Xeroderma Pigmentosum syndrome: A new model to study the role of fibroblasts in the modulation of the innate immune response against cutaneous cancer cells

Maria João Gonçalves Maia

To cite this version:


HAL Id: tel-03000465
https://theses.hal.science/tel-03000465
Submitted on 11 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Le syndrome Xeroderma Pigmentosum

Un nouveau modèle pour l’étude du rôle des fibroblastes dans la modulation de la réponse immunitaire innée contre les cellules cutanées cancéreuses.

Maria João GONÇALVES MAIA
Institut de Recherche sur le Cancer et le Vieillissement, Nice

Présentée en vue de l’obtention du grade de docteur en Interactions moléculaires et cellulaires d’Université Côte d’Azur
Dirigée par : Thierry Magnaldo
Soutenue le : 14 juin 2019

Devant le jury, composé de :
Dr. Véronique Braud, President, IPMC, Valbone
Pr. Marie-Dominique Galibert, Rapporteur, IGDR, Rennes
Pr. Alain Sarasin, Rapporteur, IGR, Paris
Pr. Alexander Steinle, Examinateur, IMM, Frankfurt
Acknowledgements

First, I would like to express my sincere gratitude to all of the members of my thesis scientific committee for accepting to evaluate my PhD work. I’m honored to have Dr. Véronique Braud as the president of the jury, Professor Marie-Dominique Galibert-Anne as a reporter, Professor Alain Sarasin also as a reporter and Professor Alexander Steinle as an examiner.

My sincere thanks to Thierry, my thesis supervisor, for giving me the opportunity to carry out the present thesis work, for sharing with me his scientific knowledge, and for granting me the freedom to explore the different research paths presented in this manuscript. I always felt how much you appreciated my work, all the confidence you had in my results and the genuine enthusiasm you expressed with the advances of this thesis project. I am truly grateful for your trust. It gave me confidence, courage, and the certainty that I could be a good researcher in the future if I chose this path. For this, I thank you.

Véronique, in addition to accepting to be the president of my thesis committee, I would like to thank you for committing so much of your time to this project during the past 3 and a half years. Everything I know about immunity I learned from you. Your dedication and enthusiasm towards this project inspired, encouraged, and gave me the motivation and strength to continue. I’m grateful that I had the opportunity to work with you. It was a real pleasure and an enriching professional experience that I won’t forget. Finally, thank you for the invaluable contribution you gave to this manuscript.

Yannick, thank you for the intellectual contribution you gave to this work since the beginning. Your interest and all our scientific (and nonscientific) discussions helped me enormously in writing this project and concluding it. Thank you for teaching me how to perform most of the laboratory techniques I used during my thesis, how to improve experimental conditions and for always taking the time to review my writings. I would like to also thank you for your friendly support. Especially for all the times you gave me the last chocolate you had in your drawer and for always caring for me and my well being. I couldn’t have done this without you. Lastly, thank you for all of the knowledge and good energy you put into improving this manuscript and for your kind corrections.

A special thanks is also needed for Sabine. I’m sure that not many people truly realize how important and fundamental your work is to our Institute. You are always the first person we call when we smell something odd, when we hear something unusual, when we see something strange. When we have a problem with door locks, with freezers, with incubators. When we need to buy a particular product, to move large equipment or to change the filter of our PSM. I
really could write a dissertation containing only the multitude of the things that you do for us on a daily basis. The most important though, is your availability to take the time to listen to us and our problems. You were a constant and reassuring presence during these past years and I know we will keep in touch for years to come. Thank you.

Margot and Julia "mes copinettes"! The first acknowledgement lines I wrote were actually to thank you two! Not only for our surrealistic discussions that reassured me that I wasn't alone in my thinking but also for your friendship, for your continuous support, and for making my days in the Pasteur tower so much fun! Finally, thank you for running with me our incredible Instagram account @lavieaulabo. I will always cherish the friendship we have created. I Love you!

I also want to thank all the former members of the laboratory especially Sahar, Sophie and Miguel. Thank you for your support and the assistance you gave towards this work.

Valérie and Nicolas, thank you for always taking the time to help me deal with the incredibly complex work of orders and shipments. I appreciate all of your help.

To the people of the second floor of the Pasteur tower, thank you for your genuine concern for my wellbeing and for all of the fun and interesting discussions we had over these past years that helped me growth as a researcher and most importantly as a person.

A particular thanks also goes to Marina Shkreli that generously let me use one of her team’s desks for almost a year now, and to all of Paquis team, for the laughter and good moments we shared together.

To my family, the ones with whom I share a bloodline and the ones with whom I chose to share life with. For them, I’m the cousin, the niece, the godmother, the best friend, the chief, the lucky one, the fearless... For me, they are the reason I wake up happy every morning. Having you in my life has made this path a lot easier. Thank you for your love and friendship, for all of your understanding that I am a bit too far away to be able to see you as much as I would like and for coming to visit as much as you can. I feel blessed to have you in my life.

Ivo, thank you for your support, encouragement and understanding of my goals and my aspirations. Your love is a blessing and has been my major source of strength. Without your help, I would not have been able to complete much of what I have done and become who I am today. Thank you for being with me every single day and for never letting me feel lonely in this solitary journey. There are no words to express how much I value and love you.

