shown in Figure 27B.

Figure 27 - Acute UV exposure and epidermal apoptosis in βACT-CPD-photolyaseXPA/HR mice



A) Acute UV irradiation induced basal epidermal cell death, which was not prevented by CPD removal in XPA/HR mice (One-way Anova: $p<0.0001$). B) The Apoptotic level in the suprabasal epidermis was increased by acute UV irradiation, even when CPDs were removed (One-way Anova: $p<0.0001$).

4.3.2 Chronic UV exposure

In the experiments Chronic Exposure 1, 2 and 3 (CE1, CE2 and CE3) mice were UV irradiated with increasing UV doses up to 1 MED, followed by 3 h of photoreactivation after each UV exposure, for 25 (CE1 and CE2) or 36 (CE3) consecutive days. Table 8 summarizes the CE1, CE2 and CE3 animal groups.

Table 8 - Chronic Exposure 1, 2 and 3 (CE1, CE2 and CE3) experimental groups

Experiment	*Group*	*Mice genotype*	*# animals*	*UV dose*	*Experiment duration*	*Remaining photolesions in the genome*
	A	βACT-CPD-photolyase/XPA/HR and XPA/HR	7	0 MED		Ø
CE1	*B*	βACT-CPD-photolycase/XPA/HR	5	1 MED	25 days	6-4PP
	C	XPA/HR	7	1 MED		CPD & 6-4PP
	A	βACT-CPD-photolyase/XPA/HR and XPA/HR	5	0 MED		Ø
CE2	*B*	βACT-CPD-photolyase/XPA/HR	3	1 MED	25 days	6-4PP
	C	XPA/HR	3	1 MED		CPD & 6-4PP
	A	βACT-CPD-photolyase/XPA/HR and XPA/HR	6	0 MED		Ø
CE3	*B*	βACT-CPD-photolyase/XPA/HR	6	1 MED	36 days	6-4PP
	C	XPA/HR	6	1 MED		CPD & 6-4PP

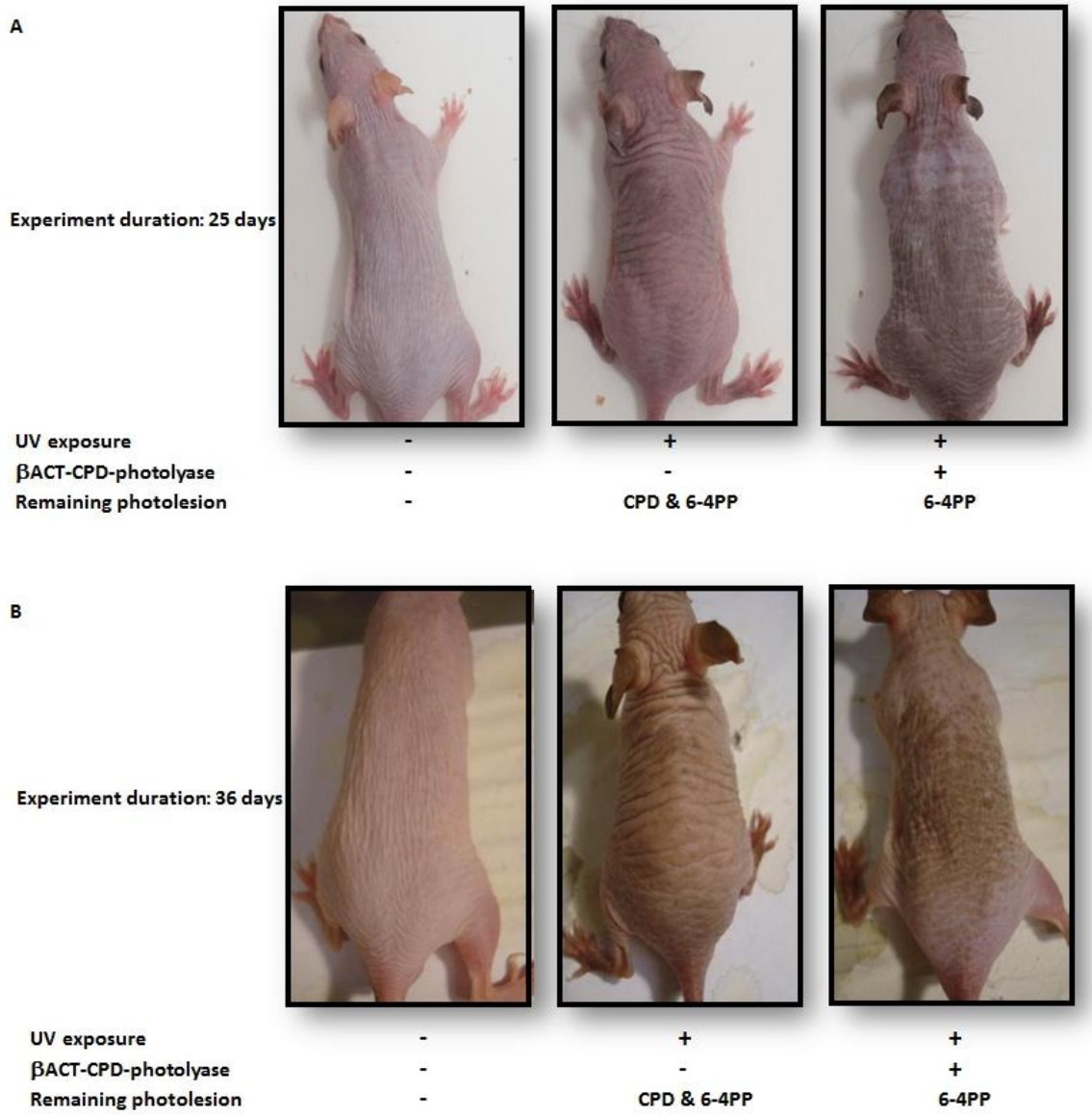
4.3.2.1 Macroscopic skin alterations

Twenty-four hours after the second UV exposure, erythema and edema were present in all UV irradiated animals, with different intensities. Animals where CPD lesions were removed by CPD-photolyase, presented lighter skin redness and swelling in comparison with the UV irradiated control group. These responses gradually faded up to the 7th day, when they were no longer present.

On the 5th day after the first UV exposure, macroscopic epidermal thickening could be observed only in UV irradiated control animals. On the 8th experimental day, dark skin pigmentation was observed in animals UV irradiated and where CPD lesions were removed. On the 14th experimental day, dark skin pigmentation was also observed in animals with no photolesion removal. These responses seemed to increase gradually in their respective groups up to the end of the experiment (data not shown).

Figure 28 presents pictures of one mouse from each animal group from experiments CE2 and CE3, as a representation of the macroscopic alterations observed on the 25th and 36th experimental days.

Figure 28 - Macroscopic alterations of βACT-CPD-photolyase/XPA/HR mice after chronic UV irradiation



A) UV irradiation of XPA/HR animals for 25 consecutive days (CE2) induced epidermal thickening and skin pigmentation. Removal of this photolesion increased dark skin pigmentation and prevented skin thickening. B) Hyperplasia and pigmentation seemed to gradually increase over time, as can be observed in mice UV irradiated for 36 consecutive days (CE3).

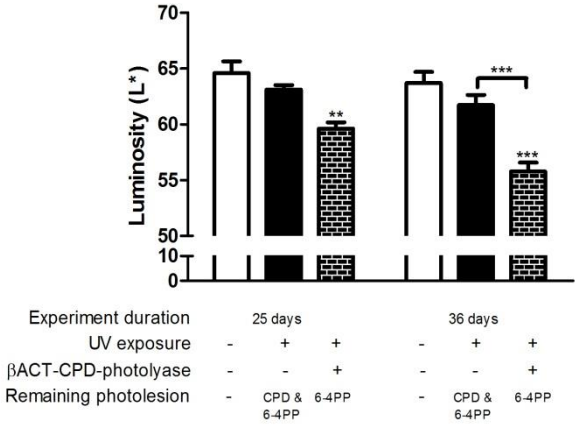
4.3.2.2 Skin darkening

Confirming macroscopic observations, skin luminosity quantification with the aid of a chromometer showed that chronic UV irradiation for 25 consecutive days (CE2) did not significantly change skin color (63.1 L*), in comparison with constitutive skin luminosity (64.6 L*). Interestingly, when CPDs were removed after each UV exposure, skin luminosity was significantly reduced (59.6 L*), which indicates that the

skin of animals with only 6-4PP lesions persisting in their genome presented a darker color, as shown in Figure 29.

Similar alterations were observed in animals UV irradiated for 36 consecutive days (CE3): skin luminosity (61.7 L*) was similar to constitutive levels (63.7 L*). Again, CPD removal after each UV exposure significantly reduced skin luminosity (55.7 L*). Interestingly, despite the macroscopic observations, there was no significant difference in skin luminosity between correlated groups of animals UV irradiated for 25 or 36 consecutive days. Furthermore, despite a tendency towards a darker skin in UV irradiated control animals, their skin luminosity was not statistically different from non UV irradiated control animals, as presented in Figure 29.

Figure 29 - Chronic UV exposure and skin luminosity in βACT-CPD-photolyase/XPA/HR mice

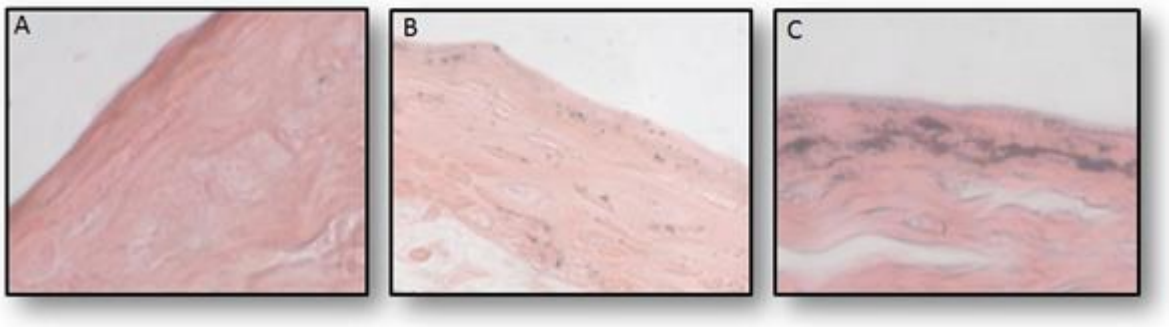


Chronic UV irradiation alone did not alter skin luminosity in XPA/HR mice. CPD removal reduced skin luminosity in animals UV irradiated for 25 (CE2) or 36 (CE3) days (Two-way Anova: p=0.0089).

4.3.2.3 Melanin content

Melanin concentration was quantified by Fontana-Masson staining followed by an arbitrary field classification on a scale from 0 to 8, representing an increase in pigment presence. Figure 30 presents epidermal sections with no melanin (A), intermediate (B) and high (C) pigment concentration.

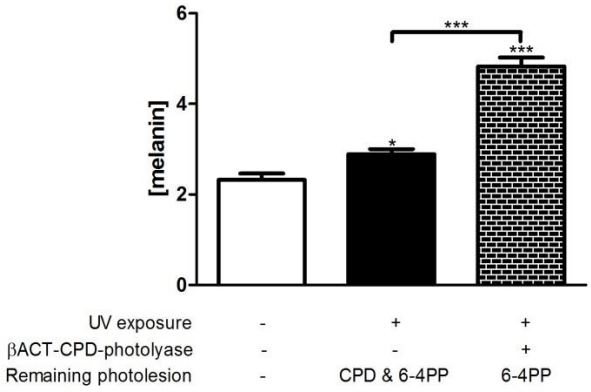
Figure 30 - Representation of epidermal sections with different melanin concentrations



Melanin was stained with Fontana-Masson in skin sections of XPA/HR mice treated with increasing UV doses up to 1 MED for 25 consecutive days. Epidermal sections were arbitrary classified according to pigment concentration: A) no pigment (scale value: 0), B) intermediate pigment content (scale value: 3) and C) high melanin concentration (scale value: 8). Magnification: 400x.

Analysis of melanin concentration after 25 consecutive days of low UV irradiation in the CE1 experiment, showed that UV irradiation induced an increase in epidermal pigment concentration (average value: 2.8), in comparison with constitutive pigment levels (average value: 2.3), which was not observed in skin luminosity measurements, but is in accordance with macroscopic observations. Again in accordance with the data previously shown, ubiquitous CPD removal further increased the UV-induced melanogenesis, enhancing total melanin epidermal content (average value: 4.8). Epidermal melanin quantification is presented in Figure 31.

Figure 31 - Chronic UV exposure and epidermal melanin concentration in βACT-CPD-photolyase/XPA/HR mice



Chronic UV irradiation for 25 consecutive days significantly increased epidermal melanin concentration in XPA/HR mice. CPD removal further enhanced skin pigment content (One-way Anova: $p<0.0001$).

4.3.2.4 Skin hyperplasia and dysplasia

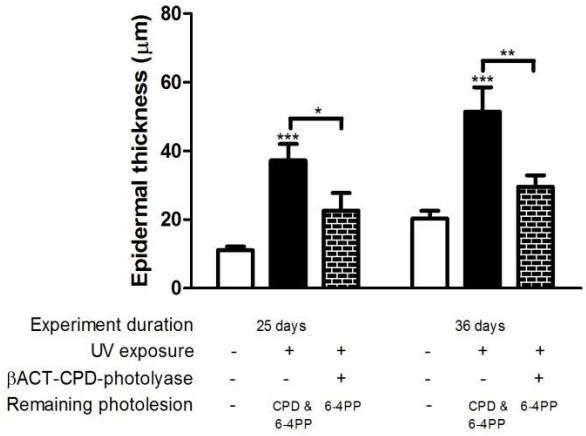
Quantification of epidermal thickness average of the two Chronic Exposure experiments (CE1 and CE2) was performed to estimate UV-induced hyperplasia in animals irradiated for 25 consecutive days. Data analysis showed that UV induced significant hyperplasia (37.2 μm) in comparison with normal epidermal thickness (11.1 μm). CPD removal was associated with intermediate hyperplasia levels (22.6 μm), which were significantly different from that observed in UV irradiated control animals, as shown in Figure 32.

Similarly, in animals chronically irradiated for 36 consecutive days (CE3), UV induced a significant increase in epidermal thickness (51.4 μm), as compared to constitutive epidermal thickness (20.3 μm). Again, CPD removal prevented UV-induced hyperplasia (29.5 μm), as presented in Figure 31.

Confirming macroscopic observations, all groups of animals irradiated for 36 consecutive days (CE3) presented a slightly thicker epidermis than animals irradiated for 25 consecutive days (CE1 and CE2). However, this increase was not statistically significant, as presented in Figure 32.

Furthermore, hyperplastic tissues also presented dysplasia.

Figure 32 - Chronic UV exposure and epidermal hyperplasia in βACT-CPD-photolyase/XPA/HR mice



Chronic UV exposure induced significant hyperplasia in XPA/HR mice. Ubiquitous CPD removal prevented UV-induced hyperplasia. Prolonged UV exposure (36 days) seemed to increase epidermal thickness but this response was not statistically significant (Two-way Anova: p=0.0068).

4.3.2.5 Cell proliferation

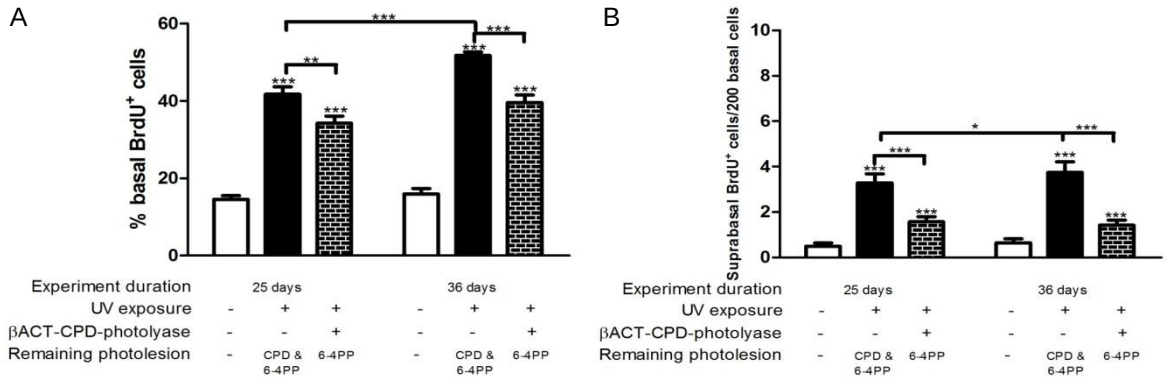
Quantification of the average BrdU incorporation in the two Chronic Exposure experiments where animals were exposed for 25 consecutive days (CE1 and CE2) was performed to estimate UV-induced cell proliferation. Results were then compared to the data obtained from animals UV irradiated for 36 consecutive days.

Chronic UV irradiation for 25 or 36 consecutive days increased cell proliferation in basal epidermis (41.8 % and 51.7 %, respectively), in comparison to normal basal cell proliferation (14.5 % and 15.9 %, respectively). CPD removal after each UV exposure was associated with intermediate levels of cell proliferation after 25 or 36 days of UV irradiation (34.2 % and 39.6 %, respectively), which were different from both controls in each case, as shown in Figure 33A.

Suprabasal constitutive cell proliferation at the end of the 25 or 36 day experiments (1 and 1.8 suprabasal $BrdU^+$ cells/100 basal cells, respectively) was also increased by chronic UV irradiation (6.5 and 9.7 suprabasal $BrdU^+$ cells/100 basal cells, respectively). CPD removal was associated with intermediate levels of suprabasal cell proliferation (3.1 and 4.7 suprabasal $BrdU^+$ cells/100 basal cells, respectively), which were different from both controls in each case, as presented in Figure 33B.

All groups from the CE3 experiment presented higher epidermal proliferation when compared to animals from experiments CE1 and CE2. However, this increase was not statistically significant, with exception of the UV irradiated control groups, where cell division was always higher in animals UV exposed for 36 consecutive days than in mice exposed for only 25 days, as presented in Figure 33.

Figure 33 - Chronic UV exposure and epidermal proliferation in βACT-CPD-photolyase/XPA/HR mice



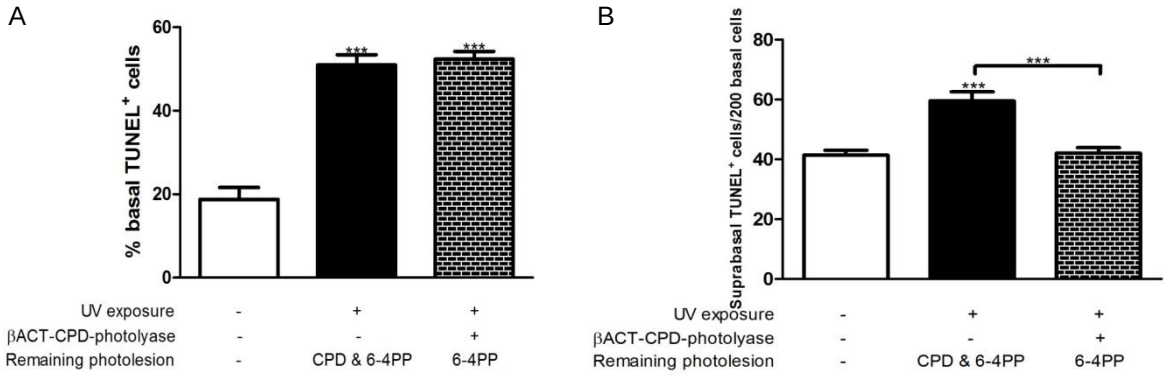
A) UV acute irradiation induced significant basal epidermal cell proliferation, which was partially prevented by CPD removal in XPA/HR mice (One-way Anova: $p<0.0001$). B) Suprabasal cell proliferation was also induced by UV exposure, which was partially prevented by CPD removal (One-way Anova: $p<0.0001$).

4.3.2.6 Cell death

TUNEL quantification showed that chronic UV irradiation for 25 consecutive days increased cell death in basal epidermis (50.9 %), in comparison with normal basal apoptosis (18.7 %). This response was not altered by CPD removal after each UV exposure (52.3 %), as shown in Figure 34A.

Suprabasal constitutive apoptosis (82.8 suprabasal TUNEL$^+$ cells/100 basal cells) was also increased by chronic UV irradiation for 25 consecutive days (119.0 suprabasal TUNEL$^+$ cells/100 basal cells). CPD removal prevented the UV-induced increase in suprabasal cell death (84.0 suprabasal TUNEL$^+$ cells/100 basal cells), as presented in Figure 34B.

Figure 34 - Chronic UV exposure and epidermal apoptosis in βACT-CPD-photolyase/XPA/HR mice

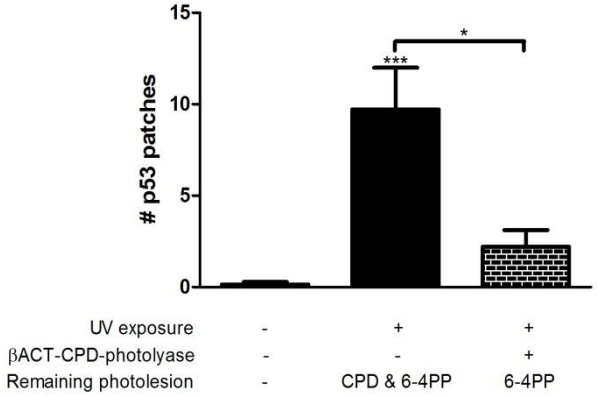


A) UV acute irradiation induced significant basal epidermal cell death, which was not prevented by CPD removal in XPA/HR mice (One-way Anova: $p<0.0001$). B) Suprabasal apoptosis was also induced by UV exposure, but not in animals with only 6-4PP lesions persisting in their genome (One-way Anova: $p<0.0001$).

4.3.2.7 p53 overexpression

Quantification of p53 overexpression showed that chronic UV irradiation for 25 consecutive days (CE1) significantly increased the number of p53 epidermal patches (9.7 patches/sheet) as compared to constitutive patch level (0.1 patches/sheet). Ubiquitous CPD removal was associated with a reduction in p53 overexpression, which was not significantly different from non UV irradiated control animals (2.2 patches/sheet), as presented in Figure 35.

Figure 35 - Chronic UV exposure and epidermal p53 overexpression in βACT-CPD-photolyase/XPA/HR mice



Chronic UV irradiation of XPA/HR mice induced epidermal p53 overexpression, which was prevented by CPD ubiquitous removal (Two-way Anova: $p=0.0007$).

4.4 6-4PP removal from basal keratinocytes in a XPA background

The experiments described in the present section (AE2, CE4, CE5 and CE6), employed XPA/HR mice transgenically expressing a 6-4PP-photolyase under the control of a K14 promoter. Therefore, 6-4PPs were removed exclusively from basal keratinocytes during photoreactivation. In addition to the test group, all experiments were also performed with UV irradiated and non UV irradiated control groups.

4.4.1 Acute UV exposure

In the experiment Acute Exposure 2 (AE2), mice were irradiated with 1.5 MED of UV, followed by photoreactivation for 3 h. Animals were observed 24 and 40 h after irradiation. Samples were collected for analysis 40 h after UV exposure. Table 9 summarizes the AE2 animal groups.

Table 9 - Acute Exposure 2 (AE2) experimental groups

Experiment	*Group*	*Mice genotype*	*# animals*	*UV dose*	*Experiment duration*	*Remaining photolesions in the genome*
AE2	A	K14-64-photolyase/XPA/HR and XPA/HR	7	0 MED	40 hours	Ø
	B	K14-64-photolyase/XPA/HR	3	1.5 MED		CPD
	C	XPA/HR	8	1.5 MED		CPD & 6-4PP

4.4.1.1 <u>Macroscopic observations</u>

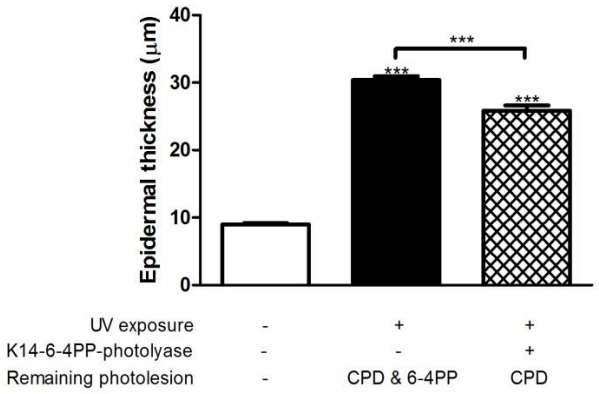
Macroscopic observation of mice 24 h after UV irradiation showed that all exposed animals presented similar erythema and edema. Forty hours after exposure, all animals presented similar, less intense, levels of these tissue responses, data not shown.

4.4.1.2 <u>Skin hyperplasia and dysplasia</u>

Acute UV exposure induced significant hyperplasia (30.3 μm), in comparison with normal epidermal thickness (8.9 μm). 6-4PP removal from basal keratinocytes

was associated with slightly lower UV-induced hyperplasia levels (25.8 µm), as presented in Figure 36. Dysplasia was also observed in hyperplastic tissues.

Figure 36 - Acute UV exposure and epidermal hyperplasia in K14-6-4PP-photolyase/XPA/HR mice



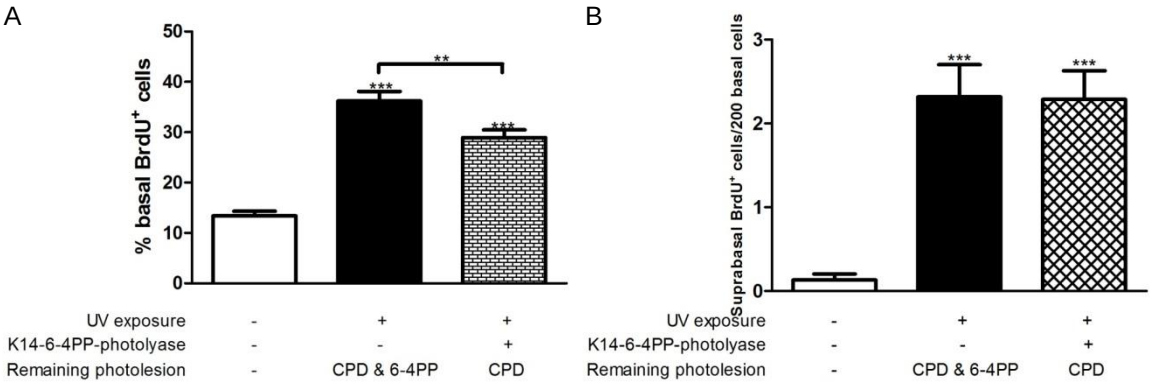
Acute UV irradiation induced significant hyperplasia in XPA/HR animals. 6-4PP removal from basal keratinocytes partially reduced UV-induced epidermal thickening (One-way Anova: $p<0.0001$).

4.4.1.3 Cell proliferation

Quantification of cell division in the basal epidermal layer demonstrated that acute UV irradiation induced a significant increase in cell division (36.1 %), in comparison with constitutive basal proliferation (13.3 %). 6-4PP removal from basal keratinocytes was associated with intermediate levels of UV-induced basal cell proliferation (28.9 %), which were significantly different from both control groups as shown in Figure 37A.

Constitutive suprabasal proliferation (0.2 suprabasal $BrdU^+$ cells/100 basal cells) was also increased by acute UV irradiation (4.6 suprabasal $BrdU^+$ cells/100 basal cells). 6-4PP removal from basal keratinocytes did not alter UV-induced suprabasal proliferation (4.5 suprabasal $BrdU^+$ cells/100 basal cells), as presented in Figure 37B.

Figure 37 - Acute UV exposure and epidermal proliferation in K14-6-4PP-photolyase/XPA/HR mice



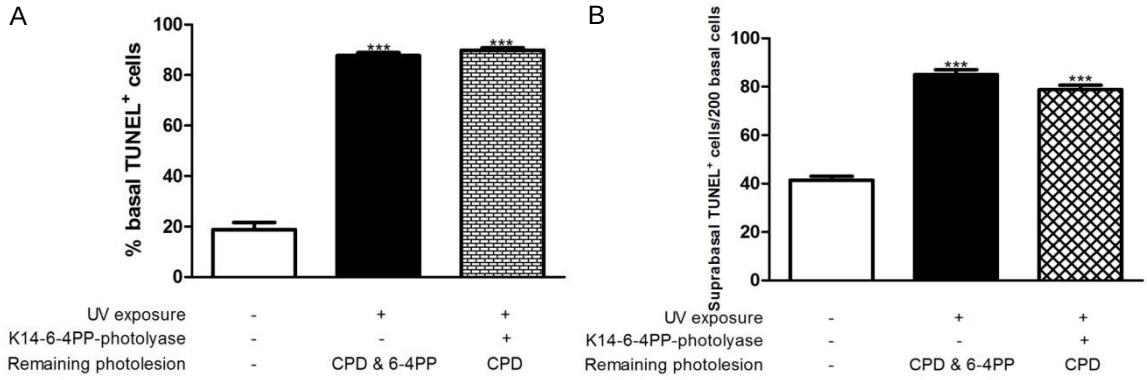
A) Acute UV irradiation induced a significant increase in basal epidermal cell division, which was partially reduced by 6-4PP removal from basal keratinocytes in XPA/HR mice (One-way Anova: $p<0.0001$). B) Suprabasal cell proliferation was induced by acute UV irradiation, which was not altered by 6-4PP removal from basal keratinocytes (One-way Anova: $p<0.0001$).

4.4.1.4 Cell death

Quantification of TUNEL positive basal epidermal cells showed a significant increase in apoptosis after acute UV irradiation (87.7 %), in comparison with constitutive basal cell death (18.7 %). 6-4PP removal from basal keratinocytes did not reduce UV-induced basal apoptosis(89.9 %), as presented in Figure 38A.

Suprabasal cell death was also significantly increased by acute UV irradiation (170.0 suprabasal TUNEL$^+$ cells/100 basal cells), as compared to normal suprabasal apoptotic levels (82.8 suprabasal TUNEL$^+$ cells/100 basal cells). 6-4PP removal from basal keratinocytes did not significantly alter UV-induced apoptosis (157.6 suprabasal TUNEL$^+$ cells/100 basal cells), as shown in Figure 38B.

Figure 38 - Acute UV exposure and epidermal apoptosis in K14-6-4PP-photolyase/XPA/HR mice



A) Acute UV irradiation increased basal epidermis apoptosis, which was not altered by 6-4PP removal from basal keratinocytes in XPA/HR mice (One-way Anova: $p<0.0001$). B) Suprabasal cell death was enhanced by acute UV exposure; 6-4PP removal from basal keratinocytes did not alter UV-induced apoptosis (One-way Anova: $p<0.0001$).