Aos meus pais, meus exemplos maiores, que são a constante da minha vida, a presença de todas as horas e a certeza de que o Amor é o único que importa. Obrigada.
Résumé de la thèse

L’étiologie des cancers cutanés est liée à des mutations génétiques résultant de l’exposition aux rayonnements ultraviolets (UV) émis par le soleil. La propagation des cellules cancéreuses dépend aussi des interactions avec les cellules présentes dans le microenvironnement circulant, notamment des fibroblastes associés au cancer (FAC) et des cellules immunitaires. Xeroderma pigmentosum (XP) est une maladie génétique qui comprend 7 groupes de complémentation génétique (XP-A à XP-G). Les patients XP présentent une déficience du mécanisme de réparation des lésions de l’ADN provoquées par les UV. Ces patients sont susceptibles au développement précoce de très nombreux cancers cutanés. XP-C est le groupe de complémentation le plus représenté en Europe. Chez ces patients, les carcinomes spino-cellulaires (CSC) sont plus fréquents que les carcinomes baso-cellulaires (CBC) (taux 5 : 1). Les CSC ont un potentiel métastatique plus élevé que les CBC. Des travaux précédents ont suggéré que la réponse immunitaire chez les patients XP pouvait être altérée, incluant un déficit de l’activité cytolytique des cellules Natural Killer (NK) et une diminution du nombre des lymphocytes T circulants.

L’objectif central de cette thèse était, d’identifier des facteurs du microenvironnement impliqués dans la progression des cancer cutanées agressifs, en prenant comme modèle de susceptibilité au cancer, des cellules de patients XP-C. Une analyse transcriptomique comparant les fibroblastes WT et des patients XP-C a permis d’identifier que CLEC2A, un ligand activateur du récepteur NKp65 des cellules NK, est exprimé par les fibroblastes WT mais pas par les fibroblastes XP-C. Nos travaux ont pu montrer une diminution du niveau d’expression de CLEC2A au cours de la sénescence réplicative ; une absence dans les FAC et dans les CSC et que, des facteurs solubles sécrétés para les CSC diminuent l’expression de CLEC2A. Ces résultats suggèrent que la perte de CLEC2A peut induire un déficit d’activation des cellules NK au sein du microenvironnement tumoral et dans les dermes des patients XP-C. Par la suite, nous avons élaboré un modèle de culture de peau 3D, dans lequel nous avons introduit des cellules NK, en présence ou absence d’anticorps bloquants CLEC2A. Ce modèles nous a permis de montrer que l’interaction CLEC2A/NKp65 régule l’invasion des CSC via un dialogue entre fibroblastes et cellules NK. Nos résultats suggèrent que l’expression de CLEC2A dans les fibroblastes WT contribue à la surveillance immunitaire dans la peau et que son absence, par des facteurs encore inconnus, favorise le développement des cancers agressifs chez les patients XP-C. CLEC2A peut être une cible dans le combat contre la progression des CSC.

Mots clés : Xeroderma pigmentosum ; carcinomes spino-cellulaires ; microenvironnement tumoral ; surveillance immunitaire ; CLEC2A
Abstract

Skin cancer etiology is related to genetic mutations arising after ultraviolet (UV) sun exposure. The propagation of cancer cells is also dependent of a crosstalk with cells present in the surrounding microenvironment, mainly cancer associated fibroblasts (CAF) and immune cells. Xeroderma pigmentosum (XP) is a genetic disease that comprises seven groups of genetic complementation (XP-A to XP-G). XP patients present a default in the mechanism responsible for the repair of UV-induced DNA lesions. They are prone to develop skin cancers with high frequencies early in their life. XP-C is the most represented complementation group in Europe and in XP-C patients squamous cell carcinoma (SCC) are more frequent than basal cell carcinoma (BCC) (ratio 5:1). SCC have high metastatic potential compared to BCC. Previous studies suggested that the immune responses in XP patients could be altered with defects in their NK lytic activity and a decrease in the levels of circulating T lymphocytes.

The main objective of this thesis was to identify microenvironment factors that could contribute to the progression of aggressive skin cancers using XP-C disease cells as a model of skin cancer susceptibility. Comparative transcriptomic analysis of WT and XP-C dermal patient’s fibroblasts revealed that CLEC2A, a ligand of the activating NK receptor NKp65 implicated in the activation of the innate immune system, is expressed in WT fibroblasts and absent in XP-C fibroblasts. Additional work showed that CLEC2A level is decreased in WT fibroblasts during replicative senescence, is absent in CAF and SCC, and is down regulated by soluble factors secreted by SCC cells. These results suggest that the loss of CLEC2A may induce a deficit of NK cell activation in the tumor microenvironment of SCC and in the dermis of XP-C patients. Elaboration of 3D skin culture models including NK cells and, in the presence or absence of blocking anti-CLEC2A antibody, allowed us to show that CLEC2A/NKp65 interaction regulates SCC cells invasion through a crosstalk between fibroblasts and NK cells. Our results suggest that the expression of CLEC2A in fibroblasts contributes to skin immune surveillance while, conversely, its absence under yet unidentified factors, favors the development of aggressive cancers in XP-C patients. CLEC2A could be a potential target in the fight against SCC progression.

Keywords: Xeroderma pigmentosum; squamous cells carcinoma; tumoral microenvironment; immune surveillance; CLEC2A
# Table of contents

**Introduction** .......................................................................................................................... 18

**Chapter I: Skin** ...................................................................................................................... 20

1. Structure of the skin .............................................................................................................. 20
   1.1. Epidermis ......................................................................................................................... 20
      1.1.a. The Basal layer (Stratum basale) ........................................................................... 22
      1.1.b. Spinous layer or Stratum spinosum ...................................................................... 22
      1.1.c. The granular layers or Stratum granulosum ......................................................... 23
      1.1.d. Cornified layers or Stratum corneum .................................................................. 23
      1.1.e. Other epithelial cells .............................................................................................. 24
   1.2. Dermal-epidermal junction ............................................................................................ 25
   1.3. Dermis ............................................................................................................................ 25
      1.3.a. Fibroblasts ............................................................................................................... 27
      1.3.b. Other dermal cells .................................................................................................... 29
   1.4. Hypodermis ..................................................................................................................... 30
   1.5. Skin appendages ............................................................................................................ 30
      1.5.a. Pilosebaceous units ................................................................................................. 30
      1.5.b. Glands ...................................................................................................................... 31
2. Function .................................................................................................................................... 31