4.4.2 Chronic UV exposure

In the experiments Chronic Exposure 4, 5 and 6 (CE4, CE5 and CE6) mice were UV irradiated with increasing UV doses up to 1 MED, followed by 3 h of photoreactivation after each UV exposure, for 25 (CE4 and CE5) or 36 (CE6) consecutive days. Table 10 summarizes the CE4, CE5 and CE6 animal groups.

Table 10 - Chronic Exposure 4, 5 and 6 (CE4, CE5 and CE6) experimental groups

Experiment	*Group*	*Mice genotype*	*# animals*	*UV dose*	*Experiment duration*	*Remaining photolesions in the genome*
CE4	*A*	K14-6-4PP-photolyase/XPA/HR and XPA/HR	7	0 MED	25 days	Ø
	B	K14-6-4PP-photolyase/XPA/HR	5	1 MED		CPD
	C	XPA/HR	7	1 MED		CPD & 6-4PP
CE5	*A*	K14-6-4PP-photolyase/XPA/HR and XPA/HR	5	0 MED	25 days	Ø
	B	K14-6-4PP-photolyase/XPA/HR	3	1 MED		CPD
	C	XPA/HR	3	1 MED		CPD & 6-4PP
CE6	*A*	K14-6-4PP-photolyase/XPA/HR and XPA/HR	6	0 MED	36 days	Ø
	B	K14-6-4PP-photolyase/XPA/HR	6	1 MED		CPD
	C	XPA/HR	6	1 MED		CPD & 6-4PP

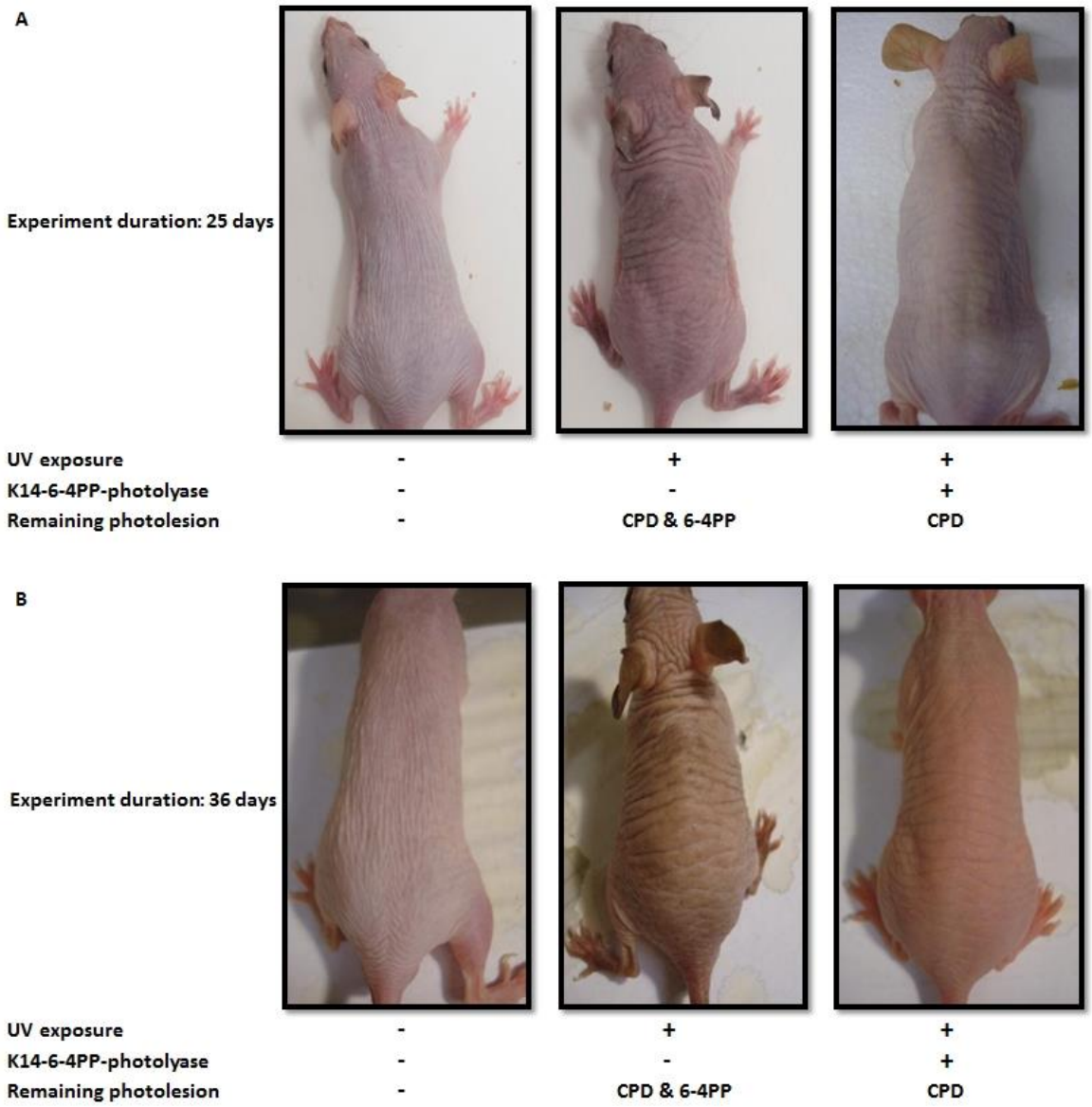
4.4.2.1 Macroscopic skin alterations

Twenty-four hours after the second UV exposure, erythema and edema were present in all irradiated animals, with similar intensities. 6-4PP removal from basal keratinocytes did not prevent skin redness and swelling. These responses gradually faded up to the 7th experimental day, when they were no longer present.

On the 5th day after the first UV exposure, macroscopic epidermal thickening could be observed in all UV irradiated animals. This response increased gradually until the end of the experiment (data not shown).

Figure 39 presents pictures of one animal from each CE4 and CE6 group, as a representation of the macroscopic alterations observed in each group on the last experimental day.

Figure 39 - Macroscopic alterations of K14-6-4PP-photolyase/XPA/HR mice after chronic UV irradiation



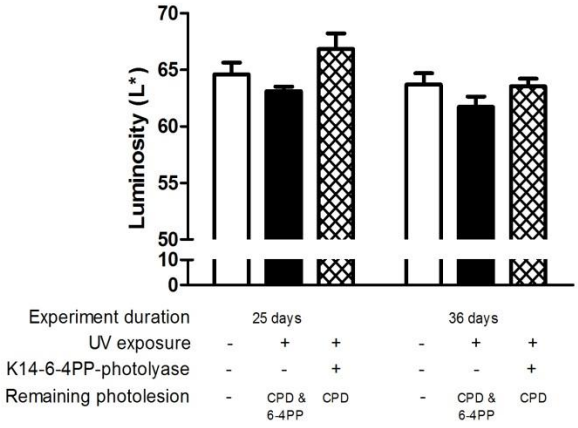
A) Chronic UV irradiation of XPA/HR animals induced epidermal thickening and skin pigmentation. 6-4PP removal from basal keratinocytes prevented pigment production but sustained skin thickening (CE4). B) Hyperplasia and skin pigmentation gradually increased over time, as can be observed in animals UV irradiated for 36 constitutive days (CE6).

4.4.2.2 Skin darkening

Skin luminosity quantification with the aid of a chromometer showed that chronic UV irradiation for 25 or 36 days did not change skin color (63.1 L* and 61.7 L*, respectively), in comparison with constitutive skin luminosity (64.6 L*, 63.7 L*, respectively). 6-4PP removal did not significantly alter skin luminosity in animals UV

irradiated for 25 or 36 days (59.6 L*, 55.7 L*, respectively), as presented in Figure 40.

Figure 40 - Chronic UV exposure and skin luminosity in K14-6-4PP-photolyase/XPA/HR mice

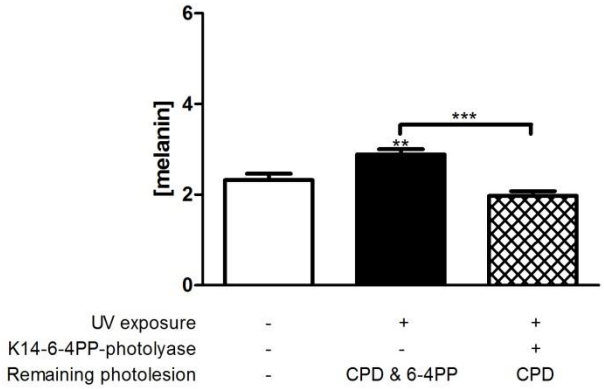


Chronic UV irradiation alone did not alter skin luminosity in XAP/HR mice. 6-4PP removal from basal keratinocytes did not reduce skin luminosity in animals UV irradiated for 25 or 36 days (Two-way Anova: p=0.0202).

4.4.2.3 Melanin Content

Analysis of the melanin concentration in the CE4 experiment showed that UV irradiation increased epidermal melanin content after 25 consecutive days of exposure (average value: 2.8), in comparison with constitutive pigment levels (average value: 2.3). 6-4PP removal from basal keratinocytes prevented UV-induced melanogenesis, sustaining low melanin levels even after UV irradiation (average value: 1.9). Epidermal melanin quantification is presented in Figure 41.

Figure 41 - Chronic UV exposure and epidermal melanin concentration in K14-6-4PP-photolyase/XPA/HR mice



Chronic UV irradiation for 25 consecutive days significantly increased epidermal melanin concentration in XPA/HR mice. 6-4PP removal from basal keratinocytes prevented UV-induced melanogenesis (One-way Anova: $p<0.0001$).

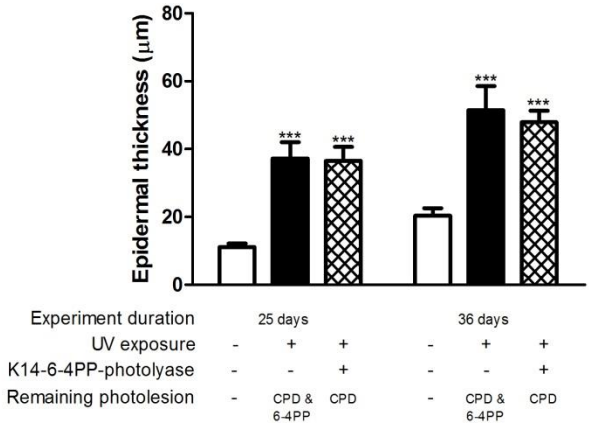
4.4.2.4 Skin hyperplasia and dysplasia

As shown in Figure 42, quantification of epidermal thickness average of the two Chronic Exposure experiments (CE4 and CE5) was performed to estimate UV-induced hyperplasia after 25 days of irradiation. Data analysis showed that UV induced a significant hyperplasia (37.2 μm), in comparison to normal epidermal thickness (11.1 μm). 6-4PP removal from basal keratinocytes did not alter UV-induced hyperplasia (36.4 μm).

Similarly, in animals chronically irradiated with UV for 36 days (CE6), UV induced a significant increase in epidermal thickness (51.4 μm), as compared to constitutive epidermal thickness (20.3 μm). 6-4PP removal from basal keratinocytes did not alter UV-induced hyperplasia (48.4 μm), as observed in Figure 42.

All groups from the CE6 experiment presented a slightly thicker epidermis when compared to animals from the CE4 and CE5 experiments. However, this increase was not statistically significant, as presented in Figure 42. Interestingly, dysplasia was also present in hyperplastic epidermis.

Figure 42 - Chronic UV exposure and epidermal hyperplasia in K14-6-4PP-photolyase/XPA/HR mice



Chronic UV exposure induced significant hyperplasia, which was not altered by 6-4PP removal from basal keratinocytes in XPA/HR mice (Two-way Anova: p=0.0014).

4.4.2.5 Cell proliferation

Quantification of epidermal proliferation average of the two chronic 25 day exposure experiments (CE4 and CE5) was performed to estimate UV-induced hyperplasia. This data was then compared with cell division levels after 36 consecutive days of UV exposure.

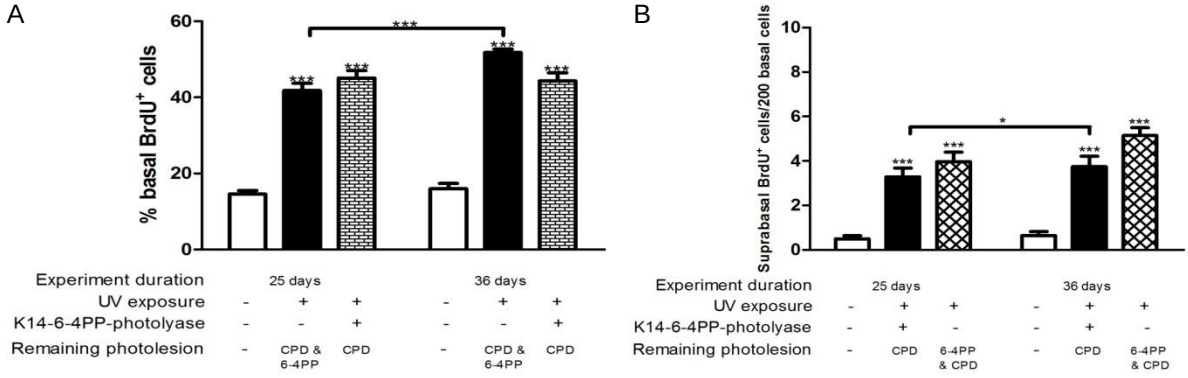
Chronic UV irradiation for 25 or 36 consecutive days increased cell proliferation in basal epidermis (41.8 % and 51.7 %, respectively), in comparison with normal basal cell proliferation (14.5 % and 15.9 %, respectively). This response was not altered by 6-4PP removal from basal keratinocytes neither in the 25 nor in the 36 irradiation day protocol (45.0 % and 44.3 %, respectively), as shown in Figure 43A.

Suprabasal constitutive cell proliferation at the end of the 25 or 36 day experiments (1 and 1.8 suprabasal $BrdU^+$ cells/100 basal cells, respectively) was also increased by chronic UV irradiation (6.5 and 9.7 suprabasal $BrdU^+$ cells/100 basal cells, respectively); 6-4PP removal from basal keratinocytes did not alter UV-induced suprabasal cell proliferation neither in the 25 nor in the 36 irradiation day protocol (7.9 and 10.2 suprabasal $BrdU^+$ cells/100 basal cells, respectively), as presented in Figure 43B.

All groups from the CE6 experiment presented a higher epidermal proliferation when compared with animals from the CE4 and CE5 experiments. However, this increase was not statistically significant, with exception of the UV irradiated control

group, where cell division was higher in animals UV exposed for 36 consecutive days then in mice exposed for 25 days, as presented in Figure 43.

Figure 43 - Chronic UV exposure and epidermal proliferation in K14-6-4PP-photolyase/XPA /HR mice



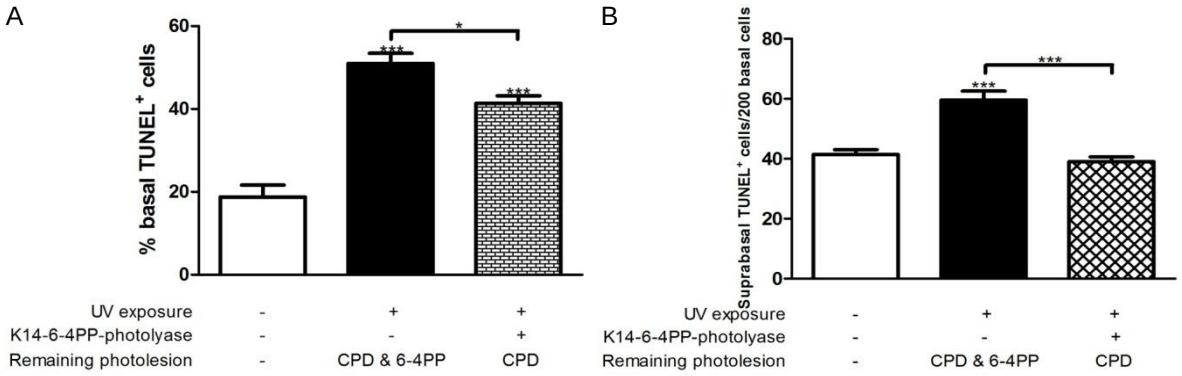
A) UV acute irradiation induced significant basal epidermal cell proliferation, which was not altered by 6-4PP removal from basal keratinocytes in XPA/HR mice (One-way Anova: $p<0.0001$). B) Suprabasal cell proliferation was also induced by UV exposure, which was not altered by 6-4PP removal from basal keratinocytes (One-way Anova: $p<0.0001$).

4.4.2.6 Cell death

TUNEL quantification showed that chronic UV irradiation for 25 consecutive days increased cell death in basal epidermis (50.9 %), in comparison with normal basal apoptosis (18.7 %). 6-4PP removal from basal keratinocytes was associated with intermediate levels of basal apoptosis (41.3 %), as shown in Figure 44A.

Suprabasal constitutive apoptosis (82.8 suprabasal TUNEL$^+$ cells/100 basal cells) was also increased by chronic UV irradiation for 25 consecutive days (119.0 suprabasal TUNEL$^+$ cells/100 basal cells). 6-4PP removal from basal keratinocytes prevented UV-induced suprabasal cell death (78.0 suprabasal TUNEL$^+$ cells/100 basal cells), as presented in Figure 44B.

Figure 44 - Chronic UV exposure and epidermal apoptosis in K14-6-4PP-photolyase/XPA /HR mice

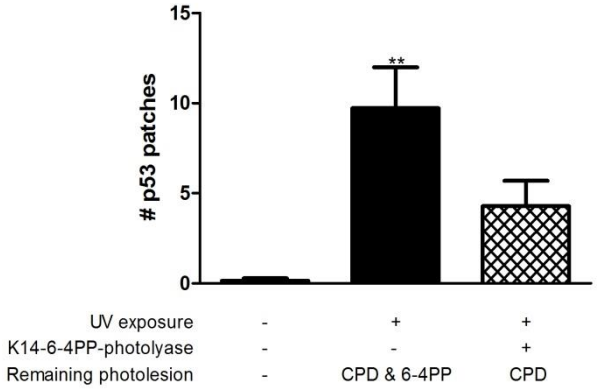


A) Acute UV irradiation induced significant basal epidermal cell death, which was partially prevented by 6-4PP removal from basal keratinocytes in XPA/HR mice (One-way Anova: $p<0.0001$). B) Suprabasal apoptosis was also induced by UV exposure, which was prevented by 6-4PP removal from basal keratinocytes (One-way Anova: $p<0.0001$).

4.4.2.7 p53 overexpression

P53 overexpression quantification showed that chronic UV irradiation significantly increased the number of p53 epidermal patches (9.7 patches/sheet) as compared with constitutive p53 patch levels (0.1 patches/sheet). Data is inconclusive about the role played by 6-4PP removal from basal keratinocytes after chronic UV exposure of XPA/HR mice since this group of animals does not statistically differ from either of the control groups. However, these mice present intermediate p53 patch levels in comparison to non UV irradiated and UV irradiated control groups, suggesting that this photolesion removal may be slightly reducing patch formation (4.2 patches/sheet), as presented in Figure 45.

Figure 45 - Chronic UV exposure and epidermal p53 overexpression in K14-6-4PP-photolyase/XPA /HR mice



Chronic UV irradiation of XPA/HR mice induces epidermal p53 overexpression, which seems to be slightly reduced when 6-4PP is removed from basal keratinocytes (Two-way Anova: p=0.0014).

4.5 CPD and 6-4PP removal from basal keratinocytes in a CSA background

The experiment described in the present section (CE7) employed CSA mice transgenically expressing a CPD-photolyase or a 6-4PP-photolyase under the control of a K14 promoter. Therefore, photolesions were specifically removed from basal keratinocytes during photoreactivation. In addition to the test group, the experiment included UV irradiated and non UV irradiated control groups. Animals were anesthetized and plucked the day prior to the first UV exposure. During the experiment, animals were shaven as necessary.

4.5.1 Chronic UV exposure

In the experiment Chronic Exposure 7(CE7), mice were UV irradiated with increasing UV doses up to 1 MED, followed by 3 h of photoreactivation after each UV exposure, for 25 consecutive days. Table 11 summarizes CE7 animal groups.

Table 11 - Chronic Experiment 7 (CE7) experimental groups

Experiment	*Group*	*Mice genotype*	*# animals*	*UV dose*	*Experiment duration*	*Remaining photolesions in the genome*
CE7	A	K14-CPD-photlyase/CSA and K14-6-4PP-photolyase/CSA and CSA	6	0 MED	25 days	Ø
	B	K14-CPD-photlyase/CSA	5	1 MED		6-4PP
	C	K14-6-4PP-photlyase/CSA	3	1 MED		CPD
	D	CSA	6	1 MED		6-4PP & CPD

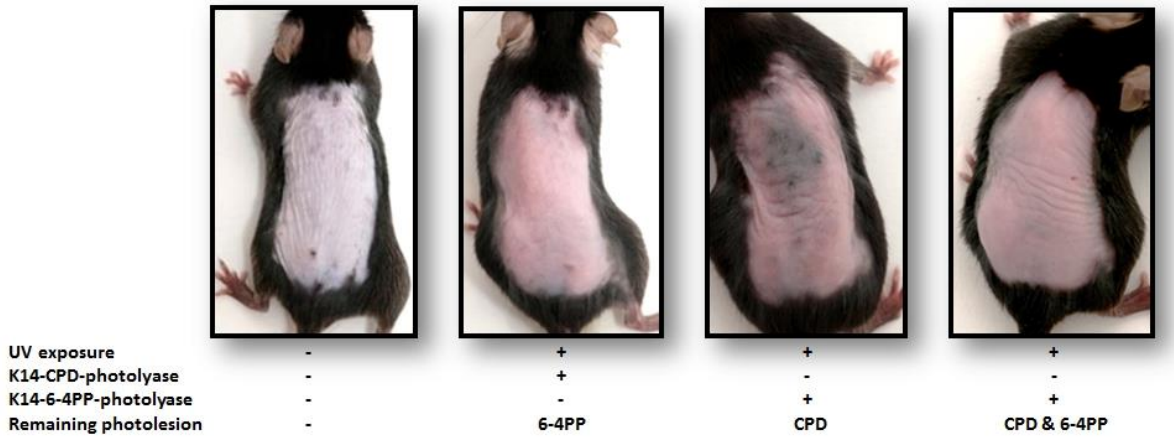
4.5.1.1 Macroscopic skin alterations

Twenty-four hours after the second UV exposure, moderate erythema and edema were present in all UV irradiated animals, with similar intensities. These responses slowly decreased until the 5th day after exposure, but were still present at the end of the experiment in all UV irradiated groups. Non UV irradiated control animals never presented skin swelling or redness.

On the 5th day after the first UV exposure, macroscopic epidermal thickening could be observed. UV irradiated control animals, and animals where only CPD lesions persisted in the genome, presented a similar intense epidermal thickness. Animals with only 6-4PP lesions persisting in the genome presented lesser epidermal thickening. From the 5th day until the end of the experiment these responses were sustained in animals UV irradiated with 1 MED and where CPD lesions were removed from basal keratinocytes, while they intensified in all the other UV exposed groups. Non UV irradiated control animals never presented epidermal thickening (data not shown).

Figure 46 presents pictures of one animal from each CE7 group, as a representation of the macroscopic alterations observed in all groups on the last experimental day.

Figure 46 - Chronic UV irradiation and macroscopic alterations in K14-CPD-photolyase/CSA and K14-6-4PP-photolyase/CSA mice

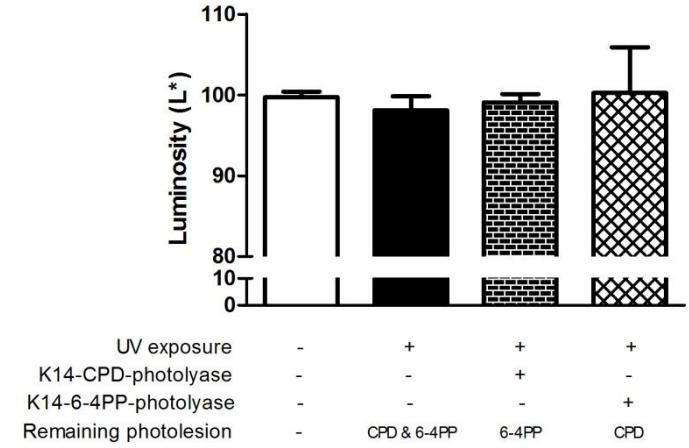


Chronic irradiation of CSA mice for 25 consecutive days with increasing UV doses up to 1 MED induced epidermal thickening and erythema which were sustained even when 6-4PPs were removed from basal keratinocytes. CPD removal from basal keratinocytes seemed capable of reducing epidermal thickening and erythema induction. Note: the dark areas observed constitute new fur growing, not skin pigmentation.

4.5.1.2 Skin darkening

Macroscopic alterations in skin pigmentation could not be observed. As shown in Figure 47, skin color analysis with the aid of a chromometer showed no differences in epidermal luminosity (L*) between groups, with values ranging from 98.10 to 100.25 %.

Figure 47 - Chronic UV exposure and skin luminosity in K14-CPD-photolyase/CSA and k14-6-4PP-photolyase/CSA mice

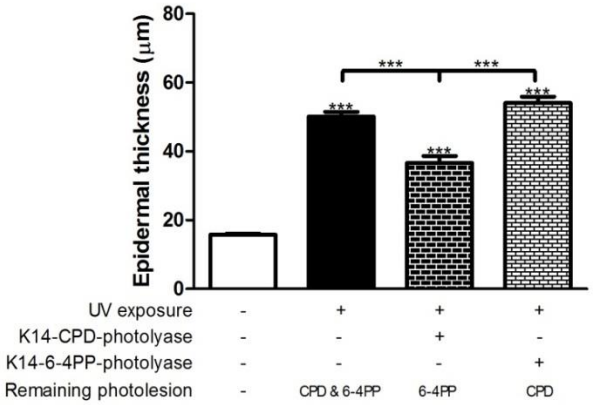


Skin color was measured with the aid of a chromometer. Chronic irradiation with increasing UV doses up to 1 MED was not capable of altering the skin luminosity (L*) of CSA animals (One-way Anova: p=0.2245).

4.5.1.3 Skin hyperplasia and dysplasia

Microscopic analyses of epidermal hyperplasia showed that UV irradiation caused a significant epidermal hyperplasia (50.1 µm), more then threefold the thickness of the normal skin (15.7 µm). CPD removal from basal keratinocytes was associated with an intermediate skin hyperplasia (36.6 µm), significantly different from both control groups. However, 6-4PP removal from basal keratinocytes did not alter the UV-induced epidermal thickening (54.1 µm), as shown in Figure 48. Dysplasia always accompanied hyperplasia in the epidermis.

Figure 48 - Chronic UV exposure and epidermal hyperplasia in K14-CPD-photolyase/CSA and K14-6-4-PP-photolyase/CSA mice



Chronic UV irradiation induced epidermal hyperplasia, which was partially prevented only by CPD removal from basal keratinocytes in CSA mice (One-way Anova: $p<0.0001$).

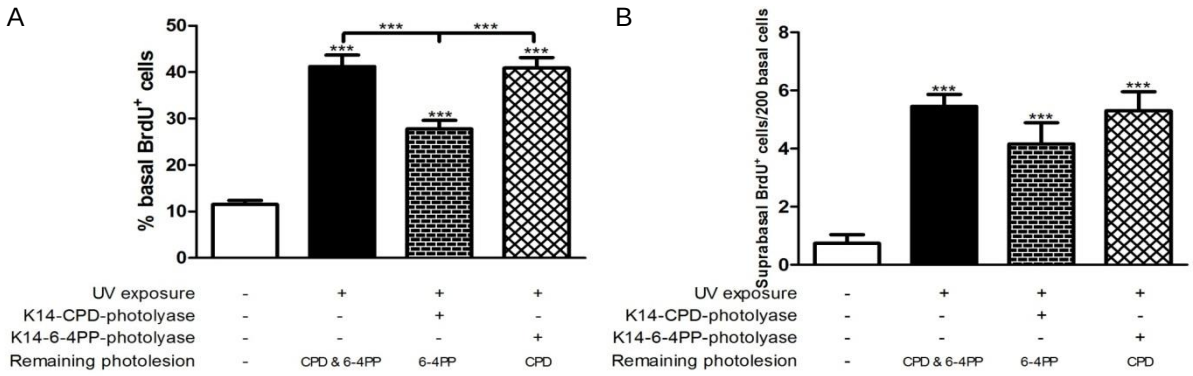
4.5.1.4 Cell proliferation

Quantification of proliferative cells in the basal epidermal layer showed that UV irradiation of CSA animals significantly increased the levels of cells in S-phase (41.2 %), in comparison with constitutive basal cell proliferation (11.5 %). CPD removal from basal keratinocytes was associated with intermediate basal proliferation levels (27.7 %), significantly different from both control groups. 6-4PP removal did not alter UV-induced basal cell proliferation (40.8 %), as shown in Figure 49A.

Quantification of BrdU positive cells on the suprabasal epidermal layer showed that UV irradiation of CSA animals significantly increased the levels of cell proliferation (10.9 suprabasal $BrdU^+$ cells/100 basal cells), in reference to normal suprabasal cell division levels (1.47 suprabasal $BrdU^+$ cells/100 basal cells). Neither CPD nor 6-4PP removal from basal keratinocytes significantly altered UV-induced

cell division in this cell layer (8.3 and 10.6 suprabasal $BrdU^+$ cells/100 basal cells, respectively), as presented in Figure 49B.

Figure 49 - Chronic UV exposure and epidermal proliferation in K14-CPD-photolyase/CSA and K14-6-4-PP-photolyase/CSA mice



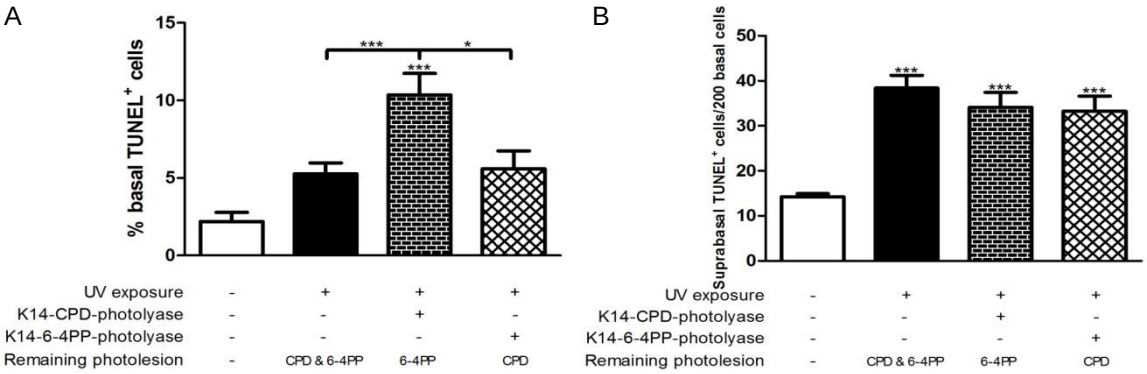
A) Chronic UV irradiation induced significant cell proliferation in basal epidermis, which was partially reduced by CPD removal from basal keratinocytes in CSA mice (One-way Anova: $p<0.0001$). B) Chronic UV induced suprabasal cell proliferation, which was not prevented by photolesion removal from basal keratinocytes (One-way Anova: $p<0.0001$).