3. Skin cancers ............................................................................................................................ 33
   3.1. Non melanoma skin cancers ......................................................................................... 33
   3.2. Melanoma skin cancer ................................................................................................. 34
   3.3. Risk factors .................................................................................................................... 36
      3.3.a. UV exposure ............................................................................................................ 36
      3.3.a. Pollution .................................................................................................................. 38
      3.3.b. Phototype ............................................................................................................... 38
      3.3.c. Aging ....................................................................................................................... 39
      3.3.d. Immunosupression ................................................................................................. 39
      3.3.e. Genetic disorders .................................................................................................... 40
   3.4. Treatment of skin cancers .............................................................................................. 41

**Chapter II: Xeroderma pigmentosum** .................................................................................. 42

1. Epidemiology and clinical traits ............................................................................................ 42
2. Etiology .................................................................................................................................... 44
   2.1. Nucleotide Excision Repair (NER) ............................................................................. 44
      2.1.a. Genotype-phenotype relationships .................................................................... 47
Chapter IV: NK cells and their receptors

3. Diagnosis and treatment ........................................................................................................ 50

4. The XP-C genetic group of complementation ..................................................................... 52

4.1. XPC in GGR .................................................................................................................... 54

4.2. Biological roles of XPC beyond NER .............................................................................. 55

4.2.a. Repair pathways ........................................................................................................... 55

4.2.b. Transcription and chromatin remodeling ..................................................................... 56

4.3. XP-C patients’ mutation profile ...................................................................................... 56

4.4. XP-C group as a model to study both non melanoma skin cancer and aging .............. 58

Chapter III: Tumor microenvironment .................................................................................. 60

1. Constituents of tumor microenvironment ......................................................................... 61

1.1. Fibroblasts associated to cancer ...................................................................................... 61

1.2. Vascular cells ................................................................................................................... 62

1.3. Immune cells ................................................................................................................... 63

1.3.a. T cells .......................................................................................................................... 63

1.3.b. B cells .......................................................................................................................... 65

1.3.c. NK cells ....................................................................................................................... 65

1.3.d. Macrophages ............................................................................................................... 67

1.3.e. Neutrophils ................................................................................................................ 67

1.3.f. Myeloid-derived suppressor cells ................................................................................. 67

1.4. Adipocytes ....................................................................................................................... 68

2. The prison break of cancer cells .......................................................................................... 68

2.1. Crosstalk between fibroblasts and cancer cells ................................................................. 68

2.2. Tumor immune microenvironment ............................................................................... 70

2.2.a. Elimination .................................................................................................................. 71

2.2.b. Equilibrium and Escape ............................................................................................ 72

3. Senescent microenvironment drives tumorigenesis ............................................................ 75

4. Microenvironment as a target for cancer therapy ............................................................... 76

4.1. Targeting CAFs in cancer therapy .................................................................................. 76

4.2. Targeting immune system in cancer therapy .................................................................. 77

Chapter IV: NK cells and their receptors ................................................................................. 80

1. NK receptors ....................................................................................................................... 80

1.1. NKRP1 subfamily ............................................................................................................. 81

1.1.a. KLRB1/CLEC2D .......................................................................................................... 82

1.1.b. KLRF1/CLEC2B .......................................................................................................... 82

1.1.c. KLRF2/CLEC2A .......................................................................................................... 82

2. The diversity of NK cell subsets in human skin .................................................................... 84
Thesis objectives................................................................. 87
Scientific article ........................................................................ 90
Materials and Methods ................................................................. 120
Results .................................................................................... 124
Discussion and Perspectives ....................................................... 136
Concluding note from the author .................................................. 146
References ................................................................................. 148
Appendix ..................................................................................... 180
Differentially expressed genes between WT and XP-C fibroblasts (2D approach) - p≤0.05 ...... 182
Genetic therapy of Xeroderma Pigmentosum: analysis of strategies and translation .......... 192
A Real-Time Cytotoxicity Assay as an Alternative to the Standard Chromium-51 Release Assay for Measurement of Human NK and T Cell Cytotoxic Activity ......................................................... 205
Basal Cell Carcinoma in Gorlin’s Patients: a Matter of Fibroblasts-Led Protumoral Microenvironment ? ............................................................................................................................... 217
List of figures