4.5.1.5 Cell death

Quantification of TUNEL positive cells in the basal epidermis showed that UV irradiation of CSA animals did not significantly increase the levels of apoptosis (5.2 %), in comparison with constitutive basal cell death (2.1 %). Surprisingly, CPD removal from basal keratinocytes increased apoptosis in this specific epidermal layer (10.3 %). However, 6-4PP removal from basal keratinocytes did not significantly alter cell death rate (5.5 %), as presented in Figure 50A.

Suprabasal cell death quantification showed that UV irradiation significantly increased apoptosis (76.7 suprabasal $TUNEL^+$ cells/100 basal cells) with reference to normal apoptotic levels (28.4 suprabasal $TUNEL^+$ cells/100 basal cells). The UV-induced increase in suprabasal apoptosis was not altered either by 6-4PP removal (66.5 suprabasal $TUNEL^+$ cells/100 basal cells) or by CPD removal (68.2 suprabasal $TUNEL^+$ cells/100 basal cells) from basal keratinocytes, as presented in Figure 50B.

Figure 50 - Chronic UV exposure and epidermal apoptosis in K14-CPD-photolyase/CSA and K14-6-4PP-photolyase/CSA mice



A) Chronic UV exposure did not significantly increase basal cell death, even when 6-4PPs were removed from basal keratinocytes. CPD removal from basal keratinocytes induced basal cell death after chronic UV exposure in CSA mice (One-way Anova: $p<0.0001$). B) Suprabasal cell death was induced by UV irradiation, which was not altered by photolesion removal from basal keratinocytes (One-way Anova: $p<0.0001$).

4.6 Summary of results from all experiments

Ubiquitous CPD removal from XPA mice partially prevented acute hyperplasia despite sustained intermediate levels of UV-induced basal proliferation. After chronic UV exposure, XPA animals presented increased melanogenesis, which was further enhanced by CPD removal. Hyperplasia and p53 patch induction were prevented by CPD removal from the whole epidermis in XPA animals, which probably correlates with reduced levels of UV-induced cell division and sustained UV-induced cell death.

6-4PP removal from basal keratinocytes in XPA mice after acute exposure reduced UV-induced hyperplasia, which probably correlates to reduced levels of UV-induced basal cell proliferation. Interestingly, 6-4PP removal from basal keratinocytes after chronic UV exposure of XPA mice did not prevent hyperplasia nor p53 patch induction but prevented UV-induced melanogenesis, which probably correlates to high levels of cell proliferation, and slightly reduced levels of UV-induced apoptosis.

In CSA animals, CPD removal from basal keratinocytes partially prevented UV-induced chronic hyperplasia. 6-4PP removal from basal keratinocytes did not alter UV-induced cell and tissue responses. Basal apoptosis was not increased by chronic UV exposure alone, only in combination with CPD-photolyase expression. UV exposure alone, or in combination with photolesion removal, did not induce melanogenesis in CSA mice.

Table 12 summarizes all the data obtained in the present work.

Table 12 - Summary of all results

Background	*Fur*	*UV*	*Photolyase*	*Persisting lesion*	*Macroscopic observations*	*Melanin*	*Hyperplasia*	*Cell proliferation*	*Apoptosis*	*p53 patches*
XPA	No	No	βACT-CPD or K14-64-PP	Ø	Erythema & edema: no Hyperplasia & Wrinkles: no Pigmentation: no	no	no	B•: low SB▲: low	B: low SB: low	no
		Acute (1 MED)	No	CPD & 6-4PP*	Erythema & edema: medium Hyperplasia & Wrinkles: high Pigmentation: no		high	B: high SB: high	B: high SB: high	
			βACT-CPD	6-4PP*	Erythema & edema: no Hyperplasia & Wrinkles: no Pigmentation: no		medium	B: very high SB: medium	B: high SB: high	
			K14-64-PP	CPD‡ + CPD & 6-4PPˣ	Erythema & edema: meduim Hyperplasia & Wrinkles: high Pigmentation: no		high	B: high SB: high	B: high SB: medium	
		Chronic (1 MED)	No	CPD & 6-4PP*	Erythema & edema: medium Hyperplasia & Wrinkles: high Pigmentation: low	medium	high	B: high SB: high	B: high SB: high	high
			βACT-CPD	6-4PP*	Erythema & edema: no Hyperplasia & Wrinkles: no Pigmentation: high	high	no	B: medium SB: medium	B: high SB: medium	low
			K14-64-PP	CPD‡ + CPD & 6-4PPˣ	Erythema & edema: medium Hyperplasia & Wrinkles: high Pigmentation: low	no	high	B: high SB: high	B: high SB: medium	high
CSA	Yes	No	K14-CPD or K14-64-PP	Ø	Erythema & edema: low Hyperplasia & Wrinkles: low Pigmentation: no		low	B: low SB: low	B: low SB: low	
		Chronic (1 MED)	No	CPD & 6-4PP*	Erythema & edema: high Hyperplasia & Wrinkles: high Pigmentation: no		high	B: high SB: high	B: high SB: high	
			K14-CPD	6-4PP‡ + CPD & 6-4PPˣ	Erythema & edema: medium Hyperplasia & Wrinkles: medium Pigmentation: no		medium	B: medium SB: high	B: high SB: high	
			K14-64-PP	CPD‡ + CPD & 6-4PPˣ	Erythema & edema: high Hyperplasia & Wrinkles: high Pigmentation: no		high	B: high SB: high	B: high SB: high	

* total epidermis; ‡ basal keratiniocytes; ˣ other epidermal cells; • B = basal; ▲ SB = suprabasal. Obs: Chronic refers to the group of all Chronic Exposure Experiment, (25 and 36 days of exposure).

5 DISCUSSION

Previous investigations successfully demonstrated that transgenically expressed photolyases were capable of virtually removing all photolesions from mouse epithelial tissue, in a specific manner: CPD-photolyase only removed CPDs while 6-4PP-photolyase exclusively removed 6-4PPs. It was also demonstrated that when photolyases were under the control of the βACT promoter, they removed the specific photolesion ubiquitously from all epithelial cells, whereas when under the control of the K14 promoter, only photolesions from basal and recently-differentiated keratinocytes were removed (209,249,250).

These works from van der Horst´s group also showed that ubiquitous CPD removal in DNA repair-proficient mice prevented acute and chronic UV responses: erythema, edema, hyperplasia, cell death, immunosuppression, RRS, mutagenesis, p53 patches and carcinogenesis (249,250). Furthermore, CPD removal exclusively from basal keratinocytes avoided UV-induced apoptosis and partially reduced hyperplasia and cancer induction (209,249).

Interestingly, in a DNA repair-proficient background, ubiquitous removal of 6-4PPs did not prevent hyperplasia, apoptosis, RRS, mutagenesis, p53 patches or carcinogenesis (250). The same skin responses were observed when 6-4PPs were removed exclusively from basal keratinocytes (209). This set of data suggested that CPDs play a major role in acute and chronic epithelial responses to UV irradiation.

Furthermore, experiments with narrow-band UVB (311 nm) and broad-band UVB (275-390 nm, with a peak at 313 nm), showed that the former produced a higher CPD/6-4PP ratio, which correlated with a higher cancer incidence. Moreover, deficiencies in 8-oxo-G DNA glycosylase (OGG1) did not alter the carcinogenic potential of each UV spectrum. Therefore, in agreement with the results obtained by van der Horst´s group, these results suggest that, in a NER-proficient background, CPDs play a major role in carcinogenesis (261).

However, these experiments were performed using DNA repair-proficient animals, which may have masked the role played by 6-4PP in these skin responses, because of their quick removal by the NER pathway (262).

Furthermore, an *in vitro* research revealed that, in XPA cells, the removal of both photolesions reduced cell death (251). Later, a more detailed study using adenoviral vectors for the delivery of photolyase transgenes in fibroblasts, showed

that removal of CPDs and 6-4PPs was able to reduce UV-induced apoptosis in XPA, XPD and XPG cells. However, in WT, XPV and CSA cell lines, only CPD removal was capable of reducing UV-induced cell death. These results indicated that, depending on the DNA repair status of the cell, only CPD or both CPD and 6-4PP may play important roles in cell responses to UV irradiation (122).

The fact that deficiencies in each NER subpathway may lead to different cell responses has been known for a long time due to the observation of the different phenotypes presented by CS and XP patients, reviewed in (263). Furthermore, previous work from de Gruijl´s group has demonstrated, using XPC and CSB hairless albino mice, that proficient GG-NER reduces carcinoma induction, whereas a functional TC-NER confers resistance to UV-induced erythema and edema (264).

To improve the comprehension of the preventive roles of each NER subpathway against different skin responses to UV irradiation, it is important to explore the specific roles of each UV-induced photolesion on local epidermal responses to acute and chronic UV exposures, either when persistent only in the transcribed strand of active genes (CSA mice), or when present in the whole genome (XPA mice).

The present study evaluated the specific roles played by each photolesion, CPDs and 6-4PPs, in epithelial responses to acute and chronic UV irradiation. DNA repair-deficient XPA and CSA mice, transgenically expressing either CPD-photolyase or 6-4PP-photolyase, were exposed to UV light for 1, 25 or 36 consecutive days, followed by lesion removal by the specific photolyase during photoreactivation. The occurrence of erythema, edema, hyperplasia, pigmentation, cell proliferation, apoptosis and p53 overexpression in the epithelium was analyzed.

Similar but not precisely equal results were obtained when CPD photolyase was expressed ubiquitously or exclusively from basal keratinocytes in previous studies (209,249,250). Therefore, in the present work, caution has to be taken when comparing the results obtained with animals from the βACT-CPD-photolyase/XPA/HR group and the other experimental groups. Furthermore, CSA animals were furred while XPA animals were hairless. Therefore, no direct correlation can be made between these groups either.

5.1 CPD removal in XPA/HR mice

5.1.1 Acute UV exposure

Present data shows that acute UV irradiation induced significant hyperplasia and dysplasia, which were partially prevented by CPD removal. CPD removal increased basal cell proliferation but reduced suprabasal cell proliferation, which is probably related to a reduced epidermal thickness.

Suprabasal cell proliferation is not observed in normal epidermis. However, it has been previously detected 48 hours (not earlier) after acute UV exposure (265). In the present study, acute UV exposure induced suprabasal cell proliferation, which may indicate a transient loss of tissue homeostasis after acute irradiation, and an accelerated epidermal turnover, resulting in the observation of dividing cells out of the basal epidermal layer. CPD removal partially prevented UV-induced hyperplasia and suprabasal cell proliferation, indicating that CPDs may play a role in the transient epidermal loss of homeostasis.

Apoptosis is expected throughout the entire epidermis, in all animals, with higher levels being observed in the suprabasal layer, due to normal tissue turnover, which normally takes 8 days in mice (188). Of note, in all groups, apoptosis is much higher than proliferation levels, which is caused by an accumulation of dying cells as keratinocytes differentiate and enter apoptosis during cell migration towards the external epidermal layer (*stratum corneum*). Acute UV irradiation increased the normal levels of basal and suprabasal apoptosis. These responses were not avoided by ubiquitous CPD removal.

Previous studies showed an increase in apoptosis starting 6 hours after UV acute exposure. Enhanced cell proliferation with consequent hyperplasia started in 2 hours and increased up to 48 hours after irradiation (265,266).

Present data indicates that acute UV-induced hyperplasia is a direct result of enhanced epidermal proliferation when the apoptosis increase is not sufficient to prevent epidermal thickening. Furthermore, CPD removal was sufficient to prevent ~56 % of the UV-induced suprabasal cell division, which resulted in a ~15 % hyperplasia reduction, indicating that although suprabasal cell proliferation contributes to hyperplasia occurrence, basal cell division also has an important role

in this skin response. These results demonstrate that CPDs play a very important role in acute responses to UV irradiation in DNA repair-deficient mice.

Previous studies with DNA repair-proficient mice exposed to a similar acute UV exposure protocol, showed an increase in hyperplasia after irradiation, which was totally prevented by CPD removal. An increase in total cell death was also observed after UV irradiation, which was totally prevented by CPD removal (209,249). These results demonstrated a major role of CPDs in skin responses to acute UV exposure. Nevertheless, it should be noted that in these experiments no photolesions remained in the genome: CPDs were removed by CPD-photolyase, and 6-4PPs were quickly removed by NER.

In the present experiments 6-4PP lesions remained in the genome, since NER is completely absent in XPA mice. This difference in photolesion persistence explains the small discrepancies between the two experiments and indicates that 6-4PP may also play an important role in acute skin responses to UV irradiation, since its persistence was enough to sustain partial hyperplasia, cell proliferation and apoptosis.

5.1.2 Chronic UV exposure

Macroscopic observations demonstrated that ubiquitous CPD removal prevented wrinkle formation, epidermal thickening, dysplasia and general skin photoaging. Hyperpigmentation was visible in all animals where 6-4PP lesions were present. Curiously, skin darkening was more intense in animals where CPD lesions were removed by CPD-photolyase.

Analysis of skin luminosity showed that the observed skin darkening increase was statistically significant only in the group of animals where CPD photolesions were removed. A slightly lower luminosity was observed in animals where both photolesions were present, but this was not statistically significant.

Confirming the skin luminosity assessment, Fontana-Masson staining showed higher melanin levels in animals UV irradiated for 25 consecutive days and where CPDs were ubiquitously removed by CPD-photolyase. Interestingly, this more sensitive and direct measurement of melanin skin content demonstrated that total epidermal pigment levels were also significantly higher in animals UV irradiated and with no photolesion removal, confirming macroscopic observations. Melanin granules

were found throughout the entire epidermis, although in a higher concentration in the basal layer. This type of epidermal melanin dispersion indicates that the observed pigment must be eumelanin, localized in heavily pigmented melanosomes, which are resistant to degradation by lysosomal enzymes, reviewed in (267).

These findings not only indicate that the dark pigmentation is induced by the presence of the 6-4PPs, but also that CPD removal intensifies this response, suggesting that CPD removal may be leading to the activation of a different melanogenesis pathway.

Microscopic epithelial analysis showed significant epidermal thickening and dysplasia in the irradiated control group. No significant hyperplasia was present in animals where CPDs were removed, which is also consistent with the general macroscopic observation of these animals.

To evaluate cell proliferation status of the epidermis after prolonged chronic UV exposure and a possible correlation with the observed hyperplasia, BrdU incorporation in the epidermis was analyzed. Quantification of BrdU positive cells in basal and suprabasal epidermis showed that UV irradiation increased the number of proliferative cells in both layers, which was only partially avoided by CPD removal. Therefore, the increase in the number of total dividing cells does not directly correlate with hyperplasia induction.

The apparent lack of correlation between the number of total dividing cells in the epidermis and hyperplasia occurrence could be due to an increase in epidermal cell death in the group of mice with no hyperplasia. It has been shown that, shortly after low UVB doses, there is a clear induction of apoptotic cells, which peaks around 6-10 hours after exposure (193,265,266). Furthermore, it is well known that the hyperplastic response is the result of the combination of two cell responses: death of previously existing keratinocytes and cell proliferation (192).

The number of epidermal apoptotic cells was quantified with a TUNEL assay. UV irradiation induced basal cell apoptosis, which was not prevented by CPD removal. Interestingly, in the suprabasal layer, UV-induced cell death was totally prevented by CPD removal, which may correlate with a reduction of total epidermal thickness.

A closer look at specific epidermal layer responses showed that CPD removal partially prevented cell division in the basal layer while it did not avoid basal cell death. Therefore, in the basal epidermis, after CPD removal, the number of dying

cells must have been similar to the number of proliferative cells, preventing hyperplasia induction. These results demonstrate that after chronic UV exposure, the basal epidermis is the main factor responsible for hyperplasia induction. Furthermore, it has been previously described that epidermal hyperplasia correlates with cell proliferation within the basal epidermal layer (192).

P53 is a transcription factor with a central role in controlling cell cycle, apoptosis, cellular metabolism, autophagy, cell proliferation, pigmentation and aging (227,268). Following UV skin irradiation, p53 is activated by ATM/ATR signaling after DNA damaged is recognized, peaking 12 hours after exposure. This transcription factor can then lead to a stall in cell cycle progression, followed by the recruitment of DNA repair proteins. Alternatively, if damages are too severe, p53 may initiate apoptosis in a p21/BAX/BCL-2 dependent manner (192,193).

Correct p53 functioning is essential in preventing UV-induced skin tumorigenesis, which has been demonstrated by increased cancer incidence in mice with the loss of a p53 allele (269), and by enhanced mutagenesis in the absence of p53 after UV exposure (192). Furthermore, mutations in p53 are observed in at least 50 % of all human cancers and in most skin cancers (270,271).

Interestingly, p53 deficiency delayed UV-induced apoptosis by 12 hours, and hyperplasia induction and CPD removal in the epidermis by 24 hours. These results suggest that p53 exerts a stimulatory effect in epidermal responses to UV irradiation, but is not essential (192).

Even though there is an increase in p53 expression in individual epidermal cells after UV exposure as a normal damage response (193), p53 overexpression in clusters of cells represent the clonal expansion of a cell, which sustains p53 overexpression.

A positive correlation has been demonstrated between p53 overexpression in groups of basal epidermal cells (p53 patches) and tumor incidence in mice after UV exposure (258,260). Even though this correlation is relatively low (8300-40000 p53 patches:1 tumor, depending on mouse lineage), both p53 patches and tumors have UV-signature mutations (i.e. C→T and CC→TT transitions) (271,272). Furthermore, experiments with several mouse lineages with different DNA repair deficiencies, have shown that the order in which NER-deficient mice develop patches is predictive of the order in which they develop tumors (254). Moreover, epidermal ablation with UVB overexposure ensures p53 patch elimination and reset of the carcinogenesis

process, delaying SCC onset by a month (273). Therefore, p53 epidermal patches are good indicators of the beginning of a tumorigenic process, especially for SCC in mice.

Present data shows, unequivocally, that chronic UV irradiation induced a significant number of p53 patches when compared to the non UV irradiated control group. CPD removal prevented the induction of p53 overexpression in cell clusters, through the combination of reduced cell proliferation and increased pigmentation, ensuring the prevention of the beginning of a tumorigenic process.

No significant difference was observed in pigmentation, hyperplasia, dysplasia or cell proliferation between animals UV exposed for 25 and 36 consecutive days, in animals where CPD lesions were removed by CPD-photolyase. On the other hand, a significant increase in the number of proliferative cells was observed in UV irradiated control animals irradiated for 36 consecutive days, in comparison to those exposed for 25 days. However, this enhanced cell proliferation was not sufficient to significantly increase hyperplasia, although a slightly thicker epidermis was observed in these mice, indicating a cumulative UV effect.

Data shows that in a XPA background, where both TC-NER and GG-NER are absent, the persistence of 6-4PP lesions was not sufficient to induce hyperplasia and p53 patches. These results indicate that CPD is a major player in the development of deleterious skin responses after chronic low UV doses *in vivo*, especially through the induction of cell proliferation. However, 6-4PP persistence sustained partial cell proliferation and apoptosis, indicating that CPDs are not the only factor responsible for all skin responses to chronic UV exposure.

The present work shows, for the first time, that 6-4PPs play an important role in the induction of skin pigmentation, through an increase in melanogenesis. Interestingly, this skin response is enhanced by CPD removal. Although this seems to be a protective (and thus potentially benign) effect, the induction of this pigmentation raises the question of how this can be related to menalocytic proliferation and, eventually, to melanoma.

5.2 6-4PP removal in XPA/HR mice

5.2.1 Acute UV exposure

Acute UV exposure induced significant hyperplasia and dysplasia, which were partially prevented by 6-4PP removal from basal keratinocytes. UV irradiation induced basal and suprabasal epidermal proliferation. Basal proliferation was reduced by 6-4PP removal from basal keratinocytes.

Basal and suprabasal epidermal apoptosis were induced by acute UV exposure, which were not prevented by 6-4PP removal from basal keratinocytes.

Therefore, reduced proliferation levels associated with sustained apoptosis ensured intermediate hyperplasia induced by acute UV irradiation followed by 6-4PP removal from basal keratinocytes.

Interestingly, previous *in vivo* studies with DNA repair-proficient mice did not show alterations in skin responses to acute UV exposure after either ubiquitous or specific 6-4PP removal from basal keratinocytes (209,250). However, the fast removal of 6-4PPs by NER in these animals may have masked the roles played by this type of photodamage in skin responses to UV light (262).

5.2.2 Chronic UV exposure

Macroscopic observation of mice chronically exposed to UV light showed a significant epidermal thickening, wrinkle formation and general skin photoaging, which were not prevented by 6-4PP removal from basal keratinocytes. Skin luminosity quantification of animals chronically exposed to UV light demonstrated that 6-4PP removal from basal keratinocytes did not alter skin color.

As previously discussed, Fontana-Masson staining confirmed that total epidermal pigment content was significantly higher in animals UV irradiated and with no photolesion removal. Interestingly, 6-4PP removal from basal keratinocytes was capable of preventing UV-induced melanogenesis, suggesting that the presence of 6-4PP lesions is necessary to the activation of a UV-induced melanogenesis pathway.

Unlike what was observed after acute UV exposure, microscopic evaluation of epidermal thickness demonstrated that chronic UV irradiation induced significant hyperplasia, which was not prevented by 6-4PP removal from basal keratinocytes.

Epidermal proliferation was quantified aiming towards a better understanding of the hyperplastic response to chronic UV exposure after 6-4PP removal. Chronic UV exposure induced significant cell proliferation in basal and suprabasal epidermis, which were not prevented by 6-4PP removal from basal keratinocytes.

Chronic UV irradiation also increased basal and suprabasal epidermal apoptosis, which were partially prevented by 6-4PP removal from basal keratinocytes. Previous *in vitro* works had already suggested an important role for 6-4PP lesions in the induction of apoptosis in XPA null cells (122,251).

These results indicate that while 6-4PPs do not seem to have an important role in cell proliferation, these lesions may play a significant role in apoptosis induction. However, the reduction observed in apoptosis induction was not sufficient to further increase hyperplasia, which is sustained by high cell division rates.

Furthermore, 6-4PP removal from basal keratinocytes did not significantly prevent p53 overexpression in clusters of epidermal cells (although it may have reduced it), indicating that 6-4PPs may not play an important role in the beginning of the tumorigenenic process.

Interestingly, no difference was observed in, hyperplasia, dysplasia or cell proliferation in animals UV exposed for 25 or 36 consecutive days and where 6-4PP lesions were removed by 6-4PP-photolyase in basal keratinocytes. As previously discussed, a significant increase in the number of proliferative cells was observed in 36 day UV irradiated control animals, in comparison to those UV exposed for 25 consecutive days. Interestingly, 6-4PP removal from basal keratinocytes was capable of preventing this increase in cell proliferation after prolonged UV exposure.

5.3 CPD and 6-4PP removal in CSA mice

5.3.1 Chronic UV exposure

Macroscopically, chronic UV irradiation of CSA animals induced low levels of edema and erythema throughout the whole experiment. Hyperplasia was also observed in all UV exposed groups. CPD removal from basal keratinocytes in animals exposed to 1MED of UV light seemed to reduce all of these skin responses. No skin pigmentation was macroscopically observed, which was confirmed by skin luminosity quantification.

It is well known that furry adult mice only present melanocytes in their hair bulbs. Interfolicular melanocytes are exclusively found in the early weeks after birth, with the exception of glaborous skin, such as the ear, nose, foot and tail, which present these pigment-producing cells spread throughout the epidermis even in adulthood, reviewed in (274). The lack of melanocytes in the shaved dorsum of the animals may help explain why photolesion removal did not alter melanogenesis in these mice. However, no increase in pigmentation was observed in the areas with glaborous skin, suggesting that melanogenesis either requires ubiquitous photolesion removal, or that photolesion removal by GG-NER is sufficient to suppress pigment production.

Epidermal thickness quantification showed that chronic UV irradiation of CSA mice induced significant hyperplasia, which was partially prevented by CPD removal from basal keratinocytes. 6-4PP removal from basal keratinocytes did not prevent UV-induced epidermal thickening.

UV exposure increased basal and suprabasal cell proliferation. Basal cell division was partially prevented by CPD removal from basal keratinocytes. 6-4PP removal was not capable of preventing UV-induced cell proliferation.

Interestingly, chronic UV irradiation did not significantly increase basal apoptotic levels, which must be prevented by photolesion removal by GG-NER, indicating that lesions persisting in the transcribed strands of active genes are not a sufficient signal for this pathway. Curiously, CPD removal from basal keratinocytes induced basal apoptosis. All irradiated groups presented significantly higher levels of suprabasal cell death.

Therefore, it can be concluded that partial reduction in basal cell proliferation associated with elevated basal cell death is responsible for partial hyperplasia prevention in CSA mice UV irradiated with 1 MED and with only 6-4PPs persisting in their keratinocytes. Sustained cell proliferation associated with no significant increase in basal apoptosis is responsible for ensuring hyperplasia in UV irradiated control animals and in mice with 6-4PP removal from basal keratinocytes.

Constitutive epidermal thickness of CSA furred animals is almost two fold that of XPA, hairless mice, despite similar proliferation rates. Interestingly the apoptotic rates present in each and every epidermal layer of CSA mice are lower than that of XPA mice, probably accounting for the observed difference in the constitutive epidermal thickness.

5.4 General considerations

The animals used in the present study, despite their NER deficiencies, are proficient in TLS mechanisms of both CPDs (by POL η) and 6-4PPs (POL ζ and possibly POL κ and POL ι). TLS may help cells cope with the presence of photolesions, but they do not remove lesions from the DNA. Therefore, the total amount of DNA damage is not altered. However, it cannot be discarded that these mechanisms and their relative efficiency in replicating through the lesion in an "error-free" manner may affect the observed cell responses to the persistence of each individual photolesion. The importance of TLS is reinforced by the existence of at least one known human syndrome related to a deficiency in this mechanism: XP variant form (263).

The present work used a UV source that emits 54 % of UVB (200 – 315 nm) and 46 % of UVA (316 – 400 nm). It is known that UVA induces not only photolesions, but also significant levels of oxidized damage and is responsible for the conversion of 6-4PPs into its photoisomer, Dewar-PP (42). The presence of these lesions could play an important part in the results obtained in the present study, explaining why CPD removal and 6-4PP removal did not present a direct additive effect.

However, the system employed in the present study induces very low UVA levels (18.4 J/m^2/day). Dewar-PP photoisomerization only occurs under UVA doses of at least 5 KJ/m^2 (275). Furthermore, UVA induces more CPDs than oxidized damages (275) and experimental animals are proficient in the removal of oxidized lesions. Therefore, the direct induction of oxidized damages and Dewar-PPs by UVA should not account for a significant amount of the observed skin responses to UV irradiation.

Nevertheless, the ratio of CPD/6-4PP induced by UVB and UVA are very different, with UVB inducing significantly more 6-4PPs than UVA (42). The presence of UVA may have increased the CPD/6-4PP ratio, aiding to the apparently more important role played by CPDs than 6-4PPs in local skin responses to acute and chronic UV exposure.

UVA and UVB-induced melanogenesis are significantly different: UVA-induced melanogenesis requires the presence of pigment and oxygen, whereas UVB-induced pigment production needs the presence of keratinocytes in addition to melanocytes.

Despite their different induction mechanisms, UVA-induced and UVB-induced melanin present an additive effect in protecting cells from UV rays and their consequences (224).

Melanin is capable of blocking UVA and UVB with the same efficiency. It is widely known that melanin is capable of not only physically blocking UV light, but also of scavenging ROS formed mostly through UVA interactions with proteins and lipids, therefore protecting cells from deleterious UV effects (276), reviewed in (231,277). In fact, DNA damage levels and skin cancer inversely correlate with total melanin in skin, reviewed in (231).

Previous studies performed with similar animal models used the same UV source, but with the addition of a filter that totally blocked UVA, thus preventing UVA-related responses. This difference may help to explain why the present work shows melanin production after UV exposure while others failed (209,249,250). Furthermore, the use of different mouse models may also explain why the aforementioned studies did not demonstrate an increase in skin pigment after UV exposure: the present study is the only one to our knowledge to apply XPA and CSA mice transgenically expressing photolyases.

No alteration in skin pigmentation was observed in UV irradiated CSA mice. This absence of melanogenesis induction may be due to the lack of melanocytes in their dorsal interfolicular epidermis (274). However, no increase in pigmentation was observed in the areas with glaborous skin, suggesting that other factors should be considered, such as the fast lesion removal by GG-NER and/or photolesion removal exclusively from basal keratinocytes.

In XPA mice, melanogenesis was observed after chronic irradiation and was enhanced by ubiquitous CPD removal. Interestingly, 6-4PP removal from basal keratinocytes prevented melanin production. These results suggest that the presence of 6-4PPs in basal keratinocytes is somehow essential for melanogenesis induction.

It is known that the presence of excised DNA damaged fragments by NER increases melanin production, which does not take place in the XPA mouse model. Therefore, this data demonstrates that the mere presence of DNA damage, and not its removal, is sufficient to elicit melanogenesis. However, it would be of interest to study if in this scenario POMC is overexpressed in a p53 dependent manner, or if other molecules are responsible for melanogenesis induction, such as NO, PG, ET-1 or KIT1.

Interestingly, in fair-skinned individuals, most of the UV blocking happens in the *stratum corneum*, whereas in dark-skinned individuals, melanin in the Malpighian layer blocks most of UV rays (278). Furthermore, it has been shown that photoprotection against UVB comes primarily from UV absorption in the *stratum corneum*, whereas UVA protection is mainly achieved by UV blocking by melanin (279,280).

In a similar manner, XPA animals where CPD lesions were ubiquitously removed and that presented tanning, efficiently block UVA, leaving the upper epidermal layers susceptible to UVB-induced damages. The other groups of animals must efficiently block UVB in their thick *stratum corneum*, leaving the basal cells partially exposed to UVA. Since most known skin cancers in humans arise from basal cells, skin pigmentation seems to be a more efficient photoadaptive response in the present scenario, which is reinforced by observed lower levels of p53 patches presented by the tanned mice.

Melanogenesis and hyperplasia are intimately connected and the observation that CPD removal prevented chronic hyperplasia, whereas 6-4PP removal from basal keratinocytes did not, should be taken into account when evaluating these different melanogenic responses to chronic UV irradiation.

Many hypotheses could explain how CPD removal in XPA mice prevents hyperplasia and dysplasia and enhances skin pigmentation. For instance, melanin production, in the presence of only 6-4PPs, could be so quick in preventing UV light from reaching basal keratinocytes that hyperplasia never takes place. However, low hyperplasia levels are still seen after acute UV exposure followed by CPD removal, which discards this hypothesis.

A second possibility could be that the persistence of both photolesions in a XPA background leads to melanocyte apoptosis, withholding pigment production. However, since an increase in melanin production was seen in the UV irradiated control group, this cell response is not likely to occur. Furthermore, melanocytes are known to be particularly resistant to UV-induced, p53-mediated, apoptosis, probably due to enhanced BCL2 (BAX suppressor) levels and upregulation of the growth arrest and DNA damage-inducible protein alpha (GADD45a), an apoptosis inhibitor, reviewed in (236).

Therefore, the most plausible explanation is that CPD removal after acute UV irradiation reduces immediate hyperplasia induction, while still allowing enough UV

light to reach melanocytes and therefore induce melanogenesis. This leads to an increase in skin pigment content, which physically blocks part of the UV rays from reaching basal keratinocytes, as previously demonstrated (281,282).

Once the damaging agent is reduced, basal cell proliferation also decreases. The low UV levels which still reach the basal layer are sufficient to sustain melanogenesis and provoke significant apoptosis. The combination of lower cell proliferation and sustained apoptosis prevents chronic hyperplasia induction. According to this hypothesis, a mild hyperplasia is seen after acute UV irradiation because melanin production has not yet taken place and a high amount of damaging rays still reach the basal epidermal layer.

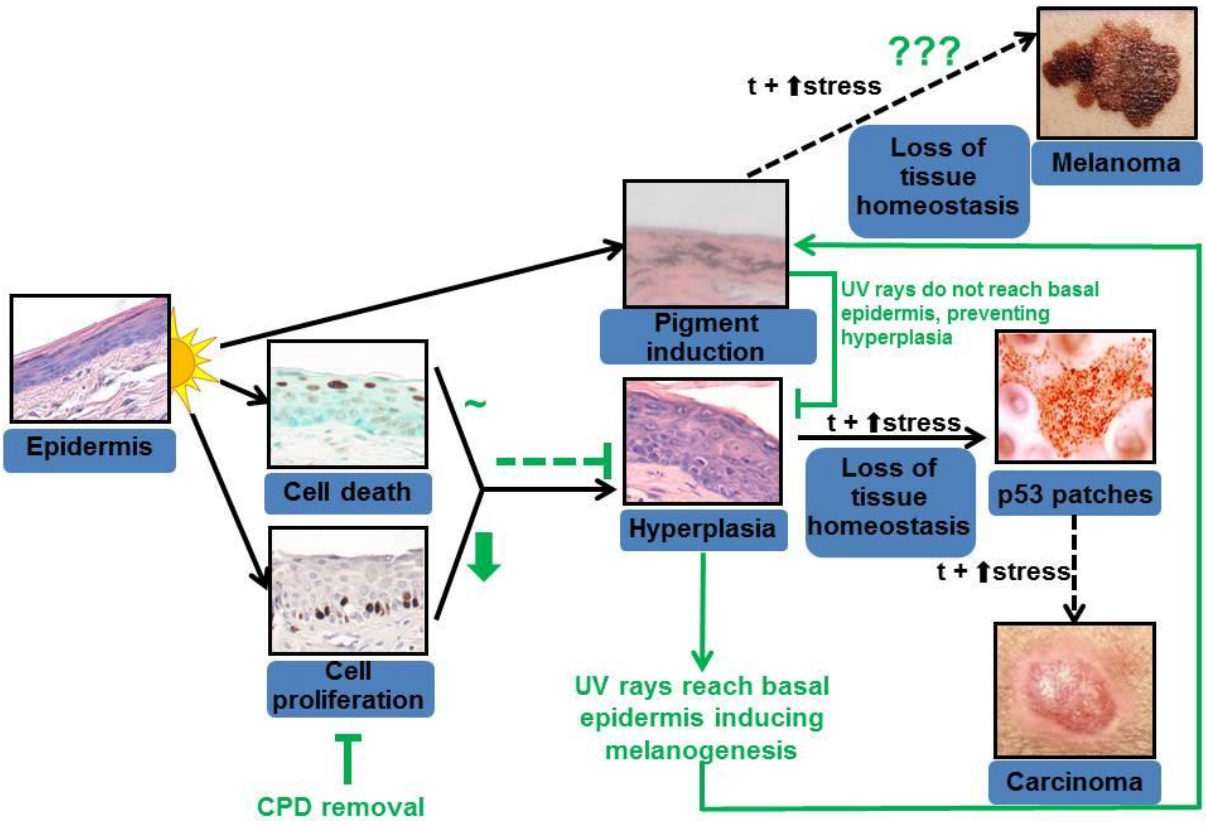
In the UV irradiated control animals, the immediate hyperplasia blocks a significant amount of UV rays from reaching melanocytes and keratinocytes, and only low levels of melanin are produced. However, the UV light that reaches keratinocytes is still enough to induce significant cell proliferation, sustaining hyperplasia. This difference in keratinocyte and melanocyte responses to UV exposure is a consequence of the higher melanin concentration in melanocytes, since melanin provides protection against UV-induced injuries (283).

In the animals where 6-4PPs are removed from basal keratinocytes, despite a slight reduction in acute hyperplasia, photolesion removal may be preventing the synthesis of signaling molecules, withholding melanogenesis induction. The high levels of UV rays that reach the basal keratinocytes lead to cell proliferation and consequent chronic hyperplasia. Chronic hyperplasia also blocks a significant amount of UV rays from reaching keratinocytes and melanocytes, reducing the signaling molecules that induce melanogenesis. In addition, photolesion removal from basal keratinocytes further reduces the signaling molecules they release, culminating in the prevention of melanogenesis induction. Again, hyperplasia is sustained due to the UV rays which still reach basal keratinocytes.

The fact that the removal of both photolesions is capable of reducing acute hyperplasia but only ubiquitous CPD removal enhances melanogenesis and prevents chronic hyperplasia whereas 6-4PP removal from basal keratinocytes not only does not prevent chronic hyperplasia but prevents UV-induced melanogenesis suggests that each photolesion may be inducing a different melanogenesis pathway. Furthermore, these results also suggest that lesion location may also be fundamental in tissue responses to UV light.

Figure 51 presents a representative model of how melanogenesis is induced after chronic UV exposure, enhanced by CPD removal and prevented by 6-4PP removal in XPA mice.

Figure 51 - Representative model of the specific roles of each photolesion in melanogenesis induction after chronic UV exposure of XPA mice



UV irradiation induces pigmentation, cell proliferation and apoptosis. The combination of the levels of cell division and death define epidermal thickness. As time (t) passes and stress accumulates, damaged skin may lose its homeostasis (represented as p53 overexpression in clusters of cells), which may culminate in carcinomas (derived from keratinocytes) or melanomas (derived from melanocytes). Alternatively, CPD removal reduces UV-induced hyperplasia, through the reduction of cell proliferation and sustained apoptosis, allowing more UV rays to reach the basal epidermal layer, leading to an increase in melanogenesis, which prevents UV rays from reaching basal keratinocytes, reverting hyperplasia. On the other hand, 6-4PP removal from basal keratinocytes could reduce the production of signaling molecules secreted by these cells, withholding melanogenesis, increasing chronic hyperplasia.

Similarly, in the CSA animals, the fast photolesion removal by GG-NER must prevent the release of signaling molecules which would induce melanogenesis. Further specific photolesion removal from basal keratinocytes would not change this response.

This set of data indicates that a minimum level of damage has to be present in the lower epidermal layers for a prolonged period of time (acute low exposure is not sufficient) to ensure activation of melanogenesis. Results suggest that the presence of 6-4PPs may be essential for the development of this skin response. However, it cannot be discarded that it is not the nature of the lesion but the total amount of DNA damage (associated with epidermal thickness), the location of the lesion on the DNA, or the damaged cell that controls pigment production.

Since CPDs are approximately 3 times more frequent in the genome than 6-4PPs after UV irradiation with the same dose (24), it is hard to assess which cell responses are consequences of the nature of the lesion and which are due to the total amount of DNA damage.

However, the same amount of CPDs and 6-4PPs could be expected in animals UV irradiated with 1 MED and 3 MEDs, respectively. After specific lesion removal with photolyases, the first group of animals would have the same amount of CPDs as the second group would have of 6-4PPs, allowing for a direct analysis of the cell responses to the two different types of lesions when present in the same quantity.

Interestingly, CSA animals transgenically expressing CPD-photolyase and UV irradiated with 1 or 3 MEDs presented different tissue responses. In animals treated with 1 MED of UV, partial reduction in basal cell proliferation, associated with elevated basal cell death, guaranteed partial hyperplasia prevention. However, in animals UV irradiated with 3 MEDs, even high apoptotic levels were not able to prevent the intense hyperplasia induced by the high levels of basal (and suprabasal) cell proliferation (data not shown).

Furthermore, CSA mice irradiated with 1 MED of UV and 6-4PP removal from basal keratinocytes presented a very similar response to that of CSA animals irradiated with 3 MEDs of UV followed by CPD removal from basal keratinocytes: high apoptotic levels were not able to circumvent the high basal (and suprabasal) cell proliferation, leading to a significant hyperplasia. Therefore, the only distinction among these two groups is that CPD removal increased apoptosis in basal epidermal cells, but still presented a higher hyperplasia (data not shown).

These results indicate that not only the nature of the lesion (CPD or 6-4PP) plays an important role in cell responses to chronic UV exposure, but also that the number of lesions persisting in the genome is crucial to define cell fate.

Comparison between the levels of acute and chronic skin responses in XPA mice showed that chronic exposure ensured a higher hyperplasia and higher proliferation levels but lower apoptosis when compared to acute UV irradiation. These results indicate that prolonged UV exposure led to an increase in cell proliferation, resulting in higher hyperplasia. Interestingly, prolonged UV exposure also led to a reduction in apoptosis, which is probably a result of the lower UV levels that reach basal keratinocytes.

Curiously, it has been shown elsewhere that previous chronic UV exposure is capable of preventing acute UVB effects even long after the chronic irradiation had ceased. Photoadaptive responses may include pigmentation, hyperplasia, and expression of UV-blocking or quencher molecules. These photoadaptive responses seemed to be more efficient when generated by chronic UVB exposure (in comparison to UVA) and were higher against CPDs than against 6-4PPs (284).

In the present study no signs of efficient photoprotection by photoadaptive responses were observed, when comparing skin responses between acute and chronic UV exposure: hyperplasia was higher but cell proliferation was similar and apoptosis was higher after prolonged UV irradiation. However, only photolesion quantification would confirm if the photoadaptive responses were efficient in protecting the skin from photolesion induction or persistence.

On the other hand, comparing photoadaptive responses between chronically irradiated mice with different genotypes, it seems that ubiquitous CPD removal in XPA mice led to the development of an efficient photoadaptive response: tanning. In the other animal groups, hyperplasia was the observed adaptive mechanism. Curiously, this photoadaptive response was accompanied by dysplasia in chronically UV irradiated mice. Again, only photolesion quantification would confirm how much each of these photoadaptive responses protects the skin from UV.

Interestingly, it has been previously demonstrated that despite a higher accumulation of CPDs and p53 levels after UV, lower apoptotic levels are observed in fair-skinned individuals (278,281,285,286). This difference in TUNEL positive cells after low UV exposure may be due to melanin-specific photothermolysis, reduced function of melanophages or other yet unknown mechanisms (286). It has also been demonstrated that in mice of different coat colors, the levels of sunburn cells and caspase 3 expressing keratinocytes were similar after UV exposure. However, the levels of TUNEL positive cells was higher in black and yellow animals than in albinos,

especially in the upper portion of the hair shaft, where melanin levels are higher (287).

Based on these observations, it can be speculated that the reduced apoptosis seen in XPA animals with 6-4PP removal from basal keratinocytes after chronic UV exposure is due to the absence of melanin. However, similar apoptotic levels were observed between animals with dark skin (ubiquitous removal of CPD) and intermediate tanned (control UV irradiated). Therefore, there is no apparent correlation between the presence of melanin and apoptosis induction after chronic UV exposure in the results presented here. Of note is the fact that chronic UV irradiation did not increase basal apoptotic levels in CSA mice, which may be related to photolesion removal by GG-NER. Curiously, CPD removal from basal keratinocytes in these animals induced basal apoptosis.

Importantly, the number of TUNEL positive cells observed in all UV irradiated mice is elevated, even in the basal layer. Animals with this amount of epidermal apoptosis would be expected to have macroscopic lesions, which were never observed. It is therefore possible that the staining is recognizing non-apoptotic cells. In the TUNEL technique, the TdT enzyme recognizes 3´-OH free DNA ends (typically abundant in apoptotic cells) and incorporates EdUTP, which is latter recognized by a specific antibody. If TdT recognizes non-apoptotic 3´-OH ends, such as those found during replication (mostly in Okazaki fragments), the specificity of the staining will be compromised. To evaluate if indeed the high number of TUNEL positive cells observed is composed exclusively by apoptotic cells, a second staining capable of detecting cell death, such as caspase 3 or 9, must be performed.

Melanogenesis, as well as hyperplasia, is important not only to protect keratinocytes, but also melanocytes from the deleterious UV effects. Keratinocyte protection from UV is important to prevent cell transformation, which could lead to BCC and SCC. Similarly, melanocyte protection from UV is important since these cells present a very low self-renewal capacity and are particularly resistant to apoptosis. Therefore, maintenance of melanocyte genome integrity is essential to guarantee melanin production and to prevent its malignant transformation to melanoma, the most fatal skin cancer (236,288,289).

However, while constitutive melanin levels inversely correlate with skin cancer incidence, it has been shown that the tan response only increases skin photoprotection in 2-3 times (190). Furthermore, melanin may also act as a

photosensitizer, producing oxygen singlet, hydrogen peroxide and other ROS, reviewed in (287). This is especially true for recently-synthetized melanin (290,291). Moreover, the melanogenic process also produces high amounts of ROS, reviewed in (292). Therefore, the tanning response, especially in fair-skinned individuals, may increase the risk of melanoma occurrence (291).

CPD removal may raise the risk of melanoma induction in XPA mice by enhancing melanogenesis. However, no signs indicating that the elevated tanning response caused by CPD removal is detrimental were observed. On the contrary, animals presented lower hyperplasia and cell proliferation.

Furthermore, CPD photoremoval seems to prevent the development of skin tumors derived from keratinocytes, as indicated by the prevention of p53 patch induction in the present work, and by the reduction in BCCs and SCC in other studies (209,250).

Long term observation of skin cancer development in βCPD-photolyase/XPA/HR animals after prolonged UV exposure is necessary to confirm skin cancer prevention by CPD removal in DNA repair deficient animals. Moreover, only specific analysis can determine if, under these experimental conditions, skin exposure to UV in the presence of melanin could increase the levels of ROS formation and consequent oxidized lesions.

Further investigation of melanocyte cell number and localization is still necessary to characterize more precisely the observed melanogenesis. To address the importance of the nature of the lesion in this process, it is necessary to investigate if ubiquitous 6-4PP removal also (partially) prevents chronic hyperplasia and induces melanogenesis. Interestingly, 6-4PP removal only in keratinocytes did not result in increased pigmentation. Similarly, it is relevant to verify if CPD removal from basal keratinocytes in a XPA background is capable of inducing melanogenesis. To verify the importance of lesion removal by TC-NER, further chronic UV exposure experiments with hairless CSA mice ubiquitously expressing CPD-photolyase and 6-4PP-photolyase would also be of interest.

Furthermore, the use of albino hairless mice expressing each photolyase under the control of the same promoter could allow for the investigation of the role of each photolesion exclusively in the development of hyperplasia, in a system where the tanning response would not mask potential hyperplasia occurrence.

Photolesion quantification in all present experimental groups is of great interest to verify if photolesion reduction is indeed responsible for the observed differences in apoptosis between acute and chronic UV irradiation protocols and between tanned and hyperplastic mice.

Despite the clear major role observed for CPD lesions in acute and chronic responses to UV exposure, its removal was not able to completely abrogate all of the UV-induced epidermal responses, suggesting that other lesions may be important in the development of these events. The minor role observed for 6-4PPs in cell proliferation and hyperplasia may be due to the fact that these lesions were only removed from basal keratinocytes. In fact, previous works suggest that photolesion location may interfere with epidermal responses to UV light (249).

The essential roles of CPDs on the UV effects in the skin have long been accepted, as can be demonstrated by the development of sunscreens with enzymes which remove CPDs. These sunscreens are presently commercialized by pharmaceutical industries.

T4 endonuclease V (T4 endo V) is an endonuclease from T4 bacteriophage that infects *E. coli* and is capable of aiding in the repair of CPDs. In the 1980´s Yarosh´s laboratory showed that this enzyme could be delivered into mammalian cells using liposomes, which (partially) recovered XP cells phenotype. Later the same group showed that these T4N5 liposomes can be delivered through the skin in a 1 % hydrogel lotion, efficiently removing CPD lesions present in the epidermis and consequently reducing UV-induced mutagenesis. Clinical trials showed that this enzyme was efficient in XP patients, reducing 30 % of the BCC rate, reviewed in (121). A sunscreen with enveloped T4 endo V is now being produced by a Japanese company (Synergy Worldwide) and is sold in some countries.

A product which is capable of removing CPDs (Eryfotona® AK-NMSC, ISDIN) due to the addition of CPD-photolyase in Repairsomes^{TM} (liposomes) to a 100 FPS sunscreen is available worldwide (293). This cream is capable of reducing >40 % of the CPDs present in the epithelium after UV exposure, and also of reducing p53 expression 2.5 fold after a one year treatment.

Published data shows that in a DNA repair-proficient background, CPD is the major component responsible for acute and chronic skin effects of sunlight (209,249,250). These results were obtained in DNA repair-proficient mice. However, the response of XP patients in a phase III clinical trial of a cream which efficiently

removes only CPDs, indicates that, even in a DNA repair-deficient background, CPDs still have an important role in the deleterious UV effects (294).

The present work confirms the major role played by CPDs in acute and chronic responses against UV irradiation, especially in cell proliferation, hyperplasia and p53 patch induction. 6-4PPs appear to have an important role in the induction of melanogenesis and apoptosis.

6 CONCLUSIONS

The present work shows an intimate connection between cell proliferation and apoptosis in the development of acute and chronic UV-induced hyperplasia and dysplasia. Chronic hyperplasia prevention depends on reduced basal cell proliferation and high basal apoptosis.

Ubiquitous CPD removal partially prevented acute hyperplasia and dysplasia despite an increase in basal proliferation, through reduced suprabasal proliferation and sustained apoptosis. After chronic UV exposure, XPA mice presented increased melanogenesis, which was further enhanced by CPD removal. Hyperplasia and p53 patch induction were prevented by CPD removal from the whole epidermis in XPA animals, through reduced cell proliferation and sustained cell death.

6-4PP removal from basal keratinocytes partially prevented acute hyperplasia and dysplasia through the reduction of basal and suprabasal cell proliferation. However, this photolesion removal did not prevent chronic hyperplasia and p53 patch induction. Melanin production was refrained by 6-4PP removal from basal keratinocytes in XPA mice.

In CSA animals, CPD removal from basal keratinocytes partially prevented chronic hyperplasia and dysplasia, through reduced basal proliferation and increased basal apoptosis. 6-4PP removal from basal keratinocytes did not alter UV-induced cell and tissue responses. UV exposure alone or in combination with photolesion removal did not induce melanogenesis in CSA mice.

These results demonstrate that, in a DNA repair-deficient background, CPDs play a major role in skin responses to acute and chronic UV irradiation, especially in cell proliferation and p53 patch induction. However, CPDs are clearly not the sole player in skin responses to UV exposure. 6-4PPs seem to play a minor role in skin responses to low UV doses, being mainly involved in apoptosis and melanogenesis.

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APPENDIXES – SCIENTIFIC PAPERS

Appendix A – Da Costa RMA, Quayle C, De Fátima Jacysyn J, Amarante-Mendes GP, Sarasin A, Menck CFM. Resistance to ultraviolet-induced apoptosis in DNA repair deficient growth arrested human fibroblasts is not related to recovery from RNA transcription blockage. Mutat Res. 2008 Apr 2;640(1-2):1–7.

Appendix B – Eker APM, Quayle C, Chaves I, Van der Horst GTJ. DNA repair in mammalian cells: Direct DNA damage reversal: elegant solutions for nasty problems. Cell Mol Life Sci. 2009 Mar;66(6):968–80.

Appendix C – Quayle C, Menck CFM, Lima-Bessa KM. Recombinant viral vectors for investigating DNA damage responses and gene therapy of Xeroderma Pigmentosum. In: Vengrova S, editor. DNA Repair and Human Health. New York: InTech; 2011. p. 145–74.

APPENDIX A

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Fundamental and Molecular Mechanisms of Mutagenesis
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Resistance to ultraviolet-induced apoptosis in DNA repair deficient growth arrested human fibroblasts is not related to recovery from RNA transcription blockage

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Abstract

The impact of ultraviolet (UV-C) photoproducts on apoptosis induction was investigated in growth arrested (confluent) and proliferating human primary fibroblasts. Confluent fibroblasts were more resistant to UV-C-induced apoptosis than proliferating cells, and this was observed for normal human cells and for cells from patients with Cockayne and trichothiodystrophy syndromes, deficient in transcription coupled repair. This resistance was sustained for at least seven days and was not due to DNA repair efficiency, as the removal of CPDs in the genome was similar under both growth conditions. There was no correlation between reduced apoptosis and RNA synthesis recovery. Following UV-C treatment, proliferating and confluent fibroblasts showed a similar level of RNA synthesis inhibition and recovery from transcription blockage. These results support the hypothesis that the decrease of DNA replication, in growth arrested cells, protects cell from UV-C-induced apoptosis, even in the presence of DNA lesions.

Keywords: Nucleotide excision repair; Apoptosis; Ultraviolet; Transcription coupled repair; Replication; Quiescent cells

1. Introduction

DNA damage may result in cellular dysfunctions, such as genetic instability, mutagenesis or cell death, considered hallmarks of biological processes such as cancer and aging. However, cells are equipped with efficient systems that are responsible for maintaining the integrity of their DNA. These mechanisms are responsible for removing and tolerating DNA damage, and failures are very hazardous for cells, their consequences being observed in several human diseases.

Xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) patients present photosensitivity, although at different levels, due to failure in removing UV-induced DNA lesions by the nucleotide excision repair (NER) mechanism [1]. XP patients denote strong pigmentation, actinic keratoses, loss of elasticity and multiple skin cancers, in sun exposed areas of the skin. Seven complementation groups (XPA-G) and one variant (XPV), which is deficient in lesion bypass by the translesion synthesis DNA polymerase eta, have been identified. CS and TTD patients are not particularly susceptible to skin cancer development, and their clinical features have little in common with XP patients. CS patients are characterized by reminiscent traits of normal aging, such as systemic growth failure, neurological degeneration and cataracts. Moreover, a small number of XP patients (from XP-B, XP-D and XP-G complementation groups) display a combination of cutaneous abnormalities together with the severe neurological and developmental anomalies of Cockayne's syndrome. Two complementation groups have been identified (CS-A and CS-B), and besides their importance on lesion removal, the corresponding proteins have an important role in RNA transcription. TTD is very heterogeneous, and groups different patients with

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sulphur-deficient brittle hair. About half of TTD patients exhibit photosensitivity, due to their deficiency in removing UV-induced DNA lesions, ichthyotic skin, small stature and mental retardation. There are three complementation groups of photosensitive TTD patients (TTD-A, XP-B and XP-D), and all of the corresponding proteins are components of the transcription factor H of RNA polymerase II (TFIIH) [2].

NER is considered the most flexible and versatile DNA repair pathway, as it deals with a wide range of structurally unrelated DNA lesions. The major injuries induced by UV light are cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4 PPs), which are removed by NER. The NER process involves the coordinated action of at least 30 proteins, and its basic mechanism consists of the removal of the DNA segment containing the lesion and gap polymerization using the intact strand as template [3]. Two NER subpathways have been identified: transcription-coupled repair (TCR), selectively acting on lesions present in the transcribed strand of expressed genes, and the global genome repair (GGR) that acts upon the rest of the genome. The transcribed strand of expressed genes is repaired faster than other genomic regions, including the non-transcribed strand of active genes [3]. CS cells are exclusively deficient in TCR of CPD but are proficient for GGR. On the other hand, XP-C and XP-E cells are exclusively deficient in GGR of both CPD and 6–4 PPs. The other XP and TTD cells are deficient for both subpathways, TCR and GGR [1].

NER-deficient cells are hypersensitive to UV light, specially those defective in TCR (XP-A, XP-B, XP-D, XP-F, XP-G, CS and TTD), which show high levels of apoptosis induction following exposure to low doses of this genotoxic agent [4,5]. The TCR-deficient cells do not recover normal transcription following UV exposure, probably as a consequence of blockage of RNA polymerase II by the remaining lesions on the transcribed strand of active genes [4,6]. Based on this evidence, transcription was proposed to act as a "damage dosimeter", where the severity of the DNA damage, the ability to remove the lesions and the kinetics of mRNA synthesis recovery, determine whether the cell lives or dies [7]. However, this is not a universal consensus. Results obtained with serum starvation and high density seeding protocols indicate that DNA replication plays a role in UV-induced apoptosis [8]. Accordingly, McKay et al. [9] have shown that the repair of UV-induced lesions is of particular importance in proliferating fibroblasts before DNA replication in the S phase. Similar results were found in human keratinocytes. Rapidly proliferating keratinocytes were more susceptible to UV-light-induced apoptosis, whereas those induced to undergo growth arrest/early differentiation were relatively resistant to apoptosis [10,11]. In agreement with the idea that replication of damaged DNA also signals for apoptosis, it was recently shown that hamster cells that had their DNA replication blocked by aphidicolin were more resistant to UV-induced apoptosis [12]. Most probably the replication of UV-C lesions generates secondary lesions that potentially lead to cell death. In fact, a balance between transcription and replication blockage must be involved in DNA damage signaling pathways responsible for the decision as to whether a cell dies or lives after DNA insult.

The impact of growth arrest and RNA transcription in apoptosis induction by UV-C-lesions was investigated in this work. These studies were performed in primary human skin fibroblasts, which sustain a contact inhibited proliferative state. This condition has the advantage of keeping low levels of DNA replication without disturbing the cells with chemicals or starvation, which certainly interfere in their normal metabolism. Moreover, most of the cells in the human body are probably in a quiescent state, similar to confluent cells. Previous experiments have indicated that XP-C confluent cells were more resistant to UV-C-induced apoptosis than proliferating ones [13]. The present report extends these findings to TCR-deficient cells (CS and TTD cells) which are also more resistant to UV-C-induced apoptosis when irradiated in confluent conditions at low doses. Protection was observed for long periods, indicating that it does not postpone cell death. NER efficiency was evaluated, and no significant difference was found for CPD removal from DNA under both growth conditions. Moreover, RNA synthesis inhibition by UV-C-light was similar in confluent and proliferating cells, despite the different apoptosis levels. Altogether, these results show a correlation between UV-C-induced apoptosis and DNA replication, and calls attention to the different apoptotic responses depending on the cell cycle status.

2. Materials and methods

2.1. Cell culture

Human primary fibroblasts derived from skin biopsies were employed in this study: normal (gently donated by Dr. Claudimara Lotfi, ICB, USP-SP, Brazil), CS1AN (mutated in *CSB*, gently donated by Dr. Jaspers NG, Department of Cell Biology and Genetics, Erasmus University Rotterdam, Netherlands), CS1VI (mutated in *CSB*), TTD1VI (mutated in *XPD*). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corporation, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS, Cultilab, Campinas, SP, Brazil), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin and 0.25 mg/ml amphotericin (Invitrogen Corporation). An atmosphere of 5% CO_2 was maintained in a humidified incubator at 37 °C. Proliferating cells corresponded to cells plated in 1.2×10^5 cells per 60 mm Petri dishes, and after 3 days approximately, with less than 80% of confluence, they were irradiated. Confluent cells were obtained after seeding 2.4×10^5 cells in 30 mm Petri dishes, and they were employed only 5 days after reaching confluency.

2.2. Cell irradiation

Confluent and proliferating cells were washed with PBS and irradiated with UV-C, using a germicidal lamp (emitting mainly at 254 nm, dose rate of 1 J/m^2 s). UV-C dose was monitored by the radiometer VLX 3W, monochromatic sensor CX-254 (Marne la Vallee, France). It should be noted that due to a change in the dosimeter, the values of UV-C doses of this work correspond to approximately 4.0 times the values previously published in this laboratory [13]. After irradiation, the cell medium was replenished and cells were incubated under normal conditions for the desired period.

2.3. Cell cycle flow cytometry

Apoptosis was measured by quantifying a sub-G1 population, following cell cycle analysis of propidium iodide staining. Briefly, floating and attached cells were harvested by trypsinization, collected and resuspended with 70% ethanol/PBS and stored at 4 °C. Cells were then stained with 50 μg/ml propidium iodide for 1 h in the presence of 40 μg/ml of RNAse A (DNAse-free). Measurements were carried out in a FACScalibur flow cytometer (Becton Dickinson,

Franklin Lakes, NJ). Results were analyzed with CellQuest software (Becton Dickinson).

2.4. Cell viability assay

Cell viability of human fibroblasts was performed 7 days after UV-C-irradiation, by employing a XTT assay (Cell Proliferation Kit II, XTT; Roche Molecular. Biochemicals, Mannheim, Germany). The procedure was followed as recommended by the supplier.

2.5. DNA synthesis quantification

Proliferating and confluent cells were pulse-labeled by incubating in medium containing 4.0 μCi/ml of [^{3}H]-methyl-thymidine (specific activity of 89.0 Ci/mmol, GE Healthcare, USA) for 1 h. Cells were then washed once with PBS, 5% trichloroacetic acid and twice with hydrated alcohol, before 0.3 M NaOH was added. Part of this cell lysate (50% of the total volume) was applied on Whatman 17 paper, which was again washed once with 5% trichloroacetic acid, twice with ethanol and once with acetone. Radioactivity was measured with a liquid scintillation spectrometer (Beckman LS 7000). The other part of the lysate was used to measure absorbance at 260 nm, for data normalization. The ratio between radioactivity and absorbance expresses the amount of [^{3}H]-methyl-thymidine incorporated by cells during DNA synthesis.