Figure 1. The skin and its appendages. .................................................................................................................. 21
Figure 2. Epidermal structure and keratinocyte differentiation ........................................................................... 21
Figure 3. Dermal extracellular matrix components ............................................................................................. 26
Figure 4. Incidence rates of non-melanoma skin cancer in 2018 ................................................................. 33
Figure 5. Incidence rates of melanoma in 2018 ................................................................................................. 35
Figure 6. UV light and the skin. ......................................................................................................................... 37
Figure 7. Structures of the UV-induced DNA lesions cyclobutane pyrimidine dimer (CPD) and (6-4) photoproducts ........................................................................................................................................... 37
Figure 8. Age at onset of XP symptoms and cancers ......................................................................................... 43
Figure 9. Nucleotide Excision Repair mechanism .......................................................................................... 45
Figure 10. DNA repair pathways ....................................................................................................................... 47
Figure 11. DNA Repair disorders ...................................................................................................................... 49
Figure 12 XPC gene ........................................................................................................................................... 53
Figure 13. XPC protein ....................................................................................................................................... 54
Figure 14. The hallmarks of cancer. .................................................................................................................. 61
Figure 15. Antigen presentation to T cells. .......................................................................................................... 64
Figure 16. NK cell killing ................................................................................................................................... 66
Figure 17. Afferent and efferent pathways in the crosstalk between CAFs and cancer cells. ....................... 69
Figure 18. Cancer immunoediting .................................................................................................................... 71
Figure 19. CLEC2A transcript variants. ............................................................................................................. 83
Figure 20. The NKp65–KACl binding structural interface ................................................................................ 84
Figure 21. Kinetics of CLEC2A expression in WT fibroblasts (F-WT) after incubation with LPS ... 128
Figure 22. CLEC2A immunofluorescence staining of FTM1_WT fibroblasts after incubation with 200 ng/mL LPS for 6, 7, 8 and 24 hours ................................................................................................................... 128
Figure 23. Lysosome quantification in WT and XP-C fibroblasts after stimulation with LPS for 6 hours. ......................................................................................................................................................... 129
Figure 24. CLEC2A expression in WT fibroblasts over population doublings determined by flow cytometry. ......................................................................................................................................................... 131
Figure 25. Expression of p16 and p21 in three primary cell lines of WT fibroblasts at low and high PD as determined by Western blot. Anti-tubulin antibody was used as a loading control ......................................................................................... 132
Figure 26. Senescence associated β-galactosidase staining in two primary cell lines of WT fibroblasts at low and high PD....................................................................................................................................................... 132
Figure 27. CLEC2A expression in primary WT and XP-D fibroblasts ............................................................. 133
Figure 28. Relative CLEC2A mRNA transcripts levels in FH84_WT, SS537_NBCCS and AS_587_NBCCS fibroblasts determined by quantitative RT-PCR .................................................................................................................. 134
Figure 29. Crosstalk between fibroblasts and NK cells ...................................................................................... 141
Figure 30. Schematic overview of the pathways involved in the different lipid classes biosynthesis – cholesterol, Fatty acids (FA), sphingolipids, eicosanoids, acylglycerides, phosphoinositides and phosphoglycerides ........................................................................................................................................... 145
List of tables