2.6. Immuno-slot-blot analysis of CPD

Proliferating and confluent fibroblasts were UV-C-irradiated with 15 J/m^2. At different times following irradiation (0, 3 and 24 h) their genomic DNA was extracted as described [14]. Genomic DNA was loaded onto nylon membrane under a vacuum, and the DNA was fixed by 2 h of incubation in 80 °C. The membranes were immersed in PBS containing 5% low-fat dried milk for 1 h, and then incubated overnight with anti-CPD (MBL, Japan) monoclonal antibody (dilution 1:1000). Chemifluorescent detection was performed with ECL western blotting kit (GE Healthcare) via alkaline phosphatase anti-mouse secondary antibody. Quantification of signals was obtained using Molecular Analyst software (Bio-Rad Laboratories, California, USA). The percentage of repair is expressed as a function of residual intensity of bands at post UV-C irradiation times over samples processed. Results are the average of four different assays, corresponding to two different DNA samples.

2.7. RNA synthesis recovery assay

RNA synthesis was determined based on a previously described method [15]. Cells were plated in Petri dishes containing normal medium. After reaching the desired confluency, cells were UV-C irradiated, incubated for a certain period of time, and then pulse-labeled for 1 h in DMEM with 3% dialyzed FCS and [5-^{3}H]uridine (4.0 μCi/ml, specific activity of 27.0 Ci/mmol, GE Health Care). Cells were then harvested and separated into two samples. In the first sample, cells were lysed (NaCl 0.3 M; Tris–HCl pH 8.0 20 mM; EDTA 2 mM; SDS 1% and K proteinase 200 μg/ml), and transferred to Whatman 17 paper, then being washed twice with 15% trichloroacetic acid and hydrated ethanol for 30 min, for radioactivity measurement. The second sample was used to determine absorbance at 260 nm for data normalization. The ratio between radioactivity and absorbance expresses RNA synthesis of these cells.

3. Results

3.1. Proficient and deficient NER cells are resistant to UV-C radiation under confluent conditions

The UV-C-induced apoptosis was determined in proliferating and confluent human primary fibroblast strains. The fraction of cells with a sub-G1 DNA content was quantified by flow cytometry in TCR proficient cells (FHN), GGR and TCR defective

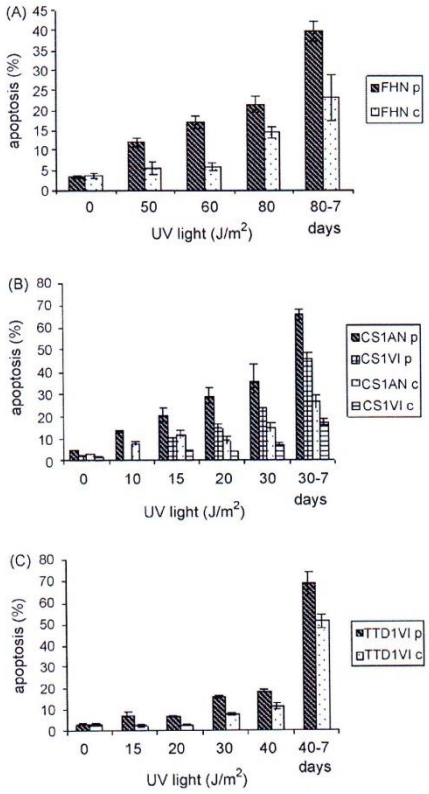

Fig. 1. Resistance of confluent cells to UV-C-induced apoptosis. Induction of apoptosis (sub-G1, as measured by flow cytometry) in normal (FHN) (A), CS-B (CS1AN and CS1VI) (B) and TTD (TTD1VI) (C) cells, 3 and 7 days (when stated) after UV-C exposure. Each result represents the mean of three duplicated independent experiments. Proliferating (p) and confluent (c) cells were employed, as indicated in the inset.

TTD (TTD1VI) cells, and TCR defective cells from two CS patients, both mutated at the *CSB* gene (CS1AN and CS1VI) (Fig. 1). Sub-G1 cells were observed only 72 h following UV-C exposure, and as cell ability to remove DNA lesions is different among the cells tested, different UV-C doses were used, corresponding to comparable cell death induction. As expected, lower UV-C doses were necessary to induce similar levels of apoptosis in NER deficient fibroblasts than normal cells. Different responses were observed in the two CS-B cell strains, probably due to either the genetic background of the cells or the effect of the different CSB mutations.

The sensitivity of these cells to UV-C-induced DNA damage was significantly reduced when they were kept in confluence. Lower levels of apoptosis were also observed in TCR deficient cells, when they are not proliferating. Similar results were

observed in XP-C fibroblasts [13], and in normal keratinocytes [10], thus indicating this to be a general phenomenon, although the protection in XP-C confluent fibroblasts seems to be stronger. Apoptosis was also checked, for the higher doses, considering seven days after UV-C irradiation. Although in both cases the levels of apoptosis increased, confluent cells were still more resistant than proliferating cells (Fig. 1). Importantly, this reduction of apoptosis in confluent cells was observed for low and high doses of UV-C. Cell viability was also investigated seven days after UV-C exposure (Fig. 2). Confirming the results for apoptosis, the confluent growth conditions dramatically increased the level of cell survival. These data indicate that the status of the cell cycle plays an important role in the decision whether a cell dies or lives after DNA insult.

The rate of DNA replication in proliferating and confluent cells was obtained by direct measurement of [H^3]-thymidine incorporation in the genome of cells under both culture conditions. Confluent cells had approximately 10% of the thymidine incorporation of proliferating cells, confirming their low rate of DNA synthesis.

3.2. CPD removal in proliferating and confluent cells

One possible explanation for the difference in cell survival observed for proliferating and confluent cells is their ability to remove DNA lesions by NER. The removal of the major DNA photoproduct, CPD, from the genome, was measured by immunoblot assays. Genomic DNA from normal (FHN), CS-B (CS1AN) and TTD (TTD1VI) cells was extracted immediately and several hours following UV-C irradiation (15 J/m^2), and CPDs were detected using specific monoclonal antibody (TDM-2) (Fig. 3). As expected, TTD cells displayed lower CPD repair levels, when compared to normal and CS-B cells. It is important to recall that this type of assay is not appropriate for distinguishing lesion removal in the transcribed strand by TCR. This explains the similar rates of repair in both normal and CS1AN cells, although this seems to be slightly lower in CS1AN cells. Curiously, all cell strains displayed slightly faster CPD removal under confluent conditions, at lower times following UV-C exposure. However, it is difficult to correlate these results with the higher survival of growth arrested cells, whereas after 24 h the percentage of remaining CPDs in TTD1V1 (~60%) and CS1AN (~20%) was similar in both proliferating and confluent cells. On the other hand, only growth arrested normal cells removed almost 100% of CPDs from their genome compared to 80% from proliferating cells.

3.3. Quantification of RNA synthesis

It is well known that UV-C-induced lesions cause transient inhibition of RNA synthesis [7]. The more efficiently the cell removes these lesions from actively transcribed genes, the faster it recovers its RNA synthesis. We were interested in evaluating the relationship between apoptosis and RNA synthesis recovery, as growth arrested cells were shown to be more resistant to UV-C-induced cell death. The RNA synthesis was measured through the incorporation of ^{3}H-uridine in different periods of time following low and high UV-C exposures in normal (FHN), CS-B (CS1AN) and TTD (TTD1VI) cells. The low-dose condition (15 J/m^2) does not induce significant apoptosis in TTD1VI and normal cells, whereas it induces approximately 20% of apoptosis

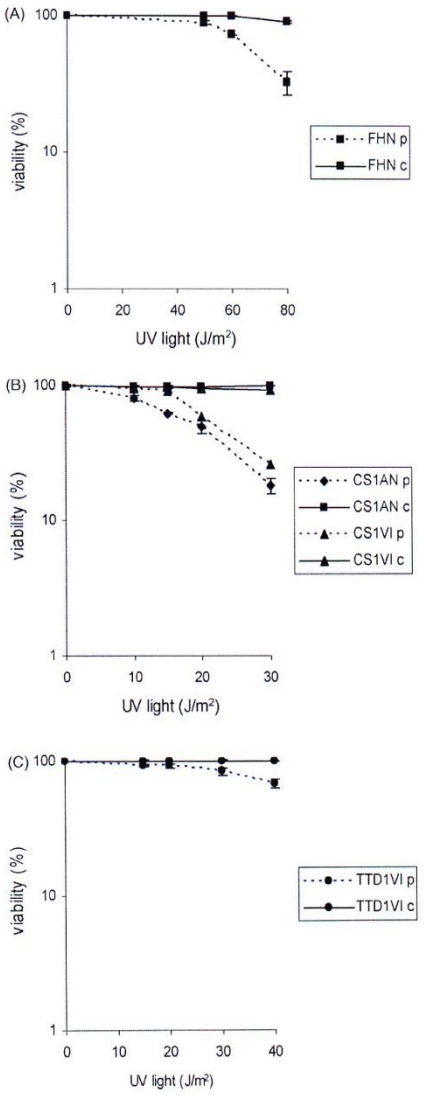

Fig. 2. Higher cell viability of confluent cells to UV-C treatment. Viability was assessed by XTT assay seven days following UV-C exposure in normal (FHN) (A), CS-B (CS1AN and CS1VI) (B) and TTD (TTD1VI) (C) fibroblasts. Each result represents the mean of three duplicated independent experiments. Proliferating (p) and confluent (c) cells were employed, as indicated in the inset.

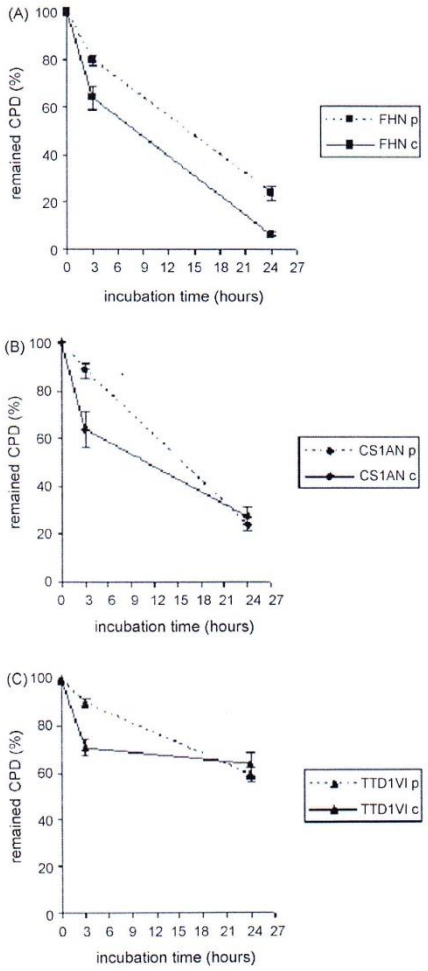

Fig. 3. Kinetics of CPD removal in proliferating and confluent fibroblasts. Cells were harvested at the indicated time after UV-C irradiation (15 J/m^2), genomic DNA was extracted, and CPDs were detected by immuno-slot blot, as described in Material and Methods: normal (FHN) (A), CS-B (CS1AN) (B) and TTD (TTD1VI) (C) fibroblasts. Experimental data are from two independent experiments, each performed in duplicate. Proliferating (p) and confluent (c) cells were employed, as indicated in the inset.

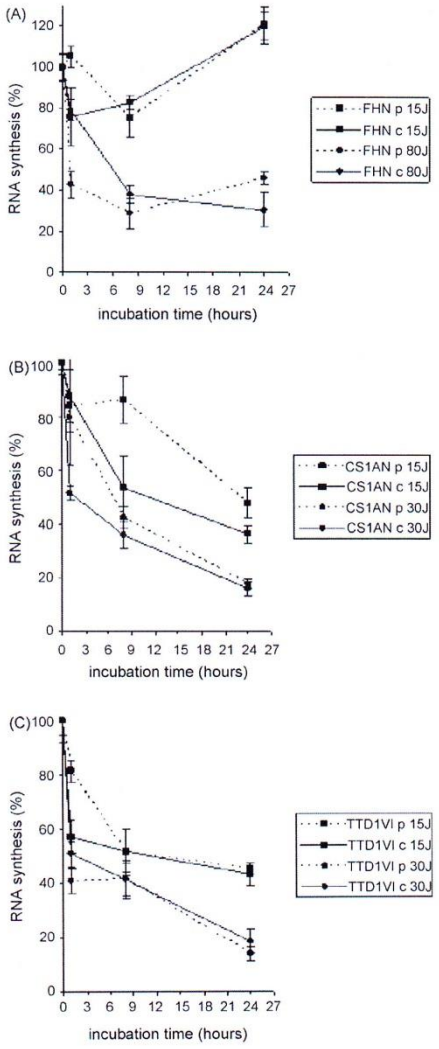

Fig. 4. RNA synthesis recovery following UV-C treatment. Fibroblasts were exposed to different UV-C doses and the RNA synthesis measured at the indicated times after irradiation. (A) Normal (FHN) fibroblasts were exposed to 15 and 80 J/m^2; and (B) CS-B (CS1AN) or (C) TTD (TTD1VI) fibroblasts were exposed to 15 and 30 J/m^2. Each result represents the mean of two duplicated independent experiments. Proliferating (p) and confluent (c) cells were employed, as indicated in the inset.

in CS1AN proliferating cells (Fig. 1). The high-dose employed for normal cells (80 J/m^2) was considered too high for TTD1VI and CS1AN cells, thus a different dose (30 J/m^2) was chosen as the high-dose for these cells, as the reduction of RNA synthesis was roughly similar (~60%, 9 h after UV-C).

The level of RNA synthesis reduction was dependent both on the UV-C dose employed and on the ability of cells to remove DNA lesions (Fig. 4), confirming that defective TCR cells, TTD1VI and CS1AN, were inefficient to recover the RNA synthesis following low (15 J/m^2) and high (30 J/m^2) UV dose irradiation. Curiously, the RNA transcription inhibition and lack of recovery was similar in TTDIVI and CS1AN, although

TTDIVI showed lower levels of UV-C-induced apoptosis. On the other hand, normal cells efficiently recovered RNA synthesis following 24 h only after a low UV dose (15 J/m^2). Surprisingly, all cell strains did not display any significant difference of UV-C-induced RNA synthesis inhibition or recovery when proliferating or confluent cells were compared. Therefore, these results indicate that the reduction of UV-C-induced apoptosis observed in confluent cells is independent on RNA synthesis recovery.

4. Discussion

Apoptosis induction by UV irradiation has been associated with the persistence of unrepaired DNA lesions. This has been confirmed in NER deficient fibroblasts expressing CPD and 6–4-PP photolyases, where photoremoval of these lesions prevented UV-induced apoptosis [16,17]. However, the mechanisms through which DNA lesions trigger the apoptosis cascade are not completely understood. This work investigated apoptosis induction by UV-C irradiation in normal and TCR-deficient cells (CS-B and TTD), in two different cell culture conditions, proliferating and growth arrested by confluence.

The results indicate a relationship between UV-C-induced apoptosis and DNA replication. Confluent fibroblasts, which have a reduced DNA synthesis rate, were more resistant to UV-C-induced apoptosis than proliferating cells. This protection was also observed in cell survival experiments, indicating that it could not result from a simple delay of cell death. The kinetics of CPD removal for proliferating and confluent cells was also analyzed. Although all lineages displayed a slightly faster removal of CPD under confluent condition, this was not enough to explain the higher survival of growth arrested cells, whereas after 24 h the percentage of remaining CPDs was similar in proliferating and confluent cells. The impact of DNA replication on CPD removal under the analyzed condition must be low, as it has been observed by several authors that high UV-C doses causes a strong blockage of DNA replication [18–20]. Therefore, the effects of DNA lesion dilution by cell division and DNA synthesis should also be low in these cells, and most of CPD reduction in Fig. 3 is due to damage removal by DNA repair.

The link between susceptibility to apoptosis and cell proliferating activity was also observed in DNA repair proficient keratinocytes [10]. Keratinocytes that were under cell cycle arrest or senescence became resistant to UV-C-induced apoptosis. The results presented here extend these observations to TCR defective fibroblasts, which were also less susceptible to apoptosis induction when arrested. Studies using human cells with TCR defects showed higher inability to restore normal transcription following UV irradiation, leading to p53 accumulation and apoptosis induction after low UV dose irradiation [4–6,21–23]. The results linking RNA synthesis inhibition and apoptosis induction favor the hypothesis that transcription acts as a damage dosimeter. However, the data presented here do not support this concept, at least for cells maintained quiescent by confluence. The inhibition of RNA synthesis following UV-C treatment was evaluated in growth arrested cells, and the results clearly indicated that the lower apoptosis was not accompanied by a more efficient RNA synthesis recovery. In fact, the kinetics of RNA synthesis inhibition and recovery were similar in growth arrested and proliferating cells. Moreover, TTD1VI cells, which were more resistant to UV-C-induced apoptosis than CS1AN cells, were similarly unable to recover RNA synthesis. Preliminary studies showed that normal and CS1AN cells also accumulated p53 under non proliferative conditions after UV-C irradiation, this accumulation not being associated with apoptosis induction (data not shown). However, the participation of transcription blockage in UV-C-induced apoptosis, especially in proliferating cells, cannot be discarded, as TCR defective cells were more sensitive to UV-C-induced apoptosis than normal cells.

Recently, further evidence pointing to the involvement of DNA duplication in UV-C-induced apoptosis was reported. The decrease of DNA synthesis by serum starvation treatment leads to a reduction of 50% in UV-C-induced apoptosis for both NER deficient and proficient rodent cells [8]. Moreover, inhibition of DNA replication by aphidicolin, following UV-C treatment, prevented apoptosis induction when compared to proliferating cells [12]. McKay et al. [9] have also observed that apoptosis induction in UV-irradiated fibroblasts only occurred after cells have entered S-phase and replicated their DNA. Working with synchronized hamster cell lines, proficient and deficient in TCR, Proietti De Santis et al. [18] have observed that at high doses of UV-C the cells underwent apoptosis without the entry into S-phase. Although this is in apparently contrast with the role of DNA replication in UV-C-induced apoptosis, the increase of G1-phase cells, observed in that work, could be due to a strong blockage of DNA replication at the beginning of S-phase. This blockage would lead to a decrease on the number of cells in S-phase, but could still be triggering apoptosis. Again, it should be emphasized that the confluent cells were more resistant even when irradiated at low UV-C doses.

The UV-C-induced apoptosis may result from DNA replication machinery blockage by DNA photoproducts, or by secondary lesions caused by this obstacle. It has been proposed that the collapsed replication fork in response to UV photoproducts generates double strand breaks (DSBs) [8], and non repaired DSBs may trigger apoptosis [24]. The ATR kinase, a member of the superfamily of phosphatidylinositol-3-kinase-related kinase (PIKK), appears to be the major activator of the replication stress response [25]. This kinase plays an essential role during normal replication [26] and is required for cellular checkpoint responses to stalled replication forks [27]. When activated, this kinase displays a series of signals which control cell cycle transition, DNA repair activity and, in some cases, the induction of apoptosis [28]. Therefore, it is possible that DNA replication of a damaged template would first activate ATR, and subsequent cell events would lead to an active program of cell death. In confluent cells, the lower levels of DNA synthesis should reduce ATR activation, thus increasing cell resistance to UV-C irradiation. Another possibility is that the apoptotic signals depend on both DNA replication and RNA synthesis, consistent with the model proposed by the Ljungman group [7,9], where UV-induced apoptosis results from the collision of transcription blocked complexes with oncoming replication forks. This model fits in with the observations of this work, as the levels of DNA

replication in confluent cells are low, and there would be lower levels of collisions, even though RNA synthesis is still highly inhibited in the growth arrested conditions.

5. Conclusions

These results demonstrate that normal or TCR-deficient fibroblasts are more resistant to UV-C-light when kept in a growth arrested state, a condition which did not significantly affect DNA damage removal, or RNA synthesis recovery. This strongly supports the idea that DNA synthesis of damaged template is necessary for apoptosis induction by UV-C light. Considering that most of the cells in the skin are normally quiescent, and not replicating their DNA, the results observed for confluent cells may correlate as to how cells respond to the UV component (especially UV-B) of sunlight *in vivo*. Although this work was performed in fibroblasts, it is possible that other cell types (such as keratinocytes) may present similar behavior, and thus the differences in apoptosis induction observed in growth arrested cells may have direct implications with photocarcinogenesis. A more extensive work on how growth arrested cells deal with DNA damages is necessary for the understanding of genetic instability and apoptosis mechanisms.

Acknowledgments

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APPENDIX B

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Cellular and Molecular Life Sciences

Direct DNA damage reversal: elegant solutions for nasty problems

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Abstract. The genomic integrity of all living organisms is constantly jeopardized by physical [e.g. ultraviolet (UV) light, ionizing radiation] and chemical (e.g. environmental pollutants, endogenously produced reactive metabolites) agents that damage the DNA. To overcome the deleterious effects of DNA lesions, nature evolved a number of complex multi-protein repair processes with broad, partially overlapping substrate specificity. In marked contrast, cells may use very simple repair systems, referred to as direct DNA damage reversal, that rely on a single protein, remove lesions in a basically error-free manner, show high substrate specificity, and do not involve incision of the sugar-phosphate backbone or base excision. This concise review deals with two types of direct DNA damage reversal: (i) the repair of alkylating damage by alkyltransferases and dioxygenases, and (ii) the repair of UV-induced damage by spore photoproduct lyases and photolyases. (Part of a Multi-author Review)

Keywords. DNA repair, photolyase, methyltransferease, dioxygenase, spore photoproduct lyase, UV damage, alkylation damage.

Introduction

The integrity of the genome of every organism is continuously threatened by environmental agents [e.g. ultraviolet (UV) light, ionizing radiation, chemicals] and endogenously produced cellular metabolites (i.e. reactive oxygen species and other radicals) that damage DNA. Replication of damaged DNA can cause mutations, which ultimately can lead to cancer, while DNA lesions that obstruct replication and transcription can lead to cellular senescence or apoptosis, which are believed to contribute to diseases of aging [1–4]. In contrast to other biomolecules, DNA cannot be replaced, only repaired. Therefore, cells have acquired a variety of DNA repair mechanisms (i.e. nucleotide excision repair, base excision repair, cross-link repair, homologous recombination and non-homologous end joining) with broad (often overlapping) substrate specificities to counteract the harmful effects of DNA injuries (for review see [1, 3]). In addition to these complex, often error-prone, genome maintenance systems (e.g. nucleotide excision repair, involving over 30 proteins), nature has evolved single enzyme mechanisms that can repair lesions without incision of the DNA sugar-phosphate backbone or base excision. The relative simplicity of these repair mechanisms, referred to as direct damage reversal, predicts essentially error-free repair, with, however, a very narrow substrate range as a trade-off. This concise review deals with the two main types of direct DNA damage reversal systems: (i) repair of alkylating damage by alkyltransferases and dioxygenases, and (ii) repair UV light-induced photolesions by spore photoproduct lyases and photolyases.

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Removal of alkylation damage

Alkylation damage

Alkylation damage could be the result of environmental exposure of DNA to compounds like N-

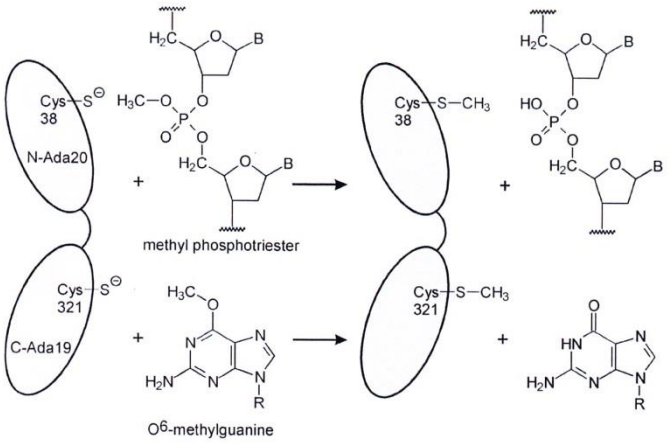

Figure 1. Reaction mechanism for *E. coli* Ada alkyltransferase. Both repair functions are depicted: demethylation of methylphosphotriesters in DNA by the N-terminal part and repair of O^6-meG by the C-terminal part. The methyl groups are transferred to a Cys residue in an irreversible suicide reaction.

methyl-N′-nitro-N-nitroso-guanidine (MNNG), N-methyl-N-nitrosurea (MNU), or methyl methanesulfonate (MMS), which cause a variety of O-alkylated and N-alkylated adducts. Apart from these powerful exogenous alkylating agents, alkylation damage may arise from endogenous sources like S-adenosylmethionine (SAM), a metabolite involved in many biochemical processes (e. g. the immune system, cell membrane maintenance, neurotransmitter degradation) and known to weakly methylate DNA. Biologically important lesions formed in double-stranded DNA (dsDNA) are O^6-methylguanine (O^6-meG), 7-methylguanine (7-meG), and 3-methyladenine (3-meA). In addition, phosphodiester groups in DNA might become methylated. Lesions like O^6-meG are mutagenic and carcinogenic as misincorporation during replication gives rise to G:C → A:T transitions. Although alkylated bases can be removed by multistep excision repair processes, several lesions are also subject to effective and highly specific direct repair mechanisms.

Alkyltransferases

In *Escherichia coli*, the *ada* gene encodes the 39-kD multifunctional repair protein O^6-alkylguanine-DNA alkyltransferase (O^6-Agt), composed of an N-terminal and a C-terminal part separated by a hinge region, and each containing a reactive center. While the 19-kD C-terminal part (C-Ada19) repairs O^6-meG or O^4-methylthymine, the 20-kD N-terminal part (N-Ada20) demethylates methyl phosphotriester lesions in the DNA. In both cases the methyl group is transferred in an irreversible reaction to a cysteine residue, Cys38 in N-Ada20 and Cys321 in C-Ada19 [5, 6] (Fig. 1). The Ada protein is strictly speaking not an enzyme, as it participates in an irreversible suicide reaction and therefore should be considered as a reactant rather than a catalyst.

Further adding to the multifunctionality of the Ada protein, methylation of Cys38 of N-Ada20 converts it into a transcriptional activator that specifically binds to the promoters of the *ada-alkB* operon and the *alkA* and *aidB* genes [7, 8]. The *alkA* gene encodes a 3-meA-DNA glycosylase (involved in base excision repair) and *alkB* a 1-meA/3-meC-DNA dioxygenase (see below). *E. coli* has a second O^6-meG-DNA methyltransferase encoded by the constitutively expressed *ogt* gene. Thus, low levels of methylation damage can be handled by the Ogt protein while in an adaptive response the relatively innocuous methyl phosphotriester lesions serve as a sensor for high methylation levels, invoking a large increase in Ada activity in the cell [9]

In humans, direct damage reversal of O^6-meG lesions requires O^6-meG-DNA methyltransferase (MGMT) also referred to as alkylguanine transferase (AGT). This homolog of C-Ada19 has Cys145, present in a conserved Pro-Cys-His-Arg active site motif, as a methyl recipient [10]. The crystal structure of an MGMT-substrate complex (Fig. 2A) shows that the O^6-meG lesion is flipped out of the DNA duplex with the side-chain of an arginine residue replacing it in the base stack [11]. Methylated MGMT cannot be reused and disappears rapidly from cells by ubiquitin-mediated degradation [12]. The dealkylation activity in human cells might interfere with effective action of alkylating agents used in chemotherapeutic treatment of cancer. The MGMT activity, however, can be

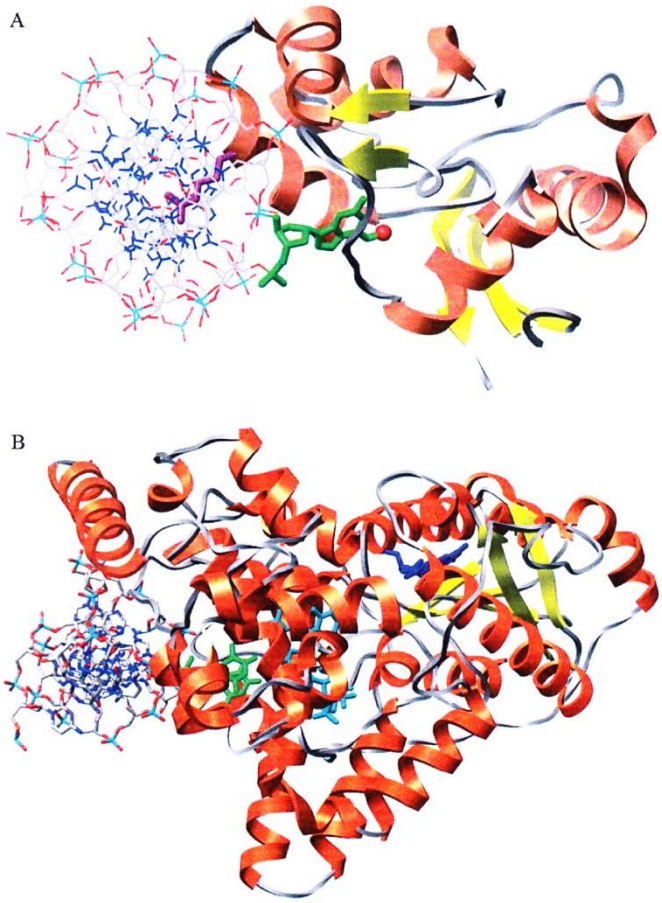

Figure 2. Base flipping in direct repair. (*A*) crystal structure of human MGMT-substrate complex (pdb: 1t38). The MGMT protein is inactive due to a Cys145Ser mutation but still capable of substrate binding. The DNA substrate has a flipped out O^6-meG lesion (green, methyl group in red). Also shown is the side-chain of the Lys128 residue (purple) intruding the DNA duplex to replace O^6-meG. (*B*) Crystal structure of an *Anacystis nidulans* CPD photolyase-substrate postrepair complex (pdb: 1tez). The photolyase chromophores FAD (cyan) and 8-HDF (blue) are shown. The thymine dimer (green) is flipped out of the DNA substrate: its cyclobutane ring is actually broken, probably by the synchrotron radiation used for structure determination. (Figures were prepared using Chimera; α-helices are in orange, sheets in yellow-green and DNA substrates in CPK colors).

reduced by inhibitors like O^6-benzylguanine and combinations of alkylating drugs and MGMT inhibitors are currently tested in clinical trials [10, 13].