Table 1 Characteristics of Papillary and reticular dermis................................................................. 28
Table 2. The ABCDE criteria allows the self-diagnosis of melanoma............................................. 36
Table 3. Xeroderma Pigmentosum complementation groups: insight in frequency, proneness to skin cancer development, neurological involvement and cellular repair capabilities. ......................... 48
Table 4. Patients and cell descriptions.................................................................................................. 122
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPC</td>
<td>Xeroderma pigmentosum complementation group C gene</td>
</tr>
<tr>
<td>XPC</td>
<td>Xeroderma pigmentosum complementation group C protein</td>
</tr>
<tr>
<td>XP-C</td>
<td>Xeroderma pigmentosum complementation group C</td>
</tr>
<tr>
<td>6-4 PP</td>
<td>6,4 pyrimidine-pyrimidone</td>
</tr>
<tr>
<td>8-oxoG</td>
<td>7,8-dihydro-8-oxoguanine</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>ABCDE</td>
<td>Asymmetry Border Color Diameter Evolution</td>
</tr>
<tr>
<td>AICL</td>
<td>Activation-induced C-type lectin</td>
</tr>
<tr>
<td>AK</td>
<td>Actinic keratoses</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3 related</td>
</tr>
<tr>
<td>AZ</td>
<td>Azathioprine</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCC</td>
<td>Basal cell carcinomas</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAA</td>
<td>Cancer associated adipocytes</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer associated fibroblast</td>
</tr>
<tr>
<td>CAF-1</td>
<td>Chromatin assembly factor 1</td>
</tr>
<tr>
<td>CCL5</td>
<td>(C-C motif) chemokine ligand 5</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDK7</td>
<td>Cyclin-dependent kinase 7</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional dendritic cells</td>
</tr>
<tr>
<td>CLEC</td>
<td>C type lectin receptor</td>
</tr>
<tr>
<td>CLEC2A/CLEC2A</td>
<td>C-Type Lectin Domain Family 2 Member A</td>
</tr>
<tr>
<td>cNK</td>
<td>circulating natural killer cells</td>
</tr>
<tr>
<td>COFS</td>
<td>Cerebro-oculo-facio-skeletal syndrome</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CPD</td>
<td>Cyclobutane pyrimidine dimer</td>
</tr>
<tr>
<td>CS</td>
<td>Cockayne syndrome</td>
</tr>
<tr>
<td>CSA/B</td>
<td>Cockayne syndrome group A/B protein</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-lymphocyte–associated antigen 4</td>
</tr>
<tr>
<td>CTLD</td>
<td>C-type lectin-like domain</td>
</tr>
<tr>
<td>CTLR</td>
<td>C-type lectin like receptors</td>
</tr>
<tr>
<td>CXCL</td>
<td>(C–X–C motif) ligand</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DDB</td>
<td>DNA damage-binding protein</td>
</tr>
<tr>
<td>DEJ</td>
<td>Dermal-epidermal junction</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAM-1</td>
<td>DNAX accessory molecule 1</td>
</tr>
<tr>
<td>EB</td>
<td>Epidermolysis bullosa</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EdU</td>
<td>5-Ethynyl-2′-deoxyuridine</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERCC</td>
<td>Excision repair cross-complementing</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cells</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FAP-α</td>
<td>Fibroblast-activation protein α</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food &amp; drug administration from the United States</td>
</tr>
<tr>
<td>FGF7</td>
<td>Fibroblast growth factor 7</td>
</tr>
<tr>
<td>FM</td>
<td>Familiar Melanoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FPC</td>
<td>Fitzpatrick phototype classification</td>
</tr>
<tr>
<td>FSP-I</td>
<td>Fibroblast specific protein 1</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GGR</td>
<td>Global genome repair</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgSF</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukine</td>
</tr>
<tr>
<td>JAK-STAT</td>
<td>Janus kinases/Signal transducer and activator of transcription proteins</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer immunoglobulin-like receptors</td>
</tr>
<tr>
<td>KLR</td>
<td>Killer cell lectin like receptor</td>
</tr>
<tr>
<td>LB</td>
<td>Lamellar bodies</td>
</tr>
<tr>
<td>LLT-1</td>
<td>Lectin like transcript 1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>LRC</td>
<td>Leukocyte receptor complex</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC1R</td>
<td>Melanocortin 1 receptor</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi drug resistant</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>ME</td>
<td>Microenvironment</td>
</tr>
<tr>
<td>MED</td>
<td>Minimal erythema dose</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MHC-1</td>
<td>Major histocompatibility complex class I</td>
</tr>
<tr>
<td>MIC-A/B</td>
<td>MHC-I-related chain A/B</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Acronym or Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>M-MDSC</td>
<td>Monocytic Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPG</td>
<td>Methylpurine-DNA glycosylase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate hydrogenase</td>
</tr>
<tr>
<td>NCR</td>
<td>Natural cytotoxicity receptors</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end-joining</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKG</td>
<td>Natural killer group receptor</td>
</tr>
<tr>
<td>NKR1</td>
<td>NK receptor protein subfamily 1</td>
</tr>
<tr>
<td>NMSC</td>
<td>Non melanoma skin cancers</td>
</tr>
<tr>
<td>NOS1</td>
<td>Nitric oxide synthase 1</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>OGG1</td>
<td>8-Oxoguanine glycosylase 1</td>
</tr>
<tr>
<td>OTUD4</td>
<td>Ovarian tumor protease domain containing protein 4</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PD-L1/2</td>
<td>Programed cell death protein ligand 1/2</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PFD</td>
<td>Pirfenidone</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin 2</td>
</tr>
<tr>
<td>PHA</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PMN-MDSCs</td>
<td>Polymorphonuclear - Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>Pol eta</td>
<td>Polymerase eta</td>
</tr>
<tr>
<td>PTCH</td>
<td>Patched</td>
</tr>
<tr>
<td>RARβ2</td>
<td>Retinoic acid receptor β2</td>
</tr>
<tr>
<td>RDEB</td>
<td>Recessive dystrophic form of epidermolysis bullosa</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RNA pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SA-β-gal</td>
<td>Senescence-associated beta-galactosidase</td>
</tr>
<tr>
<td>SASP</td>
<td>Senescent-associated secretory phenotype</td>
</tr>
<tr>
<td>SDF1α</td>
<td>Stromal cell-derived factor 1α</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SMO</td>
<td>G-protein coupled receptor smoothened</td>
</tr>
<tr>
<td>SMUG1</td>
<td>Single-strand-selective monofunctional uracil-DNA glycosilase 1</td>
</tr>
<tr>
<td>srNK</td>
<td>Skin-related natural killer cells</td>
</tr>
<tr>
<td>SS</td>
<td>Single strand</td>
</tr>
<tr>
<td>SSB</td>
<td>Single stranded binding proteins</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWItch/Sucrose Non-Fermentable</td>
</tr>
<tr>
<td>TA cells</td>
<td>Transient amplifying cells</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor associated macrophage</td>
</tr>
<tr>
<td>TAN</td>
<td>Tumor associated neutrophil</td>
</tr>
<tr>
<td>TC-NER</td>
<td>Transcription coupled-nucleotide excision repair</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell antigen receptor</td>
</tr>
<tr>
<td>TDG</td>
<td>Thymine-DNA glycosylase</td>
</tr>
<tr>
<td>TFIIH</td>
<td>Transcription factor II Human protein complex</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th cells</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rypamycin</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor-necrosis-factor related apoptosis inducing ligand</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TTD</td>
<td>Trichothiodystrophy</td>
</tr>
<tr>
<td>TTD-A</td>
<td>Trichothiodystrophy protein A</td>
</tr>
<tr>
<td>UDS</td>
<td>Unscheduled DNA synthesis</td>
</tr>
<tr>
<td>ULBP</td>
<td>UL16 binding protein 1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVSS</td>
<td>Ultraviolet sensitive syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>XCL1</td>
<td>X-C Motif Chemokine Ligand 1</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray repair cross-complementing protein 1</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma pigmentosum</td>
</tr>
</tbody>
</table>
Introduction
Chapter I: Skin

*Is there anything that skin can’t do?* - Albert Kligman (Chuong, Nickoloff et al. 2002)

The word skin has roots in both Greek and Latin and ultimately means surface envelope. Greek and Latin medicine pointed the skin as being purely an enveloping tissue of the internal organs, therefore underestimating its true importance. Partially because of that, the study of skin and its diseases did not have an official name - dermatology - till the 18th - 19th centuries (Santoro 2017). We know today that besides from “enveloping” the entire body, skin is an organ that plays vital functions in the mammalian body.

Skin is commonly described as the largest human organ. It has a surface area of 1.2 to 2 m² and accounts for about 12 to 15% of total body weight of an adult (Millington 2009, Lai-Cheong and McGrath 2017). Recent studies enlightened that even if this estimate surface area is accurate when talking about heat, water loss or topical drug delivery calculations, the measurement fails by not considering the approximately 5 million of appendages openings. These appendages openings put the total surface area of the skin between 25 and 30 m² (Gallo 2017).

1. Structure of the skin

Skin comprises three main layers: the epidermis, the dermis and the hypodermis. The epidermis is a stratified cellular layer separated from a dermis of connective tissue by a dermal-epidermal junction. The hypodermis is the deepest layer of the skin and is mainly composed of adipose cells (figure 1) (McGrath and Uitto 2016).