Several polymorphisms (mutations) are known for the MGMT gene in the human population, some affecting protein activity or expression level, but associations with cancer risk are not consistent [14]. Some tumor-derived cell lines are very sensitive to alkylating agents. These mex^-/mer^- cells are deficient in O^6-meG repair due to a very low level level of MGMT activity [15]. This repression of MGMT could be linked to extensive CpG methylation in the promoter and transcribed region [16, 17]. Treatment with 5-azacytidine, which reverses CpG methylation, results in upregulation of MGMT activity [18]. The methylation status of the MGMT promoter could be a predictor for the success of tumor treatment with alkylating agents [19, 20]

The role of alkyltransferases in carcinogenesis has been investigated in various transgenic mouse models. Mice expressing the *E. coli ada* gene are protected from hepatocarcinogenic events induced by low doses of alkylating agents [21]. Transgenic mice carrying the human MGMT gene behind a bovine cytokine promoter, which expresses selectively in the epidermis, exhibited a significantly lower tumor incidence in a two-stage topical tumor induction protocol compared to nontransgenic animals (Fig. 3) [22]. Likewise, overexpression of human MGMT in the thymus protects against MNU-induced lymphomas [23],

while brain/liver-specific overexpression of the human gene in a genetic background that predisposes to spontaneous hepatocellular carcinoma reduces tumor prevalence [24].

In contrast, MGMT-deficient mice have been shown to be hypersensitive to alkylating agents like N-methyl-N-nitrosourea and chemotherapeutic drugs as evident from lower LD_{50} values, reduced bone marrow cellularity, and impaired reproductivity of hematopoetic stem cells [25, 26]. Taken together, these experiments show that MGMT protects against carcinogenic events.

Oxidative dealkylation (dioxygenases)

The *E. coli* AlkB protein and its human homologs ABH2 and ABH3 preferentially repair 1-methyladenine and 3-methylcytosine lesions by oxidative demethylation. AlkB has a broader substrate range compared to ABH2/3 as it also repairs e.g. 1-methylguanine and 1-ethyladenine [27]. A polymeric substrate structure is not required as a trinucleotide dTp1-meApT and even 5′-phosphorylated 1-meAMP are demethylated [28]. AlkB and ABH3 prefer single-stranded DNA (ssDNA) or RNA substrates while ABH2 preferentially acts on dsDNA [29].

The subcellular localization in HeLa cells indicates that ABH2 and ABH3 are nuclear enzymes [30], and it has been suggested that ABH2 repairs DNA close to replication forks while ABH3 is involved in repair of nuclear ssDNA and RNA associated with transcriptionally active genes. ABH2 and ABH3 knockout mice as well as double knockout mice are viable and do not show an obvious phenotype. ABH2, but not ABH3, deficient mice accumulate 1-methyl-adenine lesions in the absence of exogenous methylating agents. ABH2-deficient embryonic fibroblasts could not remove MMS-induced lesions [31].

The AlkB and ABH2/3 proteins, belonging to the superfamily of 2-oxoglutarate/iron-dependent dioxygenases [32], contain a Fe^{2+} cofactor and use 2-oxoglutarate and molecular oxygen as co-substrate. The methyl-group of e.g. 1-meA is hydroxylated (Fig. 4) with concurrent formation of succinate and CO_2, followed by the spontaneous release of formaldehyde.

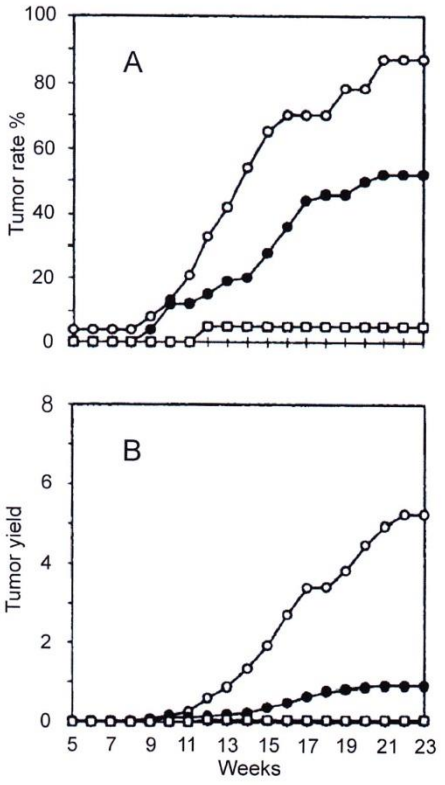

Figure 3. Effect of MGMT expression on tumor response in mice. Transgenic (closed circles) and nontransgenic (open circles) mice were initiated with a single subthreshold dose of MNU (N-nitroso-N-methylurea) followed by treatment with TPA twice a week. Transgenic animals carried the human MGMT gene expressed from the epidermis-specific Ck promoter. Control mice were treated with TPA only (open squares). Tumor response is shown as tumor rate (panel *a*, no. papilloma-bearing mice/no. survivors) and tumor yield (panel *b*, no. tumors/no. survivors). Adapted with permission from [22].

Several crystal structures are available which sustain this reaction mechanism. In the ABH3 structure [33],

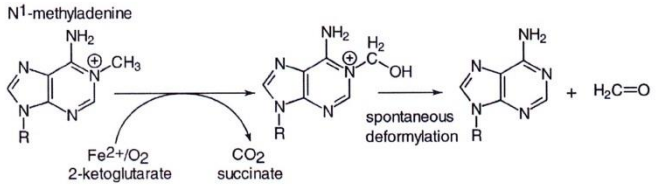

Figure 4. Reaction mechanism for oxidative dealkylation by *E. coli* alkB or human ABH2/ABH3 dioxygenases. The methyl group is hydroxylated using 2-ketoglutarate and O_2 as co-substrates and is then removed as formaldehyde.

a Fe^{2+} ion is coordinated to a conserved His-X-Asp-X_n-His motif and 2-oxoglutarate. Moreover, a flexible hairpin was identified which is probably involved in substrate binding. The AlkB-substrate structure [34] shows, in addition to the flexible lid, a putative tunnel for O_2 diffusion to the active site. The AlkB-substrate structure [35] also shows a base-flipping mechanism with the lesion flipped out by squeezing together the bases flanking the lesion. In the ABH2-substrate complex structure an additional flexible loop is present to bind the DNA strand opposite to the lesion [35].

Reversal of photoproducts

UV-induced photoproducts in DNA

UV radiation induces mainly two types of lesions in DNA, both originating from neighboring pyrimidine bases: cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6–4')pyrimidones [(6–4)photoproducts or (6–4)PPs]. (6–4)PPs, and to a lesser extent CPDs, distort the DNA double helix [36, 37] and both interfere with ongoing transcription and replication. In eukaryotes, the yield and distribution of DNA lesions may be influenced by a higher-order DNA structure as CPD lesions are found predominantly in nucleosomal DNA [38] while (6–4)PPs are primarily found in linker DNA [39].

Interestingly, CPDs are not observed in UV-irradiated bacterial spores [40]. Due to dehydration, spore DNA is forced in an A-like conformation and small acid soluble proteins (SASPs), expressed during spore formation, are bound to the DNA [41]. The very special conditions within the spore suppress the induction of CPDs, and instead promote the formation of a spore photoproduct (SP) which was identified as 5-thyminyl-5,6-dihydrothymine [42]. Moreover, spores contain large amounts of dipicolinic acid [43], which acts as a photosensitizer, improving the yield of spore photoproduct [44].

Despite the fact that most organisms have acquired the nucleotide excision repair system for removal of helix-distorting lesions and other bulky DNA adducts, highly specific direct damage reversal mechanisms exist for the three types of photoproducts.

Spore photoproduct lyase

SPs are specifically repaired by SP lyase in a light-independent process [45]. Spore formation involves the generation of a forespore within the bacterial cell. During this process, SP lyase is synthesized and packed ready for use in the forespore. As the dormant spore is unable to repair its DNA, UV-induced DNA damage will accumulate. Notwithstanding this deficit, spores are highly UV-resistant, as early in spore germination the DNA-bound SASPs are degraded by a specific protease, allowing efficient reversal of SPs into two thymines by SP lyase or, to a lesser extent, removal by nucleotide excision repair.

SP lyase is a 41-kD protein, encoded by the *spl* gene. The SP lyase amino acid sequence of several *Bacillus* and *Clostridium* species is known [46]. All SP lyases contain a conserved Cys-X_3-Cys-X_2-Cys motif characteristic for the radical SAM (S-adenosylmethionine) protein family (recent review [47]), where it is involved in the binding of a [4Fe-4S] cluster. SP lyase was indeed shown to contain such a Fe-S cluster [48]. His_6-tagged SP lyase can be overexpressed in *Bacillus subtilis* spores or *E. coli* cells and purified under anaerobic conditions [49].

SP lyase specifically binds to SP, but not to CPD, lesions in UV-irradiated DNA [50]. Although SP lyase usually repairs SPs in dsDNA, assays with synthetic substrates showed that the enzyme also acts on single-stranded oligonucleotides. Even the minimal substrates SP-TpT and SP-TT (lacking the phosphodiester bond) are converted [51]. The repair reaction is initiated by the ligation of SAM to the [4Fe-4S] cluster of SP lyase (Fig. 5). Electron transfer from the [4Fe-4S] cluster to SAM generates a free 5′-deoxyadenosyl radical, while the methionine part remains bound to the cluster [52]. The 5′-dAdo· radical abstracts the C6-H atom from the SP [53]. Next, the C-C bond between the thymines undergoes scission, followed by the transfer of a H atom from 5′-dAdoH back to the thymine monomer radical. Finally, SAM is formed and released. Although irreversible splitting of SAM has been reported, it most likely acts as a catalytic cofactor for the reversible generation of the reactive 5′-dAdo· radical rather than as (co)substrate [49].

Photolyases

Photolyases are 50-55-kD single-chain flavoproteins that lesion-specifically bind to UV-induced CPDs or (6–4)PPs in the DNA (reviewed in [54, 55]). When these photolyase + UV-DNA complexes are illuminated with visible (blue) or near-UV light, photon energy is used to repair DNA damage. In nature this is very effective: lesions induced by the UV-component of sunlight are repaired by visible light from the same source.

Absorbtion of visible light requires the presence of a chromophoric group. In fact, photolyases contain two, functionally different, chromophoric cofactors. The first chromophore, FAD, acts as the photochemical reaction center. Various FAD redox states have been found in purified photolyases: oxidized FAD, the half-reduced neutral semiquinone radical $FADH^{\cdot}$, and the deprotonated fully reduced form $FADH^-$. Only the

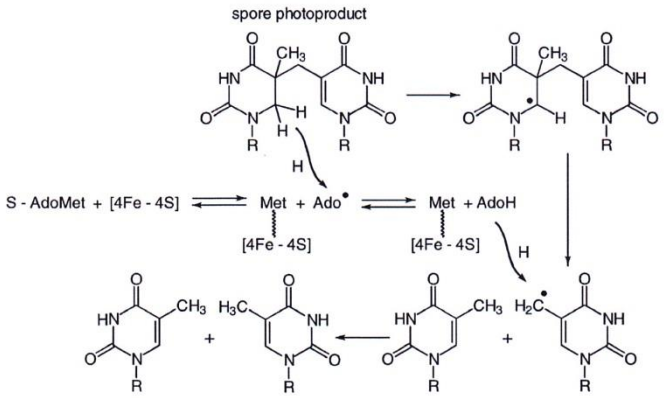

Figure 5. Reaction mechanism of *B. subtilis* spore photoproduct (SP) lyase. A 5′-deoxyadenosyl radical (Ado·), generated by electron transfer from the Fe-S cluster, abstracts an H atom from the SP, which induces scission of the bond between the thymines. The reaction is completed by H-atom transfer from AdoH to the thymine radical.

fully reduced form is biologically active in the repair of UV lesions [56]. It is possible, however, to convert the inactive forms of FAD into $FADH^-$ by photoreduction where an electron is transferred from an exogenous photoreductor through a chain of three tryptophans to FAD [57]. The second chromophore, which is either reduced folate (5,10-methenyl-tetrahydrofolate, MTHF) or 8-hydroxy-5-deazaflavin (8-HDF), acts as an auxiliary light-harvesting antenna. Although the antenna chromophore binding site is specific for either MTHF or 8-HDF, recently a 'promiscuous' binding site was found in *Thermus thermophilus* photolyase which could bind FMN, riboflavin, or 8-HDF [58]. The presence of a second chromophore is not an absolute requirement for activity, as apophotolyases lacking this chromophore are still able to repair UV lesions. Its presence, however, improves the efficiency of photolyase considerably.

Photolyases are specific for either CPD or (6–4)PP lesions. Yet the reaction mechanisms are largely the same: shuttling an electron to the lesion in order to destabilize it. The driving force for this reaction is the energy acquired by the chromophores by absorption of a photon, yielding excited fully reduced $FADH^-$ (Fig. 6). For (6–4)PP photolyases a light-independent step precedes electron transfer. Binding of the (6–4)PP lesion to photolyase induces a rearrangement and an oxetane-ring is formed [59] with the help of two His residues in photolyase [60]. Once a lesion radical is formed, the bond(s) between the pyrimidine rings are broken and finally an electron is transferred back to the catalytic co-factor.

Several crystal structures are available e.g. for *E. coli* [61] and *Anacystis nidulans* [62] photolyase. Structures are very similar, containing an α/β domain with five parallel β-strands and several α-helices and a helical domain containing α-helices only. A similar structure was obtained for the core region of cryptochrome (see below). A photolyase-substrate complex structure [58] clearly shows the dimer lesion completely flipped out of the DNA (Fig. 2B). For a detailed description of reaction mechanism and structures see a recent review [55].

Recently the crystal structure of a *Drosophila* (6–4)PP photolyase-substrate (and product) complex became available [63]. Based on these structures a reaction mechanism has been proposed without an oxetane intermediate.

The photolyase/cryptochrome protein family

At present a vast number of photolyase and photolyase-like genes are known, their gene products sharing a well-conserved core domain of about 500 amino acids to which the two chromophores are bound. Phylogenetic analysis of the amino acid sequence of the core domain of all members of the photolyase family indicates a clear relationship [64]. Yet they are functionally very different, as the phylogenetic tree (Fig. 7) not only comprises genuine photolyases involved in DNA repair but also photolyase-like cryptochromes, which differ from photolyases in having a unique C-terminal extension of varying length. Despite their sequence and structural homology to photolyases, cryptochromes are not capable of repairing UV-induced DNA damage. These findings suggest that multiple gene duplications of an ancestral gene, probably encoding a primordial CPD photolyase, led to functionally diverged proteins [65]. Because of the high UV irradiance due to an anoxic atmosphere, such a photolyase would be essential for survival in early stages of evolution.

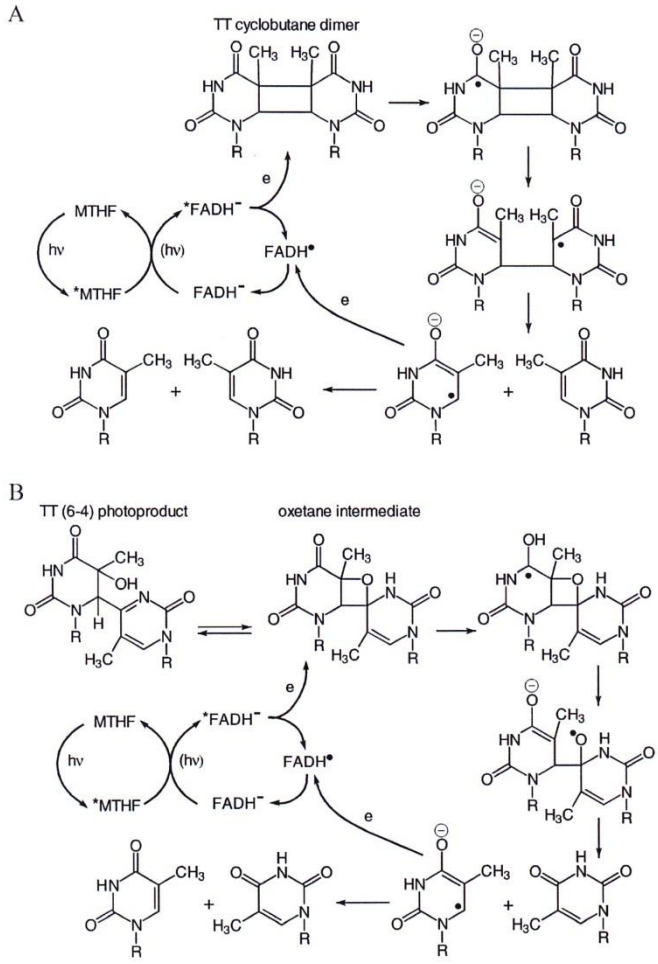

Figure 6. Reaction mechanism of photolyases. In CPD photolyase (*A*) upon absorption of a photon by the MTHF or 8-HDF antenna chromophore energy is transferred to the catalytic co-factor $FADH^-$. Alternatively excited $*FADH^-$ is obtained by direct absorption of a photon. Then an electron is donated from $*FADH^-$ to the dimer lesion which induces breakage of the cyclobutane bonds. Finally, an electron is transferred back from the thymine monomer radical to $FADH^•$, returning photolyase to the initial state. A similar mechanism has been proposed for (6–4)PP photolyase (*B*), but previous to electron transfer an oxetane ring (azetidine ring in case of TC(6–4) photoproduct) is formed in an enzyme-mediated reaction.

Based on amino acid sequences, two phylogenetically somewhat distantly related groups can be distinguished (Fig. 7), each containing organisms from all three primary kingdoms of life. The first group comprises a single main cluster with class II CPD photolyases, mainly but not exclusively, from animals and plants. Placental mammals, in contrast to marsupials, do not possess photolyase genes. Among other members of this cluster are several viral photolyases [66]. The other group is quite heterogeneous. Separate clusters can be distinguished containing class I CPD-photolyases, (6–4)PP photolyases, and animal cryptochromes, plant cryptochromes and cryptochromes-DASH.

Cryptochromes were first identified in plants as blue light photoreceptor proteins involved in light-regulated hypocotyl elongation, induction of flowering and photoentrainment of circadian expression of genes (reviewed in [67]). Animal cryptochromes share a variable C-terminal extension in addition to the photolyase-like core and have a function in the circadian clock. This biological clock generates daily rhythms in metabolism, physiology and behavior, and as the intrinsic periodicity of this internal timekeeper

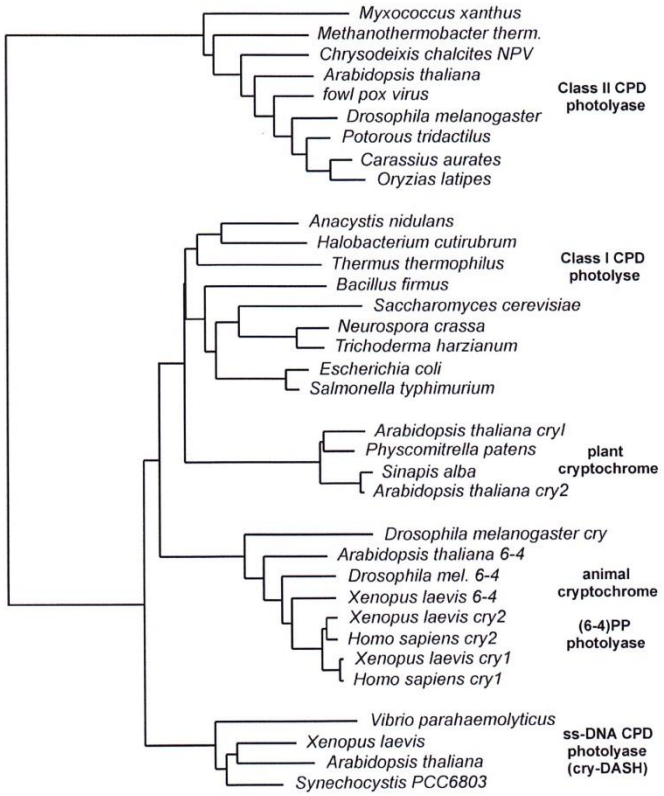

Figure 7. Unrooted phylogenetic tree of the photolyase/cryptochrome family. Amino acid sequences were aligned with ClustalX and a tree was obtained for the core region with the neighbor-joining method (PHYLIP package vs. 3.67 by J. Felsenstein) using the Jones-Taylor-Thornton matrix.

is not exactly 24 h, the clock needs to be reset every day by light. As in plants, *Drosophila* cryptochrome protein acts as a photoreceptor, synchronizing this biological clock to the day-night cycle. In peripheral tissues, but not in the brain, the fly CRY protein is also involved in rhythm generation [68]. Mammals contain two cryptochrome genes (*Cry1* and *Cry2*), inactivation of which, surprisingly, accelerated (*Cry1*) or slowed down (*Cry2*) behavioral rhythms of the mouse [69]. Interestingly, knocking out both genes resulted in a complete loss of rhythmicity, showing that the CRY proteins are indispensable core components of the molecular oscillator underlying circadian rhythmicity [69, 70]. Structure-function analysis of the unique tails of mammalian [71], *Xenopus* [72], *Drosophila* [73], and *Arabidopsis* [74] cryptochromes revealed that the acquirement of different species-specific C-terminal extensions separated cryptochromes from photolyases. Moreover, it caused diversity within the cryptochrome protein family, making cryptochromes act as photoreceptor protein (as in *Arabidopsis*), as combined photoreceptor and core oscillator protein (as in *Drosophila* and zebrafish), or a pure core oscillator protein (as in *Xenopus* and mammals). Intriguingly, the *Drosophila* CRY protein was recently shown to be required for sensing magnetic fields, thus adding a third (clock-independent) function to this class of flavoproteins [75].

The function of cryptochromes-DASH was uncertain as they show aspecific DNA-binding and weak photolyase activity [76, 77] while FAD and MTHF chromophores are present [78, 79]. Eventually they were shown to be photolyases with a clear preference for CPDs in single-stranded nucleic acids [80].

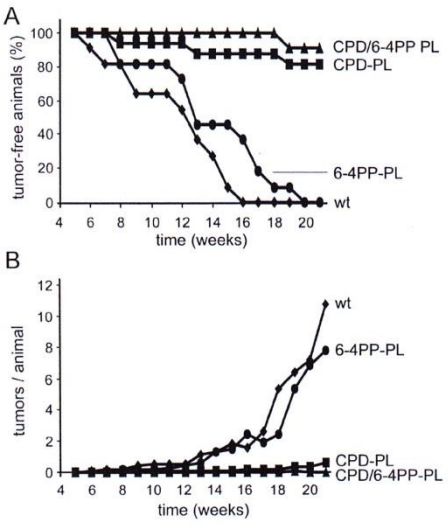

Figure 8. The effect of photoreactivation of CPDs and (6–4)PPs on skin carcinoma occurrence. CPD-photolyase (squares), (6–4)PP-photolyase (circles), and CPD-photolyase/(6–4)PP-photolyase (triangles) transgenic mice, as well as their wild-type littermates (diamonds), received daily treatments of UVB light (500 J/m^2/day), followed by exposure to photoreactivating light for 3 h. (*a*) The fraction of tumor-free mice in time after the first UV treatment. (*b*) The average number of squamous-cell carcinomas per mouse in time after the first UV treatment. Reprinted with permission from [100].

Photolyase in mammals

Except for marsupials, data on the occurrence of photoreactivation in mammals have been conflicting. However, as the only two photolyase-like genes in the human and mouse genome encode cryptochromes (see previous section) [69], the absence of photoreactivating activity in placental mammals seems now generally accepted. Presumably, placental mammals have gone through a nocturnal phase during evolution and consequently lost this important genome protection mechanism against sunlight.

Like most other eukaryotes, mammals remove photolesions from the DNA by the versatile and evolutionary highly conserved nucleotide excision repair (NER) pathway. NER is a complex, multistep process involving the concerted action of 30 or so proteins to sequentially execute damage recognition, chromatin remodeling, incision of the damaged DNA strand on both sides of the lesion, excision of the 27–29mer oligonucleotide containing the damage, and gap-filling DNA synthesis followed by strand ligation. Two NER sub-pathways exist: global genome NER repairs helix-distorting lesions in the entire genome, while transcription-coupled NER specifically repairs transcription-blocking lesions in the transcribed strand of active genes ([1, 3] and a review in this issue by S. Tornaletti). Inborn defects in NER, as in xeroderma pigmentosum, can give rise to striking UV sensitivity and a strong cancer predisposition of the exposed skin [81].

Before the introduction of transgenic technology, mammalian studies on photoreactivation were restricted to photolyase containing non-placental mammals [82, 83]. *Monodelphis domestica* has an efficient photoreactivation pathway which leads to an approximately 80 % reduction in pyrimidine dimers after skin UVB irradiation and to the prevention of UVB-induced local and systemic immunosupression and epidermal Langerhans cell depletion [84]. To date, *M. domestica* and other marsupials are still used as mammalian models for photoreactivation studies since their UV responses have proven to be similar to those of placental mammals [85, 86].

Taking advantage of the absence of photolyases in placental mammals, photolyases have been widely expressed in heterologous mammalian cell systems. This approach led to a better understanding of the specific effects and cellular responses provoked by CPDs and (6–4)PPs. In addition, the structure of CPD and (6–4)PP lesions, their DNA distorting properties, and the interactions between photolysase and DNA has widened the scope for the biological impact of this unique repair enzyme system [87].

After a microinjected microbial photolyase was shown to rapidly eliminate CPD lesions in UV-exposed human fibroblasts [88], in the following years several research groups generated mammalian cells transiently or stably expressing photolyases. Experiments with CPD-photolyase transgenes in normal (NER-proficient) human cell lines revealed that CPDs are an instigator of UV-mediated apoptosis [89, 90]. The introduction of CPD or (6–4)PP photolyases into mouse cells carrying transgenic mutation reporter genes demonstrated that photoreactivation of CPDs, rather than (6–4)PPs, substantially lowered the mutation rate, implying that this lesion type is primarily responsible for the UVB-induced mutations in mammalian cells [91]. Likewise, expression of CPD photolyase in cells from patients with xeroderma pigmentosum complementation group A (XP-A), a cancer-prone NER-deficient syndrome with increased UV sensitivy, demonstrated that the removal of CPD lesions increased UV resistance and reduced the mutation frequency. Since UV survival was increased, but was not the same as in wild-type cells, these results suggested that CPDs, together with other DNA lesions [probably including (6–4)PPs] can lead to

cell killing and mutations [92]. Although these results partially elucidate the role of CPDs in UV-induced cell responses, it should be noted that while NER is efficient at removing (6–4)PPs and other types of helix-distorting damage, CPDs are a poor substrate. Accordingly, except for transcription-coupled NER of the template strand of active genes, CPDs are removed at substantially reduced rates by global genome NER in human cells and virtually not at all in rodent cells [93, 94].

Indeed, by expressing either (6–4)PP or CPD photolyases in XP-A and other NER-deficient cells, it was later shown that removal of the less-abundant (6–4)PPs decreased the apoptotic response to the same extent as or even better than removal of CPDs [95–97]. These studies therefore suggest that (6–4)PP lesions are more potent on the induction of apoptosis than CPD lesions, which tend to induce cell cycle arrest. These results corroborate the hypothesis that in NER-deficient cells both lesions have equally important roles on outcome after UV radiation but that on DNA repair-proficient cells only CPDs are responsible for the induction of apoptosis, probably due to the rapid repair of (6–4)PP by NER [98].

Recently, we generated *Potorous tridactylus* CPD-photolyase and *Arabidopsis thaliana* (6–4)PP-photolyase transgenic mice to assess the relative contribution of CPDs and (6–4)PPs to the detrimental effects of UV light on the skin [99, 100]. Ubiquitous expression of CPD photolyase was shown to protect the UV-exposed mouse skin from hyperplasia, apoptosis, and sunburn in a light-dependent manner [99]. Moreover, expression of CPD photolyase in keratinocytes only was sufficient to block apoptosis in keratinocytes and (non-photolyase-expressing) dermal fibroblasts, which points to intercellular anti-apoptotic signaling. Importantly (as shown in Fig. 8), photoreactivation of CPD lesions provided superior protection against UV-induced carcinogenesis, whereas direct repair of (6–4)PPs provided marginal protection [100]. In line with this finding CPDs formed the main trigger for mutagenic events and formation of mutant *p53* patches, composed of preneoplastic epidermal cells [100]. Likewise, preferential elimination of CPDs (but not of (6–4)PPs) from basal keratinocytes dramatically reduced UV skin cancer induction [101]. Apart from mutation induction, immunesurveillance plays a pivotal role in carcinogenesis [102]. Ubiquitous rather than keratinocyte-specific removal of CPDs abolishes UV-induced immunosuppression, while photoreactivation of (6–4)PPs does not have any effect. These findings suggest that CPD lesions in cells other than keratinocytes are a prerequisite for UV-induced immune suppression. In conclusion, and in line with *in vitro* studies, in repair-proficient placental mammals, CPDs are responsible for the negative effects of UV light. To reveal the true relative potential of CPDs and (6–4)PPs in these processes, the same set of experiments should be performed on photolyase transgenic mice, crossed in an NER-deficient *Xpa* background.

Photoreactivation of CPD lesions is not limited to transgenic animals. Topical application of photolyase-containing liposomes on the UV-exposed skin of volunteers, in combination with exposure to photoreactivating light, was shown not only to reduce the amount of CPDs by 40–45% but also to prevent UVB-induced immunosupression [103]. Altogether, these results indicate that placental mammalian cells still have the necessary conditions to allow the enzymatic activity of photolyases. Further investigation is still required, but in the near future we might think about the broad use of these enzymes, together with UV sunscreens, to prevent malignant UV sunlight effects.

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APPENDIX C

6

Recombinant Viral Vectors for Investigating DNA Damage Responses and Gene Therapy of Xeroderma Pigmentosum

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1. Introduction

1.1 The dark side of the sun

The genome of all living organisms is constantly threatened by a number of endogenous and exogenous DNA damaging agents. Such damage may disturb essential cellular processes, such as DNA replication and transcription, thereby resulting in double-strand breaks (referred to as 'replication fork collapse'), which can lead to chromosomal aberrations and/or cell death, ultimately contributing to mutagenesis, early aging and tumorigenesis (Ciccia & Elledge, 2010). One of the most important exogenous sources of DNA damage is the ultraviolet radiation (UV) component of sunlight, since it is responsible for a wide range of biological effects, including alteration in the structure of biologically essential molecules, such as proteins and nucleic acids. Indeed, UV is one of the most effective and carcinogenic exogenous agents that act on DNA, threatening the genome integrity and affecting normal life processes in different aquatic and terrestrial organisms, ranging from prokaryotes to mammals (Rastogi et al., 2010). In addition, UV is the major etiologic agent in the development of human skin cancers (Narayanan et al., 2010).

Sunlight is the primary UV source, whose spectrum is usually classified according to its wavelength in UVA (320-400 nm; lowest energy), UVB (280-320 nm) and UVC (200-280 nm; highest energy). Although these three UV bands are present in sunlight, the stratospheric ozone layer entirely blocks the UVC and most of UVB, thus the solar UV spectrum that reaches the Earth's ground is composed by UVA and some UVB, even though ozone layer depletion can cause changes in this spectral distribution (Kuluncsics et al., 1999).