1.1. Epidermis

The epidermis is a self-renewing, stratified squamous terminally differentiated epithelium. Keratinocytes are the major cell type present in the epidermis accounting for 90 to 95% of the cells. Keratinocytes migrate from the epidermal basement membrane to the outermost part of the epidermis during a differentiation process and form four main different layers during the transit: basal layer, spinous layers, granular layers and cornified layers (figure 2). These different layers contribute to maintain of the structure of the skin.
Figure 1. The skin and its appendages. (McGrath and Uitto 2016)

Figure 2. Epidermal structure and keratinocyte differentiation. (Eckhart, Lippens et al. 2013)
1.1.a. The Basal layer (Stratum basale)

The basal layer of the epidermis consists of a single layer of small and cuboidal keratinocytes. These keratinocytes express keratins K5, K14 and K15 and can be divided in three subtypes depending on their mitotic activity: epidermal stem cells, transient amplifying cells and post-mitotic keratinocytes (Lyle, Christofidou-Solomidou et al. 1998, Eckhart, Lippens et al. 2013). The epidermal stem cells are located in special niches of the basal layer. They are quiescent cells, that divide unfrequently upon request for epidermal renewal or wound healing and, when they do, they give rise to the transient amplifying (TA) cells. These TA proliferating cells divide to generate post-mitotic keratinocytes that start terminal differentiation (Blanpain and Fuchs 2006, Koster and Roop 2007, Eckhart, Lippens et al. 2013).

Potten et al. and Bickenbach et al. were among the first to identify epidermal stem cells by labeling basal cells with a nucleotide analog. Once labeled, stem cells are able to retain the label for prolonged periods of time in what had been termed as “label retaining cells” (Potten, Kovacs et al. 1974, Bickenbach and Mackenzie 1984). More recently, following the identification strategy proposed by Ami Li et al. in 1998, authors were able to estimate the percentage of stem cells in the basal layer. The identification is based on the patterns of expression of two surface markers: the adhesion molecule α6 integrin and the proliferation-associated marker CD71 also known as transferrin receptor. Epidermal cells expressing high levels of α6 integrin and low levels of CD71 are considered as stem cells, and conversely epidermal cells expressing α6 integrin and high levels of CD71 are considered as being TA cells (Li, Simmons et al. 1998). Webb et al. reported 7.44% of stem cells in normal adult skin but for Schneider et al. the percentage doesn’t go higher than 1% in mouse skin. Even with this discrepancy, in both cases, and in conformity with what it is now widely accepted, stem cells are the less present subgroup in the basal layer and inversely TA cells constitute the most present subgroup (Schneider, Barland et al. 2003, Webb, Li et al. 2004).

By constantly providing post-mitotic keratinocytes that undergo differentiation, the basal layer contributes to assure the mechanical stability and homeostasis of the epidermis.

1.1.b. Spinous layer or stratum spinosum

Post-mitotic keratinocytes move upwards to spinous layers changing in shape and protein expression. Spinous keratinocytes are adjacently attached through desmosomes and stop expressing K5 and K14 and K15. Instead, they express K1 and K10 (Fuchs 1990, Lyle, Christofidou-Solomidou et al. 1998). The cytoplasm of the upper spinous cells contains lamellar bodies (LB) enriched with the precursors of stratum corneum lipids. Enzymes involved in lipogenesis such as lipid hydrolases, proteases and antimicrobial peptides (AMPs) are also
present in these LB. Due to keratin synthesis and lipogenesis, cells from the upper spinous layers become larger and ultimately reach another differentiation stage to form granular layers (Feingold 2007).

1.1.c. The granular layers or *stratum granulosum*

The granular keratinocytes are the uppermost nucleated cells of the epidermis. They are characterized by the presence of LB and two types of keratohyalin granules containing mostly profilaggrin, and loricrin (Steven, Bisher et al. 1990). Profilaggrin is the precursor of filaggrin, known for its role as an aggregator of keratin filaments, as well as in maintaining epidermal hydration (Dale, Resing et al. 1985, Kezic and Jakasa 2016). Loricrin is the major component of the corneocyte envelope (Mehrel, Hohl et al. 1990). LB migrate from the cytoplasm to the cell membrane and ultimately release their lipid components into the intercellular space between the *stratum granulosum* and the *stratum corneum*. This process, is primarily regulated by calcium signaling (i.e. depend on minimal Ca\(^{2+}\) concentrations) and promotes the formation of a unique hydrophobic film as initially described by Gray et al. in 1975 (Gray and Yardley 1975, Feingold and Elias 2014).

1.1.d. Cornified layers or *stratum corneum*

Cornified layers are the outermost layers of the epidermis. Keratinocytes present in these layers have lost nuclei and cytoplasmic organelles and are called corneocytes. Under the influence of the now active filaggrin, keratin filaments aggregate and align and cells become flattened (Steinert and Marekov 1995, Kezic and Jakasa 2016). Corneocytes possess a cornified envelope within the plasma membrane that is composed by structural proteins - including involucrin and loricrin - cross-linked between each other through transglutaminases. The intercellular spaces between corneocytes are filled with a lipid lamellae composed by a complex series of lipids, derived from the secretion of the LB. The terminal differentiation process ultimately results on a rigid and hydrophobic structure that provides both mechanical resistance and an active permeable barrier preventing transepidermal water loss and blocking the entry of external compounds (Candi, Schmidt et al. 2005, Feingold and Elias 2014).

Corneocytes are attached to each other by a modified type of desmosomes called corneodesmosomes. During the last stages of the differentiation process, corneodesmosomes are degraded by proteases allowing the desquamation of dead corneocytes. The equilibrium between cell loss by desquamation and epidermal renewal is essential for epidermal homeostasis (Marks, Nicholls et al. 1981, Candi, Schmidt et al. 2005, Eckhart, Lippens et al. 2013).
1.1.e. Other epithelial cells

Langerhans cells

Langerhans cells reside in the basal and suprabasal layers of the epidermis and account for about 2% to 4% of epidermal cells. They are antigen-presenting dendritic cells (DC) that originate from bone marrow and derive from primitive erythro-myeloid progenitors (Collin and Milne 2016). The most important role of Langerhans cells is to detect and transport antigens to skin local lymph nodes therefore contributing to the adaptive immune response of the skin. They also have a role in maintaining immune tolerance against innocuous and self-antigens of the skin (Romani, Holzmann et al. 2003, Stoitzner 2010, Tay, Roediger et al. 2014).