The chemical nature and efficiency in the formation of DNA lesions greatly depend on the wavelength of the incident photons. Despite its lowest energy, UVA light can deeply penetrate into the cells, mostly damaging DNA by indirect effects caused by the generation of reactive oxygen species which may react with nitrogen bases, resulting in base alterations and breaks in the DNA molecule. On the other hand, UVB can be directly absorbed by DNA bases, producing two main types of DNA damage, the cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone-(6-4)-photoproducts (6-4PPs), both resulting from

covalent linkages between adjacent pyrimidines located on the same DNA strand, which leads to severe structural distortions in the DNA double helix. Interestingly, it has been recently demonstrated that UVA can also be directly absorbed by the DNA molecule, efficiently generating both CPDs and 6-4PPs (Schuch et al, 2009).
CPDs correspond to the formation of a four-member ring structure involving carbons C5 and C6 of both neighboring bases, whereas 6-4PPs are formed by a non-cyclic bond between C6 (of the 5'-end) and C4 (of the 3'-end) of the involved pyrimidines. Since those lesions induce strong distortions in the DNA molecule, they may lead to severe consequences to the cell if not properly removed, such as transcription arrest and replication blockage, thus disturbing cell metabolism, interfering with the cell cycle and, eventually, inducing cell death. DNA mutations can also result from misleading DNA processing. Long term consequences may include even more deleterious events, such as photoaging and cancer (Sinha & Häder, 2002; Narayanan et al., 2010; Rastogi et al., 2010).

1.2 DNA repair of UV lesions and related human syndromes

To ensure the maintenance of the genome integrity, several mechanisms that counteract DNA damage have emerged very early in evolution, including an intricate machinery of DNA repair, damage tolerance, and checkpoint pathways (Figure 1).

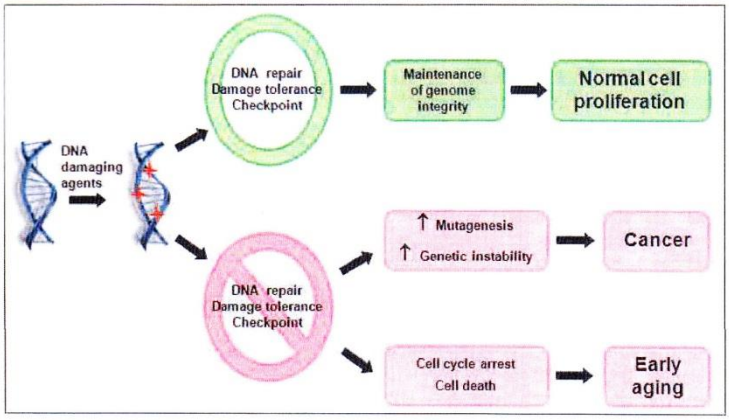

Fig. 1. Main consequences of DNA damage. DNA damage can be induced by a variety of endogenous and exogenous agents. Several mechanisms, including an intricate machinery of DNA repair, damage tolerance, and checkpoint pathways, counteract DNA damage, aiming for the maintenance of genome stability, and guaranteeing normal cell proliferation. When these mechanisms fail, errors in DNA replication and/or aberrant chromosomal segregations take place, increasing mutagenesis and genetic instability and contributing to a higher risk of cancer development. Alternatively, these damages may disturb the transcription and/or cause replication blockage, leading to cell death , thus contributing to early aging.

The nucleotide excision repair (NER) is one of the most versatile and flexible DNA repair systems, removing a wide range of structurally unrelated DNA double-helix distorting lesions, including UV photoproducts, bulky chemical adducts, DNA-intrastrand crosslinks, and some forms of oxidatively generated damage by orchestrating the concerted action of over 30 proteins, including the seven that are functionally impaired in xeroderma pigmentosum patients (XPA to XPG) (Costa et al., 2003; de Boer & Hoeijmakers, 2000). The NER pathway has been extensively studied at the molecular level in both prokaryotic and eukaryotic organisms. Depending on whether the damage is located in a transcriptionally active or inactive domain in the genome, its repair will be processed by one of two NER subpathways: global genome repair (GG-NER) or transcription-coupled repair (TC-NER). Indeed, while GG-NER is a random process, removing distorting lesions over the entire genome, TC-NER focus on those lesions which block RNA polymerases elongation, thus being highly specific and efficient (Fousteri & Mullenders, 2008; Hanawalt, 2002).

Briefly, the NER pathway involves a sequential cascade of events that starts with damage recognition, which defines the major difference between GG-NER and TC-NER. The latter is triggered upon blockage of RNA polymerase translocation at the DNA damage site, whereas GG-NER is evoked by specialized damage recognition factors, including the XPC-hHR23B heterodimer, and also XPE for certain lesions. The subsequent steps are carried out by a common set of NER factors that are shared by both subpathways and involve opening of the DNA helix around the lesion site by the concerted action of two helicases; dual incision of the damaged strand at both sides of the lesion by two endonucleases; removal of the damaged oligonucleotide (24-32 mer); gap filling of the excised patch using the undamaged strand as a template by the action of the replication machinery; and ligation of the new fragment to the chromatin by DNA ligase (Cleaver et al., 2009; Costa et al., 2003). Even though the core NER proteins that carry out damage recognition, excision, and repair reactions have been identified and extensively characterized, the regulatory pathways which govern the threshold levels of NER have not been fully elucidated (Liu et al., 2010). A schematic representation of this repair mechanism in humans is illustrated in Figure 2.

Several human autosomal recessive diseases are caused by dysfunction of the NER pathway, xeroderma pigmentosum (XP) being the prototype. Although this chapter will mainly focus on the XP syndrome, deficiencies in NER can also lead to other genetic diseases, such as trichothiodystrophy (TTD), Cockayne syndrome (CS), cerebro–oculo–facial–skeletal syndrome (COFS) and UV-sensitive syndrome (UVsS), all of which have photosensitivity as a common feature.

Xeroderma pigmentosum (XP) is a rare human disorder transmitted in an autosomal recessive fashion characterized by severe UV light photosensitivity, pigmentary changes, premature skin aging and a greater than 1,000-fold increase incidence of skin and mucous membrane cancer, including squamous and basal cell carcinomas and melanomas, with a 30-year reduction in life span (Cleaver et al., 2009; Karalis et al., 2011; Narayanan et al., 2010). In addition to cutaneous features, patients often develop ocular abnormalities, including neoplasms which may cause blindness. For most patients, often referred to as classical XP, this syndrome is caused by an impaired GG-NER activity, with or without deficiencies in TC-NER, determined by mutations in one of seven NER genes (*XPA* to *XPG*). When TC-NER is also affected (mutations in *XPA*, *XPB*, *XPD* and *XPG* genes), accelerated neurodegeneration may also occur in a substantial number of patients, suggesting increased neuronal cell death due to accumulated endogenous damage (Gerstenblith et al., 2010; Hoeijmakers, 2009). The eighth complementation group corresponds to the XP-variant

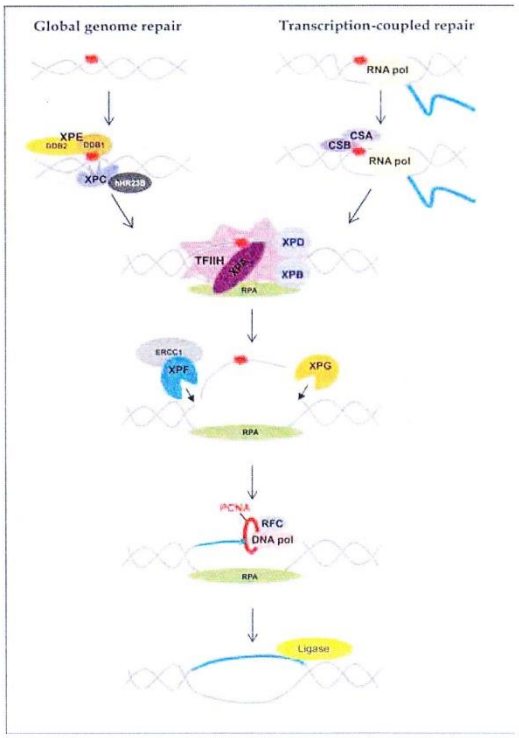

Fig. 2. Schematic representation of repair of DNA lesions by nucleotide excision repair (NER). Depending on where the DNA damage is located in the genome, it will be processed by one of the two NER subpathways: the global genome repair (GG-NER) or the transcription-coupled repair (TC-NER), that basically differ in the lesion recognition step. Lesions occurring randomly in the genome are recognized by the XPC-HR23B complex, with the participation of XPE (DDB1-DDB2) for certain lesions, both complexes are GG-NER-specific. On the other hand, lesions present in the transcribed strand of active genes that lead to the RNA polymerase arrest trigger the TC-NER subpathway, which involves the CSA and CSB proteins. The following steps are common to both subpathways. The DNA double helix around the lesion is opened by XPB and XPD (helicases belonging to the TFIIH complex) and the single strand region is stabilized by RPA, allowing damage verification by the XPA protein. The DNA around the damaged site is then cleaved by the XPF–ERCC1 and XPG endonucleases, excising an oligonucleotide of 24-32 mer, and this patch is resynthesized by the replication machinery using the undamaged strand as a template. Finally, the new fragment is sealed to the chromatin by the DNA ligase.

(XPV) patients, whose XP phenotype is related to mutations in the *POLH* gene, which encodes the translesion synthesis DNA polymerase eta responsible for the replication process on UV-irradiated DNA templates (Johnson et al., 1999; Masutani et al., 1999).
A list of NER genes, which are related to XP syndrome, with their specific functions is given in Table 1.

Gene	Protein	Protein size (A.A.)	Function	Pathway
XPA	XPA	273	Interacts with RPA and other NER proteins, stabilizing ssDNA regions and also facilitating the repair complex assembly.	GG-NER TC-NER
XPB	XPB	782	Belongs to TFIIH complex, working as a 3` → 5` helicase.	GG-NER TC-NER
XPC	XPC	940	Responsible for lesion recognition in GG-NER.	GG-NER
XPD	XPD	760	Belongs to the TFIIH complex, working as a 5` → 3` helicase.	GG-NER TC-NER
DDB2	XPE/p48 subunit	428	Forms a complex with XPE/p127 subunit, which is believed to facilitate the identification of lesions that are poorly recognized by XPC-hHR23B.	GG-NER
XPF	XPF	905	Found as a complex with ERCC1, which functions as an endonuclease 5′ of the lesion.	GG-NER TC-NER
XPG	XPG	1186	Functions as an endonuclease 3′ of the lesion.	GG-NER TC-NER

*GG-NER- global genome repair; TC-NER- transcription-coupled repair.

Table 1. List of NER genes related to xeroderma pigmentosum and their roles in human DNA repair.

The Cockayne syndrome (CS) is predominantly a developmental and neurological disorder, caused by mutations leading to a defective TC-NER, which prevents recovery from blocked transcription after DNA damage. CS patients are characterized by early growth and development cessation, severe and progressive neurodysfunction associated with demyelination, sensorineural hearing loss, cataracts, cachexia, and frailty (Weidenheim et al., 2009). Curiously, although severe photosensitivity is a common feature reported for most CS patients, it is not linked to an increased frequency of skin cancers, like it is in XP patients. Interestingly, specific mutations in one of three XP genes (*XPB*, *XPD* and *XPG*) may result in a clinical phenotype which reflects a combination of the traits associated with XP and CS (XP/CS patients). This observation indicates that simultaneous defects in GG-NER and TC-NER can cause mutagenesis and cancer in some tissues and accelerated cell death and premature aging in others (Hoeijmakers, 2009).

The hallmark of trichothiodystrophy (TTD) is sulfur-deficient brittle hair, caused by a greatly reduced content of cysteine-rich matrix proteins in the hair shafts. In severe cases, mental abilities are also affected. Abnormal characteristics at birth and pregnancy complications are also common features of TTD, which may imply a role for DNA repair genes in normal fetal development (Stefanini et al., 2010). As CS patients, TTD patients do not present a high incidence of skin cancers. Genetically, three genes were identified for this disease (*XPB*, *XPD* and *TTDA*), but most TTD patients exhibit mutations on the two alleles of the *XPD* gene (Itin et al., 2001).

Cerebro–oculo–facial–skeletal syndrome (COFS) is a disorder determined by mutations in *CSB*, *XPD*, *XPG* and *ERCC1* genes, leading to a defective TC-NER (Suzumura & Arisaka, 2010). It is characterized by congenital microcephaly, congenital cataracts and/or microphthalmia, arthrogryposis, severe developmental delay, an accentuated postnatal growth failure and facial dysmorphism.

Photosensitivity and freckling are the main features of patients with UV-sensitive syndrome (UVsS), but these patients have mild symptoms and no neurological or developmental abnormalities or skin tumors. Although other genes may be involved, mutations in the *CSB* gene were found in some of these patients, leading to defective TC-NER of UV damage (Horibata et al., 2004; Spivak, 2005).

Therefore, the general relationship between defects in NER genes and clinical disease phenotypes is complex, since mutations in several genes can cause the same phenotype, and different mutations in the same gene can cause different phenotypes (Kraemer et al., 2007).

Even though DNA repair malfunctions are autosomal recessive diseases and their incidence is therefore relatively low (~1/100,000), many of the individuals with DNA repair deficiencies die in early childhood since there is no effective treatment, only palliative care. Therefore, the search for a long-term treatment has been intense. Several strategies using recombinant viral vectors are being used in order to improve the resistance of cells from these patients to DNA damaging agents (Lima-Bessa et al., 2009; Menck et al., 2007). Also, the studies of DNA repair mechanisms have yielded a better understanding of specific cell processes which lead to human diseases such as cancer, neurodegeneration and aging (Hoeijmakers, 2009). This review will focus on the use of recombinant viral vectors for the purposes of investigating both the cellular responses to DNA damage and the perspectives of providing therapy for XP patients.

2. Recombinant viral vectors as gene delivery tools

An ideal gene delivery tool should have the ability to transduce proliferating and fully differentiated cells with high efficiency; mediate high-level, prolonged and controlled transgene expression; have little toxicity (both at cellular and organism levels); elicit small immune responses *in vivo*; and be able to accommodate large DNA fragments for transgene transduction (Howarth et al., 2010). Unfortunately, there is no single tool that fulfills all these criteria.

Viruses have had million of years to improve their capacity to infect cells with the aid of evolutionary pressures. Researchers have been trying to take advantage of this ability creating recombinant viral vectors. In general, for that purpose, the viral genome is manipulated and sequences needed to form the infective virion are deleted, opening space to insert the transgene of interest.

Several viral vectors have been created and the most widely used are: adenovirus, retrovirus (including lentivirus) and adeno-associated virus. The main characteristics of these vectors are presented in Table 2.

Virus	Nucleic acid	Genome size (Kb)	Envelope	Virion size (nm)	Integration	Transgene size (Kb)	Immune response	Transgene expression
Adenovirus	dsDNA linear	36		90	episomal	8 - 25	***	days - months
Adeno-associated virus	ssDNA linear	4.7		25	site-specific	4.7 - 9	*	months-years
Retrovirus	ssRNA (homodimer)	7 - 12	*	100	random	<10	**	years
Lentivirus	ssRNA (homodimer)	9	*	100	random	10 - 16	**	years

Table 2. Main features of viruses currently used as recombinant vectors for gene delivery.

Searching for the perfect gene delivery tool, intense modifications have been added to the vectors' genomes, nucleocapsid and envelopes, always searching for less immunogenic vectors, with higher and more specific transduction properties. Currently, recombinant viruses are the vector of choice for research and clinical trials worldwide, but still only few phase II or III trials are being conducted (Atkinson & Chalmers, 2010). All viral vectors cited here have already been used in *in vitro*, *ex vivo* and *in vivo* experiments and in clinical trials.

2.1 Recombinant adenoviral vectors

Adenoviruses (Ad) are non-enveloped double-stranded DNA viruses with tropism for the respiratory and ocular tissues. The first generation recombinant vector can carry up to 8 Kbp of DNA, while the last generation, in which the viral DNA sequence is completely deleted (also named gutless), is able to efficiently transduce over 25 Kbp of DNA (Atkinson & Chalmers, 2010).

Despite the fact that the gutless vector needs the aid of helper viral proteins supplied *in trans*, adenoviral vectors are easily produced in high titers. Once the transgene has been delivered inside the nucleus it remains episomal, reducing the risk of tumorigenesis induced by insertional mutagenesis. On the other hand, the episomal DNA is not replicated and its segregation in mitosis leads to the eventual loss of the transgene in the daughter cells. Thus, the transgene expression is short-lived. A possible solution is to add a site-specific integration sequence next to the transgene, leading to a prolonged transgene expression (Atkinson & Chalmers, 2010). Another advantage of the adenoviral vectors is their ability to transduce post mitotic cells since the transgene is already delivered in its active form, as a double-stranded DNA. This property is of particular interest when aiming for gene therapy in neurons (Atkinson & Chalmers, 2010).

The biggest challenge for the use of adenoviral vectors *in vivo* is the immunological response it elicits. This strong response is not only due to the natural immunogenicity of its components, but also to pre-existing immunity caused by previous contact with at least one of the over 50 serotypes of human infecting adenovirus (Seregin & Amalfitano, 2009). Taking into consideration that these vectors are only capable of a transient expression of the transgene and that repeated dosage might be necessary, a strong immune response is very

undesirable. Possible alternatives to circumvent this issue are: manipulation of the viral capsid proteins and DNA, making them less immunogenic; the usage of a different serotype on each application; and the use of immunosuppressants (Atkinson & Chalmers, 2010; Seregin & Amalfitano, 2009).
The great importance of the immunological response against a gene therapy vector was brought to attention when, in 1999, a patient suffering from an ornithine transcarbamylase deficiency, died due to an unexpected inflammatory response reaction to the adenoviral vector used in a clinical trial (Edelstein et al., 2007). Still, adenoviral vectors are currently the most widely used viral vectors in clinical trials, accounting for approximately 24% of all vectors used in gene therapy clinical trials (Edelstein et al., 2007; Hall et al., 2010).

2.2 Recombinant adeno-associated viral (AAV) vectors

Adeno-associated viruses (AAV) are non enveloped, single-stranded DNA, with serotype-specific tropism viruses. To date, 12 serotypes have been identified in primate or human tissues (Schmidt et al., 2008) in a total of over 100 known serotypes (Wang et al., 2011). Their productive lytic infection depends on the presence of a helper virus, adeno or herpesvirus, that provide *in trans* the necessary genes for the AAV replication and virion production. In the absence of a helper virus, the AAV establishes its latent cycle integrating specifically in the 19q13.4 region of the human genome (Daya & Berns, 2008). The site-specific integration is mainly dependent on the virus internal terminal repeats (ITRs), the integration efficiency element (IEE) and Rep 68 and Rep 78 genes. In the 19q13.4 region, several muscle-related genes are present, including some responsible for actin organization. No significant side effects have been observed due to AAV genome integration in this chromosome region (Daya & Berns, 2008).
The onset of transgene expression delivered by an AAV vector is delayed, usually starting several days after the transduction, probably due to the time invested in the synthesis of the DNA second strand (Michelfelder & Trepel, 2009). Although late, the transgene expression is long lasting and there is a very low humoral response, mainly related to previous exposure to the viral antigens (Daya & Berns, 2008). Despite the small size of the AAV nucleocapsid and genome, it has been shown that transgenes up to 7.2 Kb can be delivered by AAV vectors, but the oversized genomes reduce at least 10 fold the transduction efficiency (Dong et al., 2010). Several strategies have been developed seeking to optimize the vector capacity, such as the *trans*-splicing vector. With the simultaneous usage of two AAV vectors, this technology takes advantage of the concatamers formed by the ITRs that can recombine to form the desired transgene inside the transduced cell. These trans-splicing vectors allow the final transgene to have up to 9 Kb (Daya & Berns, 2008).
Only recently adeno-associated viral vectors started being used in gene therapy research and account for less than 4% of all vectors used in gene therapy clinical trials (Edelstein et al., 2007; Hall et al., 2010). Although these vectors do not behave as the parental virus, since they do not integrate in the genome (due to the lack of the REP protein), gene expression can be very long and elicit low immunological responses, making AAV vectors promising in gene therapy investigations.

2.3 Recombinant retroviral vectors

The *Retroviridae* family is characterized by a single-stranded RNA genome which can only replicate inside the host cell with the aid of an RNA-dependent DNA polymerase, the reverse transcriptase. This enzyme transcribes the virus' RNA into a DNA sequence that the host cell machinery can transcribe and translate (Froelich et al., 2010).

Retroviral vectors are capable of transducing a wide range of cell types, are able to accommodate extensive changes in their genome, accept long transgenes, have low immunogenicity, can be produced in high titers, and promote a prolonged transgene expression due to their ability to integrate into the host cell genome (Froelich et al., 2010). On the other hand, most retroviral vectors can only transduce replicating cells since the transport of the transcribed viral DNA to the nucleus is mitosis-dependent. Additionally, there is always the risk of insertional mutagenesis due to the semi-random integration of the vector genome in the host cell's genome (Froelich et al., 2010). Nowadays, the most widely used retroviruses as gene therapy tools are the lentiviruses (LVs), such as the human immunodeficiency virus (HIV). These vectors have the same advantages as other retroviral vectors and are capable of transducing post mitotic cells. Moreover, the LVs tend not to integrate by transcription initiation sites, reducing the risk of insertional tumorigenesis (Froelich et al., 2010).

The retroviral vectors were the first vectors used in gene therapy clinical trials in 1989 (Edelstein et al., 2007, Rosenberg et al., 1990) and are extensively used in fundamental biological research, functional genomics and gene therapy (Mátrai et al., 2010). In 2004, 28% of the clinical trials involving viral vectors included retroviral vectors (Edelstein et al., 2007); in 2010 that number dropped to approximately 23% (Voigt et al., 2008). This drawback is due to the unfortunate events of the French severe combined immunodeficiency (SCID) trial in 2002, where two out of ten children died in consequence of a leukemia, which was related to the insertional mutagenesis of the retroviral vector used (Edelstein et al., 2007).

Since then, special attention has been paid to the safety of these vectors as many are known to derive from viruses that cause severe diseases, such as the acquired immunodeficiency syndrome (AIDS). Strategies are constantly developed to prevent the risk of insertional mutagenesis. For that purpose, in addition to the virions being replication-defective, generated by *trans*-complementation, several further manipulations of the viral genome were made. The development of a self-inactivating (SIN) vector (Iwakuma et al., 1999) prevents horizontal and vertical gene transfer and diminishes the probability of the production of a replicating virion or over-expression of a host cell oncogene (Edelstein et al., 2007).

3. Investigating DNA damage responses with adenoviral vectors in human cells

3.1 *In vitro* and *in vivo* adenoviral gene transduction for the correction of DNA repair defects

The knowledge of the molecular defects in XP cells was the starting point for understanding how human cells handle lesions in their genome. So far, different techniques have been used to study DNA repair mechanisms and reverse malfunctions in this essential system. One powerful tool employed in these studies has been the use of recombinant adenoviral vectors to transduce DNA repair genes directly into human skin cells, aiming to improve the knowledge of basic mechanisms that cells use to protect their genome.

Experiments using first generation recombinant adenoviral vectors have been successfully employed in the transduction of both SV40-transformed and primary fibroblasts derived from XP-A, XP-C, XP-D and XP-V patients (Armelini et al., 2007). The expression of the respective functional proteins in all transduced defective cell populations was significantly increased, reaching levels even higher than seen for wild type cells (Armelini et al., 2005;

Lima-Bessa et al., 2006; Muotri et al., 2002). Moreover, different phenotypical analyses, including cell cycle, apoptosis and cell survival assays, have been carried out, all indicating that the protein expression mediated by the recombinant adenoviruses was clearly accompanied by the recovery of the DNA repair ability and increased resistance to UV radiation, thereby demonstrating functional correction of the XP phenotype. It is worth mentioning that, even though transgene expression mediated by adenoviruses is typically short-lived, sustainable high expression of XPA and XPC proteins with parallel increased UV-irradiation resistance was obtained even two months after cell transduction (Muotri et al., 2002).

For XP-A, XP-C and XP-D transduced cell lines, phenotypic analyses also involved assays aiming to investigate their ability to perform DNA repair after UV irradiation. This has been measured through determination of unscheduled DNA repair synthesis (UDS), which corresponds to the incorporation of [methyl-^{3}H] thymidine in cells that are not in S-phase, and is visualized by autoradiography as the presence of radioactive grains inside nuclei. Interestingly, UDS activity in all transduced deficient cell lines was restored to levels comparable to NER proficient cell lines, indicating those cells became able to efficiently remove UV lesions by restoring NER activity.

It is well known that UV radiation promotes DNA elongation delay as a result of replication blockage by UV photolesions (Cleaver et al., 1983), which can be easily seen by running pulse-chase experiments in alkaline sucrose gradients. Using this approach, it has been possible to show that XP-V transduced cells were able to elongate nascent DNA on UV-damaged DNA templates as efficiently as wild type cells (Lima-Bessa et al., 2006), once again demonstrating the great potential of recombinant adenoviruses in the transduction and expression of functional proteins.

One interesting conclusion came from the observation that even though *XPA*, *XPC* and *XPD* genes were over-expressed in all transduced cell lines when compared to NER proficient cells, this had no impact in the UV-resistance or NER capability, suggesting that neither of these proteins is limiting for NER in human cells. Another possible explanation is that once the NER pathway requires a coordinated action of several proteins, increasing only one of these proteins does not result in speeding up removal of the DNA lesions. Similarly, the excess of polη (*XPV*) mediated by adenoviral transduction has not affected cell survival nor elongation of replication products in UV-treated XP-A human cells, suggesting not only that polη is not a limiting factor for the efficient replication of the UV-damaged DNA in XP-A cells, but also demonstrating that the deleterious effects caused by the remaining DNA lesions in the genome cannot be mitigated by an efficient bypass mediated by polη.

However, the potential of such vectors is not restricted to *in vitro* assays. Indeed, another real perspective is their use to investigate the molecular mechanisms of DNA repair and their consequences *in vivo*, thus opening new avenues for a better understanding of cellular and physiologic responses to DNA damage. *In vivo* experiments may also help to establish the relationship between DNA repair, cancer and aging, as mice models for different DNA repair syndromes have been developed by different groups worldwide. Despite the extensive use of these models to broaden the understanding of several DNA repair related disorders, little work has been done *in vivo* testing gene therapy strategies for these diseases. Indeed, up to the present moment, only one study showed an efficient *in vivo* gene therapy protocol for complementation of the XP phenotype (Marchetto et al., 2004).

Exciting results by Marchetto and co-workers showed that the administration of subcutaneous injections of an adenoviral vector carrying the *XPA* human gene directly into

the dorsal region of XP-A knockout mice led to an extensive expression of the heterologous protein in different skin cells, including dermal fibroblasts, cells of the hair follicle and basal replicating keratinocytes, which are believed to be the starting point of most skin tumors. As a result, the repair capability of these transduced cells was restored, thus preventing UVB-induced deleterious skin effects, such as persistent scars, skin hyperkeratosis and, ultimately, avoiding the formation of squamous cell carcinomas (Marchetto et al., 2004).
Despite the promising results of this work, no others followed. Researchers are now aware of several possible limitations and complications of gene therapy after some unexpected severe events in clinical trials (Edelstein et al., 2007) and are spending more time improving gene targeting tools and techniques before risking *in vivo* approaches. In that sense, extreme progress has been made with experiments *in vitro,* as previously presented. A general panel showing the main uses of the recombinant adenoviral vectors carrying DNA repair genes is presented in Figure 3.

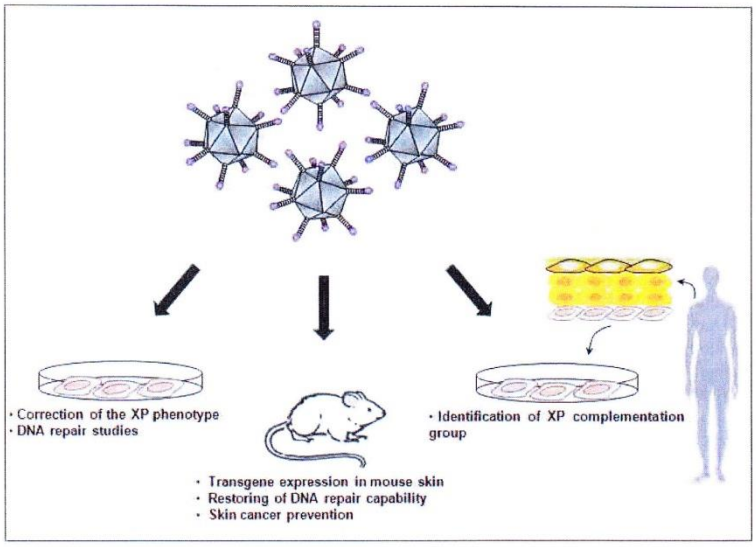

Fig. 3. DNA repair gene transduction by recombinant adenoviruses. Adenoviral vectors have been successfully employed to transduce human XP genes directly into established human cell lines (left), XP knockout mice skin (center), and fibroblasts from the skin of XP patients (right). Endpoints are indicated for each particular case.

Based on the successful complementation of the XP phenotype both *in vitro* and *in vivo*, adenoviral vectors could be proposed as an efficient tool for diagnosis and identification of XP patients' complementation groups. This hypothesis was recently tested and confirmed: with the use of adenoviruses carrying DNA repair genes, it has been possible to determine

the complementation group of three Brazilian XP patients, now characterized as XP-C patients. To that end, adenoviral transduced cells from these patients have been submitted to UV treatment and then analyzed by simple assays, such as cell survival and UDS (Leite et al., 2009). This diagnosis has been performed using the patients' skin fibroblasts but the potential use of adenoviral vectors for this purpose becomes even more exciting, considering that the adenoviral transduction could be held in cells present in the patients' blood, thus becoming a faster and less invasive technique. Besides scientific and epidemiological goals, the identification of the gene defect may help to predict clinical prognosis for the XP patients and guide appropriate genetic counseling for their families. Direct gene sequencing can be performed to identify the mutated genes, but as there are eight potential candidate genes for XP, functional complementation assays are still used for the genetic diagnosis of these patients.

3.2 Investigating UV-induced cell responses employing photolyases

Photoreactivation is a very efficient DNA repair mechanism, which specifically removes the two main UV photoproducts. Photoreactivation is carried out by flavoproteins known as photolyases. These enzymes recognize and specifically bind to UV lesions, thus reverting them back to the undamaged monomers, using a blue-light photon as energy source (Brettel & Byrdin, 2010; Sancar, 2008). Interestingly, photolyases demonstrate a great efficiency for discriminating the target lesion, either CPDs or 6-4PPs, and so far no photolyase has been shown to be able to repair both lesions. Thus, enzymes that repair CPDs are referred to as CPD-photolyases, while 6-4PP-photolyases specifically repair 6-4PPs (Müller & Carell, 2009). Both classes of photolyases are evolutionarily related, but functionally distinct (Lucas-Lledó & Lynch, 2009). Curiously, genes encoding genuine photolyases have been lost somehow in the course of the evolution of placental mammals, including humans. Instead, these organisms retain cryptochromes, photolyase-homologous proteins that participate in the maintenance of circadian rhythm, but that do not keep any residual activity related to DNA repair (Partch & Sancar, 2005).