Merkel cells

Merkel cells are neuroendocrine cells mainly located in the basal layer of the epidermis and account for about 0.2% to 5% of epidermal cells. The embryonic origin of Merkel cells has been a matter of intense debate on whether they originate from embryonic epidermal or neural crest progenitors. A recent review unify this two hypothesis and clarify that Merkel cells derive from both neural and embryonic epidermis during early intrauterine life and they only undergo further differentiation once in epidermis (Abraham and Mathew 2019). Merkel cells have mechanosensory properties and are more concentrated in high tactile and sensitive areas. Their cytoplasm contains neurosecretory granules and they are tightly connected with sensory nerve endings forming the Merkel cell-neurite complex. Throughout this association Merkel cells actively participate in touch reception (Boulais and Misery 2007).

Melanocytes

Melanocytes are DCs responsible for skin color. They derive from embryonic neural crest and are located either in hair follicles or in the basal layer of the epidermis where the ratio melanocytes : keratinocytes is 1: 10 (Cichorek, Wachulska et al. 2013). Melanocytes produce melanin pigments packed in cytoplasmic melanosome units that are responsible for the distribution of melanin to neighboring keratinocytes. Eumelanin and phaeomelanin are the two types of melanin produced in skin and hair. The different appearances of skin are not related to the number of melanocytes present in the epidermis but to the size of the melanosomes and the nature of the melanin. Melanin production is stimulated by ultraviolet (UV) radiation and contributes to skin protection against UV associated cell damage (McGrath and Uitto 2016, Yi, Su et al. 2018).
1.2. Dermal-epidermal junction

The dermal-epidermal junction (DEJ) is at the interface between dermis and epidermis. It promotes epidermal adhesion to the dermis and is essential to the crosstalk and cell migration between the two compartments.

DEJ is composed by a network of proteins, the main ones being type IV collagen and laminins, and is divided in different layers. Lamina lucida, the upper layer of the DEJ, is structurally attached to basal keratinocytes through hemidesmosomes. Lamina densa is below lamina lucida and interacts with the upper dermis through anchoring fibrils. Besides its importance in maintaining the cohesion and integrity of skin, DEJ also plays a role in the protection against mechanical shearing. Both epidermal keratinocytes and dermal fibroblasts are implicated in the synthesis of the components of the DEJ and therefore in its homeostasis (El Ghalbzouri, Jonkman et al. 2005, Has and Nystrom 2015).

1.3. Dermis

The dermis is the major component of the human skin and its thickness varies among different regions of the body. It consists of an extracellular matrix (ECM) of connective tissue that provides strength and flexibility to the skin and has a strong capacity to retain water. Unlike the epidermis, dermis is an abundantly vascularized and sparsely cellular layer with fibroblasts being the most abundant cell type. The blood vessels present in the dermis provide nutrients and oxygen to the skin and also have a role in thermoregulation (Plager, Bieber et al. 2014).

There are three classes of ECM components: fiber-forming structural molecules, nonfiber-forming structural molecules, and matricellular proteins (figure 3).
Collagens and elastin are important fiber forming molecules providing structure to the dermis. Glycosaminoglycans (GAGs) (like hyaluron, chondroitin sulfate and dermatan sulfate) and proteoglycans (like versican, decorin, dermatopontin and lumican) are important to ensure dermis hydration. Normally proteoglycans bound to a GAG core protein. Matricellular proteins act as signaling molecules principally in injured skin.

Fiber-forming molecules are the ones providing structure to the ECM. The major fiber-forming molecule is collagen comprising approximately 80% of the dry weight of the skin. There are nearly 30 different types of collagen identified so far (Ricard-Blum 2011). Type I and type III are the most abundant in the dermis while type IV is the principal type in the DEJ. Whereas collagen fibers provide tensile strength to the skin, elastic fibers are the ones responsible for skin elastic properties. The main component of elastic fibers is elastin (Kielty, Sherratt et al. 2002). Other fiber-forming proteins that are mediators in cell migration and hemostasis are fibrins, fibronectins and vitronectins (Weinstein and Boucek 1960, Bosman and Stamenkovic 2003, Tracy, Minasian et al. 2014).

The majority of the dermis interstitial space is filled by the nonfiber-forming molecules - proteoglycans and glycosaminoglycans (GAGs). Their hydrophilic nature assures the hydration of the dermis. Matricellular proteins do not affect the structure of the dermis. They are very little present in a healthy dermis but increases expression during tissue injury.
contributing to cell-matrix interactions (Bosman and Stamenkovic 2003, Tracy, Minasian et al. 2014).

ECM began to be considered as more than a structural support when it was shown that some cells needed to be anchored to ECM to grow in a process called “anchorage dependence”. We now know that besides its structural support and anchoring capacities, the complex composition of the ECM is essential to the homeostasis of the skin, by regulating certain aspects of cell behavior such as proliferation and migration (Stoker, O’Neill et al. 1968, Bosman and Stamenkovic 2003, Tracy, Minasian et al. 2014).

1.3.a. Fibroblasts

Mesenchymal dermal fibroblasts, the main cell type of the dermis, are responsible for both the synthesis and degradation of ECM components. Skin fibroblasts represent a heterogeneous population of cells. Vimentin and fibroblasts specific protein 1 (FSP-1) are two of the most known fibroblasts markers in the post-natal skin. However, they cannot be considered as specific markers as they are present in other types of cells such as endothelial and myoepithelial cells (vimentin) and hematopoietic, endothelial cells and vascular smooth muscle cells (FSP-1) (Sorrell and Caplan 2004, Kong, Christia et al. 2013, Chang, Li et al. 2014, Driskell and Watt 2015).