Previous studies have confirmed that the CPD-photolyase is active when delivered to human cells, reducing mutagenesis (You et al., 2001), preventing UV-induced apoptosis (Chiganças et al., 2000) and recovering RNA transcription driven by RNA polymerase II (Chiganças et al., 2002). These successful studies have motivated the adenoviruses-mediated expression of the CPD-photolyase from the rat kangaroo *Potorous tridactylus* and the plant 6-4PP-photolyase from *Arabidopsis thaliana* in human cells aiming to discriminate the precise role of UV-induced cellular responses in both NER-deficient and NER-proficient human cells. Employing immunofluorescence, immunoblot and local UV experiments, it has been possible to see that these enzymes are not only very specific for their lesions, but are also really fast to find them, colocalizing with regions of damaged DNA and other DNA repair enzymes in less than two minutes (Chiganças et al., 2004; Lima-Bessa et al., 2008).

Adenoviral-mediated photorepair of CPDs substantially prevented apoptosis in all UV-irradiated cell lines (both NER-deficient and NER-proficient cells), confirming the involvement of these lesions in cell death signaling, as previously reported. On the other hand, 6-4PP repair by the 6-4PP-photolyase decreased UV-induced apoptosis only in those cell lines deficient for both NER subpathways, causing minimal effect, if any, in NER-proficient cells, including those lacking polη. These results suggest that, when not efficiently repaired, 6-4PPs also have important biological consequences, triggering cell responses

leading to the activation of apoptotic cascades. Interestingly, in CS-A cells (TC-NER deficient), a substantial attenuation of apoptotic levels could be again detected when CPDs were removed from the genome by the means of CPD-photolyase, while no detectable effect was observed as a consequence of photorepair of 6-4PPs, indicating that CPD lesions are the major UV-induced DNA damage leading to cell death, also in cells that are only proficient in GG-NER, the main subpathway of NER responsible for the removal of 6-4PPs in humans (Lima-Bessa et al., 2008).

These results suggest that CPDs and 6-4PPs may play different roles in UV-induced apoptosis depending on the repair capacity of human cells. In GG-NER proficient cells, the harmful effects of UV light seem to be predominantly due to the prolonged remaining CPDs in the genome caused by their slow removal by NER, with the minor participation of 6-4PPs (Lima-Bessa et al., 2008). Indeed, it has been reported that about 80–90% of 6-4PPs are removed from the human genome in the first 4 hours following UV exposure, whereas 40–50% of CPDs still remain to be repaired 24 hours later, probably due to the higher affinity of the XPC/hHR23B complex for 6-4PPs (Kusumoto et al., 2001). Thus, the lack of noticeable effects on UV-induced apoptosis in NER-proficient cells after 6-4PPs photorepair may be simply due to their fast repair by GG-NER. On the other hand, as for CPDs, the remaining of 6-4PPs in the genome seems to cause major disturbances in cell metabolism that lead to cell death. A summary of these results is shown in Figure 4.

To further confirm the idea that the roles of CPDs and 6-4PPs in UV-killing are related to the cellular repair capacity, authors have expressed these photolyases in TTD1V1 cells, a particular TTD cell line with a slower kinetics of 6-4PPs repair, eliminating about 50% and 70% of 6-4PPs at 6 and 24 hours post-UV treatment, respectively. Once again, repair of both lesions by the respective photolyase notably reduced apoptosis in these cells, even though the 6-4PP photorepair was less effective than seen for NER-deficient cell lines (Lima-Bessa et al., 2008). These photolyases were also used to identify a defect in the recruitment of downstream NER factors on certain XPD/TTD mutated cells, slowing down the removal of UV-induced lesions. As this recruitment was recovered by treatment with the histone deacetylase inhibitor trichostatin A, the data indicated that this defect is partially related to the accessibility of DNA damage in closed chromatin regions (Chiganças et al., 2008).

Another interesting finding came from assays investigating the time-dependent kinetics of the apoptosis commitment after UV treatment. Transduced XP-A cells were UV-treated and photoreactivated (to allow photorepair of the respective UV lesions) at increasing periods of time. Surprisingly, the data suggests that the initial trigger event to cell death after UV irradiation is relatively delayed, since photorepair of CPDs or 6-4PPs was able to reduce apoptosis even when photoreactivation was performed up to 8 hours after UV irradiation. After that, photoreactivation did not prevent UV-killing in these cells, indicating a commitment by events that irreversibly lead to cell death. These results are also in agreement with the indications that fast removed lesions (such as 6-PPs) do not activate apoptosis in NER-proficient human cells (Lima-Bessa et al., 2008). The main implication of all these findings is the fact that skin carcinogenesis in XP patients may also have 6-4PP lesions as important players, suggesting that tumors from these individuals are not only quantitatively different from those of normal people, but may also have different causative lesions. Transduction of XP knockout mice with adenoviral vectors carrying photolyase genes may help to address this question.

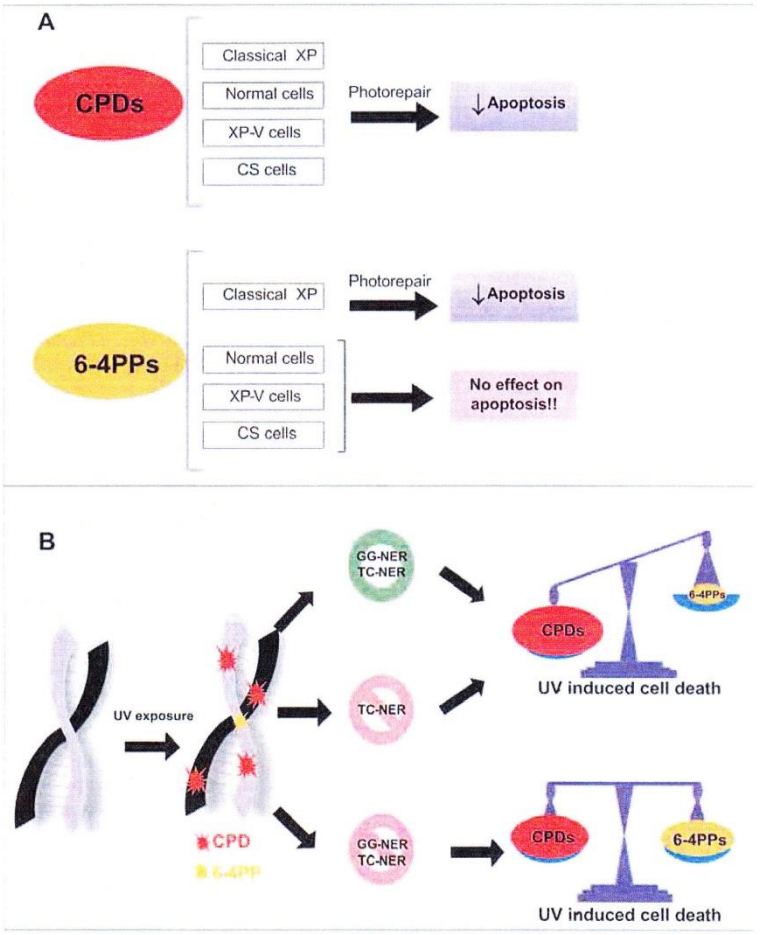

Fig. 4. Effects of photorepair of CPDs and 6-4PPs on UV-induced apoptosis. (A) Summary of the impact of the specific removal of CPDs and 6-4PPs by photorepair in human cell lines with different DNA repair capabilities. (B) Schematic representation of the main conclusions of the results shown in panel *A*. Those results clearly implicate that CPDs and 6-4PPs play different roles on UV-induced apoptosis depending on the cellular repair capacity.

4. Employing retroviral vectors for correcting XP phenotype

The first genetic analysis of XP patients was performed through somatic cell fusion followed by analysis of restoration of normal UDS. If somatic cell fusion complements XP genetic deficiency, it will then be positive for UDS activity. These experiments were able to identify the seven classical XP complementation groups and the variant group (Zeng et al., 1998). This implies that DNA repair deficiencies can, in fact, be corrected by the introduction of a normal copy of the affected gene, giving hope for the development of gene therapy protocols for XP patients. In fact, the introduction of a normal copy of the defective gene in XP cells can complement the DNA repair ability, as demonstrated by the delivery of conventional expression vectors, via calcium precipitation and microneedle injection (Mezzina et al., 1994).

In 1995, viral vectors were first used as gene delivery tools in DNA repair experiments (Carreau et al., 1995a). In this study, a LXPDSN retroviral vector carrying the wild-type XPD gene was capable of complementing primary fibroblasts of XPD patients with a long-term expression. A subsequent study showed that this complementation was gene-specific and that there was a long-term expression of the transgene (Quilliet et al, 1996). The use of retroviral vectors for DNA repair genes delivery was further validated in 1996 and 1997, when XP-A, XP-B, XP-C and TTD-D cells were also complemented with the aid of gene-specific retroviral vectors (Marionnet et al., 1996; Zeng et al., 1998).

The compilation of these results shows that the retroviral delivery of several DNA repair genes was able to specifically complement several deficiencies presented by XP, CS and TTD patients such as UDS, reduced catalase activity, UV-sensitivity, recovery of RNA synthesis, increased mutation frequency, stabilization of p53 (Dumaz et al., 1998) and deregulation of ICAM-1 (Ahrens et al., 1997).

Since XP patients already receive autologous graft transplants after massive skin tissue removal surgery (Atabay et al., 1991; Bell et al., 1983), most researches in the field of XP gene therapy focus on the three-dimensional skin reconstruction *in vitro*, using the patients' cells genetically corrected *ex vivo*. In this technique, the patients' fibroblasts and keratinocytes are cultured *in vitro* after a skin biopsy of a non-UV-irradiated area. Then, retroviral vectors are used to stably complement the genetic deficiency of these cells. Finally, the keratinocytes and the fibroblasts are used to three-dimensionally reconstruct the epidermis and dermis, respectively. This construct can then be used as a graft when the part of patient's damaged skin is removed in a necessary surgery. To that end, Arnaudeau-Bégard and co-workers managed to complement XP-C keratinocytes, recovering a wild-type phenotype and UV-resistance with the aid of a retroviral vector carrying a normal copy of the *XPC* gene (Arnaudeau-Bégard et al., 2003). Furthermore, Bergoglio and co-workers have also developed a selection method for genetically corrected keratinocytes that does not involve particles derived from microorganisms which could lead to immunological clearance of the transgene, using CD24 as an ectopic marker (Bergoglio et al., 2007).

In 2005, Bernerd and co-workers were able to reconstruct a three-dimensional skin model *in vitro* using fibroblasts and keratinocytes from a donor XP-C patient. With this model, they were able to see that the XP skin has peculiar characteristics: hypoplastic horny layers, decreased and delayed keratinocyte differentiation, epidermal invaginations, a generally altered proliferation control and fibroblasts with distinct morphology and orientation. Furthermore, the epidermal invaginations were proven to be related to alterations of both keratinocytes' and fibroblasts' functions and were characterized as epidermoid carcinoma-like structures (Bernerd et al., 2005). It is important to keep in mind that an XP skin biopsy

might give us further and more precise knowledge of the XP skin physiology, but this is a delicate procedure which requires the patients' agreement.

Since the use of common retroviral vectors in gene therapy can be dangerous due to semi-random insertional mutagenesis, researchers have developed several self-inactivating-lentiviral vectors carrying DNA repair genes. These vectors were shown to efficiently transduce primary and transformed fibroblasts, complementing in a gene-specific manner XP-A, XP-C and XP-D cells. Furthermore, the recovery of normal levels of UV-resistance in the transduced cells was shown to be persistent for at least 3 months (Marchetto et al., 2006). The reconstruction of a genetically corrected, three-dimensional XP skin followed by the implantation of the graft on a patient (Figure 5) is still an ongoing chore that has to be taken very cautiously, always prioritizing the patient's well-being.

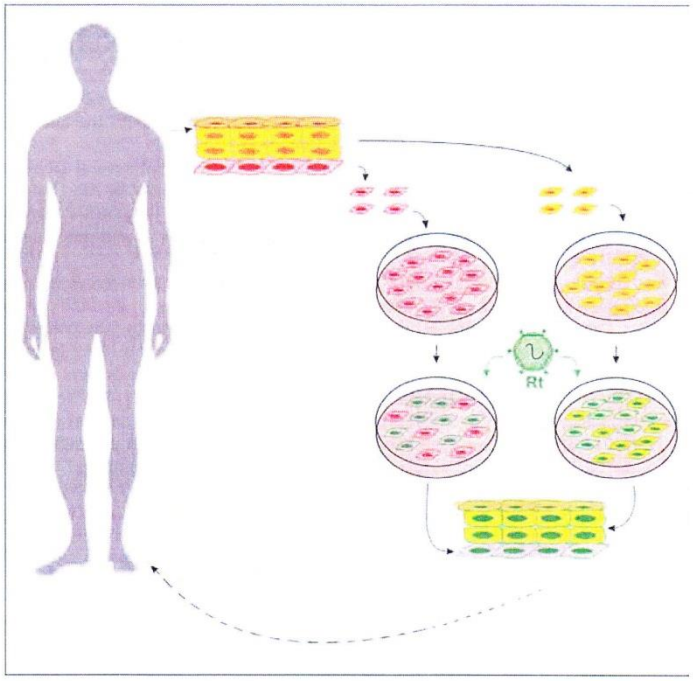

Fig. 5. Schematic representation of *ex vivo* gene therapy for XP patients using recombinant retrovirus (Rt). Skin-derived fibroblasts and keratinocytes from an XP patient are cultivated *in vitro*, and transduced with retroviral vector carrying the wild type *XP* cDNA. Transduced cells are then used to reconstruct the human skin *in vitro*, with a normal phenotype. Dashed line raises the possibility of engraftment of the reconstructed skin directly on XP patients.

It is also important to keep in mind that these grafts do not include melanocytes, responsible for the very common melanomas in these patients (Khavari, 1998), and that the skin will only be genetically complemented in the areas that receive the grafts, all the other areas of the body will still be extremely photosensitive since no paracrine effect is known for DNA repair proteins and that immunological clearance or gene silencing by cellular methylation can always prohibit a long-term transgene expression (Magnaldo & Sarasin, 2002). Importantly, several XP complementation groups also present other relevant symptoms, such as neurodegeneration, which will not be improved by the skin grafts. For those patients, another kind of gene therapy might be more efficient, such as the development of genetically corrected stem cells (ESs) (Magnaldo & Sarasin, 2002) or induced pluripotent cells (iPSCs, see below (Alison, 2009). Unfortunately, there is still no reference on that kind of research for xeroderma pigmentosum.

5. Host cell reactivation (HCR) as a tool for DNA repair research

The host cell reactivation (HCR) technique was first described in human cells by Protic-Sabljic and co-workers in 1985 (Protic-Sabljic et al., 1985). In this first work, the technique consisted of transducing cells with a plasmid containing a putative cDNA with a selective gene into XP cells to look for a reversion of the UV sensitivity due to gene complementation, allowing identification of the genes responsible for that phenotype.
Other studies have refined the technique which is now widely used as an indirect measure of cellular DNA repair capacity. Mostly, a plasmid containing a reporter gene such as luciferase (LUC) or chloramphenicol acetyltransferse (CAT) is treated with a genotoxic agent such as UV radiation and introduced in the cell where DNA repair capacity is to be evaluated. If the cell is able to remove the lesions from the plasmid, the reporter gene will be expressed. Different DNA repair rates can be addresses by differences on the amount of gene reporter expression at a certain time (Merkle et al., 2004). A schematic representation of HCR is shown in Figure 6.

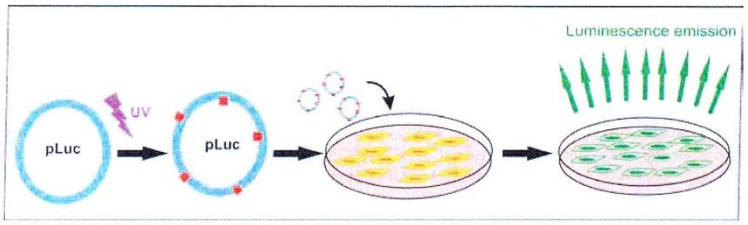

Fig. 6. Schematic representation of host cell reactivation (HCR) assay. A plasmid carrying a reporter gene (in this case, the luciferase gene) is UV-irradiated *in vitro* and then transfected into host cells. 48 hours later, the cellular DNA repair capacity is indirectly estimated by measurement of the reporter gene activity in the cellular extract.

In 1995, the HCR assay was further used to visualize the genetic complementation of mammalian expression vectors carrying the DNA repair genes *XPA*, *XPB*, *XPC*, *XPD* and *CSB*. In this study, plasmids containing LUC or CAT were UV irradiated and co-transfected with the plasmids containing each of the complementing genes of the DNA-repair deficient

cells. Again, only the cells with the correct complementation were capable of removing the DNA damage in the reporter gene, allowing the expression of that protein. This technique facilitates the identification of the complementation group of a given patient, being particularly useful in cases of CS, TTD and some XP patients such as XP-E that present a normal UDS after UV treatment (Carreau et al., 1995b).

Recent data using the HCR assay has shown that the CS proteins are essential for the reversion of oxidated lesions (Pitsikas et al., 2005; Spivak & Hanawalt, 2006; Leach & Rainbow, 2011) and evidence obtained with HCR suggests that, unlike what was previously shown with UDS assays, DNA repair capacity in fibroblasts does not decrease with aging (Merkle et al., 2004). This reduction may however be cell type-specific and DNA repair pathway-specific since blood cells repair capacity decreases approximately 0.6% per year of age (Moriwaki et al., 1996). This technique is still widely used and its great advantage is that the DNA plasmids or the viral vectors are treated in a controlled manner, not being subject to the cell's global response to the same treatment. Further technique improvements will surely allow HCR to be used in different assays such as *in vivo*, yielding a better knowledge of the DNA repair pathways and their interactions with other pathways and physiological events.

6. Other treatments for xeroderma pigmentosum

6.1 General care

There is no treatment that has been proven so far to be 100% effective in all XP cases. The only palliative measure that patients can rely on is complete sun avoidance. This includes not only avoiding going out even on cloudy days and covering all exposed body areas such as skin and eyes, but also using special artificial lights that emit no UV wavelengths (Kraemer, 2008). Premalignant lesions, such as actinic keratoses, and malignant lesions must be quickly treated with topical 5-fluoracil or liquid nitrogen, imiquimod cream, electrodesiccation and curettage, surgical excision or chemosurgery, as needed. When extensive areas are damaged and have to be surgically removed, skin grafts from sun unexposed areas of the same patient should be used. When eyes are affected, methylcellulose eye drops or contact lenses can help prevent trauma and corneal transplantations might be needed in extreme cases (Kraemer, 2008).

When caring for XP patients, it is very important to keep in mind that the total sun avoidance also prevents the production of vitamin D in the skin, so dietary supplementation might be needed. Furthermore, the DNA repair deficiencies which prevent the repair of photolesions may also make individuals sensitive to other mutagens such as cigarette smoke, so patients should be protected against these agents (Kraemer, 2008).

Aside from removal of local lesions and total sun avoidance, two other palliative treatments might help improving the XP patient's quality of life: topical use of T4 endonuclease (Yarosh et al., 2001) and oral intake of retinoids (Campbell & DiGiovanna, 2006).

6.2 Topical use of T4 endonuclease

In 1975, Tanaka and co-workers demonstrated that the bacteriophage T4 endonuclease V is capable of making an incision 5′ within a CPD lesion. The resulting DNA flap is recognized and removed by a 5′→3′ exonuclease, leaving a gap that is filled by a DNA polymerase, using the undamaged single strand as a template. A DNA ligase then joins the repaired fragment to the parental DNA (Tanaka et al., 1975).

In the 80's, Yarosh's laboratory discovered that the T4 endonuclease V could be delivered into cells using 200 nm liposomes as a delivery vehicle. The anionic liposomes not only protect the cationic enzyme inside, but also promote the escape from a clatrin-coated endosome after cellular intake by destabilizing the vesicle's membrane with an acid pH. By cleaving DNA at the site of UV-induced lesions, the enzyme reverses the DNA repair defect of XP cells (Yarosh, 2002). Further work by the same group also showed that these T4N5 liposomes in a 1% hydrogel lotion when applied in cultured human fibroblasts, mouse dorsal back or cultured human breast skin is capable of delivering the enzyme into cells in less than one hour, being almost entirely restricted to the epidermis (Ceccoli et al., 1989; Kibitel et al., 1991).

An inverse correlation was later shown between the T4N5 dose and the level of CPDs that remained in the epidermis. This curve reached a plateau (at 0.5 μg/ml), probably due to saturation of the cell machinery for further repairing the damage after the initial incision by the T4 enzyme (Yarosh et al., 1994). These studies also showed that even in the higher dose of T4N5 liposomes, only ~50% of the CPD lesions were removed but that was capable of reducing the mutagenesis rate by 99% in transformed fibroblasts and 30% in primary fibroblast cell culture. These numbers are probably not only related to the number of remaining lesions, but also to the smaller size of the repair patch filled by BER compared to that needed in NER (Yarosh, 2002; Cafardi & Elmets, 2008).

Finally, after two phase I clinical trials (Yarosh et al., 1996 as cited in Cafardi & Elmets, 2008) and three phase II clinical trials (Wolf et al., 2000 and Yarosh et al., 1996 as cited in Cafardi & Elmets, 2008), in 2001 the T4N5 liposomes were tested in XP patients. The patients were instructed to apply 4-5 ml of the lotion containing 1 mg/ml of endonuclease everyday for a year. Except for lesion removal when necessary, and daily use of sunscreens of 15 SPF or higher, no concomitant treatments were allowed. The treatment was shown to be efficient, reducing the rate of actinic keratoses and basal-cell carcinomas to 68% and 30% respectively in the placebo and treatment groups, reducing tumor promotion and progression. The treatment was also capable of reducing some immunosuppressant molecules, such as interleukin-10 (IL-10) and tumor necrosis factor-α (TNF- α). Unfortunately, the treatment was only effective for patients under 18 years-old. This might be because XP patients older than that already had too much DNA damage in their cells that could not be reversed (Yarosh et al., 2001). Despite the promising results, there are currently no topical DNA repair enzymes approved by the FDA. Clinical trials are still being conducted to analyze the application of T4N5 liposomes in other deficiencies and immunosuppressed patients (Cafardi & Elmets, 2008).

6.3 Oral use of retinoids

Despite interventions such as sunlight avoidance and tumor removal, most of the XP patients continue to develop a large number of skin cancers. These high-risk patients may suffer from field cancerization that may happen when a wide field of the epithelium has been exposed to the same genotoxic agent and adjacent but not contiguous areas present genetic and morphological alterations that may lead to a carcinogenesis process. As the whole skin area has been exposed to sunlight, inducing independent tumors with different growth rates, this hypothesis may explain why the patients have a 30% increase in the chances of having a second basal cell carcinoma (BCC) and then a 50% increase of a third BCC (Campbell & DiGiovanna, 2006).

In XP patients, the oral use of retinoids might be beneficial, regardless of the strong side effects. In chemoprevention the goal is to identify early biological events in the epithelium which may lead to a carcinogenesis process and intervene with chemicals which will help stop or reverse the process (Campbell & DiGiovanna, 2006). Retinoids, also known as vitamin A, are the most studied chemopreventive agent for skin cancers, upper aerodigestive tract and breast and cervical cancers. The exact mechanisms through which the retinoids are capable of reducing cancer incidence are still unclear, but it has been shown that they are capable of altering keratinocytes' growth, increasing their differentiation status, affecting their cell surface and immune modulation. Retinoids mediate gene transcription by binding to two families of nuclear receptors, the retinoid acid receptors (RARs) and the retinoid X receptors (RXRs). Retinoids have only a mild effect on existing tumors, but can suppress the development of new lesions (Campbell & DiGiovanna, 2006). In 1988, it was shown in a three year study that isotretinoin in a dose up to 2 mg/day/Kg was able to reduce skin cancers in XP patients by 63%. Unfortunately, in the year following the discontinuation of the treatment there was an increase of 8.5% of cancer incidence in those patients with reference to the two years of treatment (Kraemer et al., 1988).

Furthermore, the constant use of retinoids can have severe side effects ranging from inflammation in existing tumors, dry skin and mucosa and hair loss to pancreatitis, osteoporosis, hyperostosis and myalgia among others. The retinoids' toxicity is dose related and cumulative, but most of the side effects can be prevented with constant check-ups and use of local special moisturizers (Campbell & DiGiovanna, 2006). Indeed, several retinoids can be used as chemopreventives. The two most common are isotretinoin and acitretin, the first having a shorter half-life and being the drug of choice for women due to retinoids' theratogenic potential, especially in fetuses (Campbell & DiGiovanna, 2006).

6.4 Potential effects of DNA repair adjuvants

The use of DNA repair adjuvants and antioxidants may also help reducing skin cancer incidence in XP patients. Some known DNA repair adjuvants are selenium, aquosum extract of *Urcaria tomentosa* and Interleukin-12 (IL-12) (Emanuel & Scheinfeld, 2007).

Selenium seems to interact with Ref-1, activating p53, inducing the DNA repair branch of the p53 pathway, in a BRCA1-dependent manner, dealing mainly with oxidative stress (Fisher et al., 2006). On the other hand, it has been already reported that high levels of selenium can be mutagenic, carcinogenic and possibly teratogenic (Shamberger, 1985), probably due to non-specific sulfur substitution on proteins and consequent TC-NER activity decrease (Abul-Hassan et al., 2004). Thus, special attention should be taken regarding the dose of dietary selenium supplementation.

The aquosum extract of *Urcaria tomentosa* (cat's claw) seems to increase the removal of CPDs and reduce oxidative damage, either by an increase in base excision repair (BER) or by an antioxidant property, reducing erythema and blistering after UV. Despite several studies *in vitro* and *in vivo*, the precise mechanisms are still unknown (Emanuel & Scheinfeld, 2007).

Another interesting finding is that, besides IL-12 being a strong immunomodulatory molecule, able to prevent UV-induced immunosuppression through IL-10 inhibition (de Gruijl, 2008), it is also capable of increasing DNA repair by inducing NER, as shown by the RNA level increase in some NER molecules (Schwarz et al., 2005).

6.5 Gene therapy targeted approaches: The use of meganucleases for correcting XP-C cells

There are several techniques to specifically target, substitute, or correct a gene, diminishing the chances of insertional recombination, such as the use of recombinases, transposons, zinc-finger nucleases, endonucleases and meganucleases (Silva et al., 2011). Meganucleases can function as RNA maturases, facilitating the maturation of their own intron or as specific endonucleases that can recognize and cleave the exon-exon junction sequence wherein their intron resides, creating a specific double strand break (DSB), giving rise to the moniker "homing endonuclease". The meganuclease function is probably related to the current status of its lifecycle (Silva et al., 2011).

Meganucleases can be used as gene targeting tools in several ways. Ideally, they can provide a true reversion of the mutation, but the efficacy of correction is invertionally correlated to the distance of the initial DNA DSB. Alternatively, it can insert a functional gene upstream of the mutated one or in a safe location where it will not induce insertional mutagenesis. Also, meganucleases can be used for introducing specific mutations for research purposes such as understanding the role of a gene or of a specific point mutation. Furthermore, meganucleases capable of targeting viral sequences are being researched as antiviral agents (Silva et al., 2011).

Recently, the design of a specific I-CreI meganuclease targeting for the *XPC* gene was able to specifically target two *XPC* sequences, showing *in vitro* for the first time that extensive redesign of homing endonuclease can modify a specific chromosome region without lost of specificity or efficiency (Arnould et al., 2007). These results are very promising for the development of future gene therapy strategies for XP patients.

6.6 Induced Pluripotent Cells (iPSCs) as gene therapy agents

In 2006, the induction of pluripotent cells (iPSCs) by the expression of Oct3/4, Sox2, c-Myc and Klf4 in fibroblasts gave hope for a new gene therapy using pluripotent cells that would not elicit an immunological response in the patient, since his own cells would be used to induce the iPSCs and that would not be confronted by ethical issues like the use of embryonic stem cells (Takahashi & Yamanaka, 2006). Since then, this technology has been improved and iPSCs have been induced in a variety of cell types from different species. Also, iPSCs have been differentiated to several different cell types, from fibroblasts to neurons (Sidhu, 2011).

Fanconi Anemina (FA) is a DNA repair related disease, where mutations in one of fourteen genes lead to extreme sensitivity to interstrand crosslinking agents. Patients show progressive bone marrow failure, congenital developmental abnormalities and early onset of cancers, mostly acute myelogenous leukemia and squamous cell carcinomas. Bone marrow transplantation is a palliative treatment for the secondary leukemia but no cure is currently available for FA patients (Kitao &.Takata, 2011). In 2009, Raya and co-workers were able to use lentiviral vectors to genetically correct fibroblast and keratinocytes from patients with various FA complementation group deficiencies and then induce their dedifferentiation into pluripotent stem cells. Interestingly, uncorrected FA cells did not generate iPSCs, indicating a role for DNA repair in nuclear reprogramming. Thus, the generated iPSCs had normal FA genes and have the potential of being used for gene therapy of the donor patients, with no risk of inducing immunological rejection (Raya et al., 2009). Hopefully soon FA patients and others will be able to benefit from this technology as a safe gene therapy approach.

7. Concluding remarks

Recombinant viral vectors were developed more than thirty years ago, and they have provided extremely useful tools to understand cell metabolism. This chapter focuses on their use to understand cells' responses to DNA damage, especially UV-irradiated DNA repair-deficient cells. These vectors provide means to interfere in these responses, affecting DNA metabolism and revealing important aspects of the DNA repair mechanisms. The discovery of RNA interference mechanisms in human cells offer still more opportunities to modify cells' responses by silencing specific DNA repair genes. Several libraries of viral vectors for the expression of small double-stranded RNA molecules (shRNA) targeting human genes are commercially available, and are already being used for understanding gene function. The use of such vectors to make cells deficient in more than one DNA repair pathway, using cells deficient in XP genes as hosts, for example, may help us to reveal the intricate network of interactions between the different metabolic pathways that contribute to genome maintenance after damage induction (Moraes, et al., 2011; in press). Moreover, the progress that has been made towards gene therapy for xeroderma pigmentosum, using these recombinant viral vectors is also discussed. Although the results indicate a series of limitations, and it is clear that there is still a long way to go, they make researchers go forward, giving a gleam of hope to these patients and their families.

8. Acknowledgements

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