Three different subpopulations of fibroblasts can easily be identified in different locations: papillary layer, reticular layer and fibroblasts associated with hair follicles. Papillary layer is the upper layer of the dermis. This layer is thinner, has high cellular density and a poorly organized fiber bundles comparing to the reticular layer. Fibroblasts from papillary layer proliferate at higher rate, and interfere with keratinocyte viability and growth when cultured in a skin equivalent model (Harper and Grove 1979, Janson, Rietveld et al. 2017). Fibroblasts from the reticular layer are mostly involved in wound healing (Janson, Saintigny et al. 2012, Janson, Rietveld et al. 2017). Driskell et al. show that in mouse, fibroblasts from papillary and reticular dermis arise from two different lineages which may be a coherent explanation for how different and specific the functions of these two subpopulations are (Driskell, Lichtenberger et al. 2013). The characteristics of papillary and reticular fibroblasts are summarized in table 1 The third subpopulation of fibroblasts, associated with hair follicles, plays a role in the homeostasis of the hair follicle (Yang and Cotsarelis 2010).
### Characteristics of papillary and reticular dermis.

<table>
<thead>
<tr>
<th></th>
<th>Papillary fibroblast</th>
<th>Reticular fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (diameter of cells in suspension)</td>
<td>small</td>
<td>large</td>
</tr>
<tr>
<td>Shape</td>
<td>spindle</td>
<td>stellate, flattened</td>
</tr>
<tr>
<td>Edge of the cell</td>
<td>smooth</td>
<td>rough</td>
</tr>
<tr>
<td>Proliferation rate</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Collagen contraction</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Density in monoculture</td>
<td>high</td>
<td>low – contact inhibition</td>
</tr>
<tr>
<td>Focal adhesions</td>
<td>rare</td>
<td>abundant</td>
</tr>
<tr>
<td>Effect on keratinocytes</td>
<td>complete keratinocyte differentiation, thicker keratinocyte layer</td>
<td>defective keratinocyte differentiation, thinner keratinocyte layer</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>promote angiogenesis</td>
<td>no effect</td>
</tr>
<tr>
<td>Gene expression (GO term analysis)</td>
<td>genes involved in immune response</td>
<td>genes involved in cytoskeletal organization, cell motility</td>
</tr>
<tr>
<td>Collagen I</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Collagen VI alpha 1</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Collagen VI alpha 2</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Collagen XVI</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Versican</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Decorin</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>GAG</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>α-smooth muscle actin</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>MMPs</td>
<td>higher with donor age</td>
<td>constant with donor age</td>
</tr>
<tr>
<td>HGF</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>TGF-β</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Podoplanin</td>
<td>high</td>
<td>no production</td>
</tr>
<tr>
<td>Transglutaminase 2</td>
<td>no production</td>
<td>high</td>
</tr>
</tbody>
</table>

Matrix metalloproteinase (MMP), Hepatocyte growth factor (HGF), Transforming growth factor-β (TGF)

For a long time, fibroblasts were considered as only having a structural role in the homeostasis of the dermis through the synthesis and remodeling of the ECM. Although they are still a very enigmatic heterogeneous population, it is now established that fibroblasts interact with numerous distinct cell types – epidermal, endothelial, dendritic and immunocompetent cells – playing a central role in a diversity of processes. They are essential in wound healing, in mediating inflammatory and immune responses, in cancer cell progression and could be considered as a barometer for aging (Sorrell and Caplan 2009). The functional diversity of dermal fibroblasts is not totally understood. Besides of the obvious differences observed in fibroblasts arising from different lineages, Xu et al. proved that the different stimuli to which fibroblasts are subjected in different anatomic sites also contribute to the heterogeneity of
fibroblast population (Xu, Kuang et al. 2017). The role of fibroblasts in immune responses and cancer cell progression will be further discussed later in this manuscript.

1.3.b. Other dermal cells

Mast cells

Mast cells derive from myeloid progenitors and are present in connective tissues throughout the body. In skin, they are concentrated in the papillary dermis near blood vessels, cutaneous appendages and nerves. In 1950, Hellstrom and Holmgren determined that there are about 7000 to 10000 mast cells per mm$^3$ in normal skin. Mast cells play a role in immune responses and are particularly well known for their role in the immediate allergic reaction through the release of histamine. Although they reside in the dermis, mast cells can migrate to the epidermis upon inflammation (Hellstrom and Holmgren 1950, Harvima and Nilsson 2011).

Macrophages

Macrophages are phagocytic cells that derive from monocyte differentiation. Besides from their classical phagocytic properties removing dead cells and metabolites from tissues, they also play a role in wound healing and angiogenesis. Macrophages are activated after a cutaneous injury and transit between a pro-inflammatory and an anti-inflammatory phenotype. They contribute to both the synthesis of ECM and the re-epithelialization (Koh and DiPietro 2011).

Dermal dendritic cells

Dermal DCs are a heterogeneous population that can be distinguish from other dendritic cells by the expression of CD209, a dendritic cell specific intercellular adhesion molecule 3 (ICAM-3) grabbing non integrin receptor. Like Langerhans cells in the epidermis, dermal DCs are antigen-presenting cells (APC), playing a role in both the initiation of primary immune responses and in the maintenance of immune tolerance (Geijtenbeek, Torensma et al. 2000, Ebner, Ehammer et al. 2004).

Lymphocytes

T cells are the main type of lymphocytes present in the dermis. There are about 20 billion T cells in skin, making it the largest reservoir of T cells in body tissues. Some T cells remain resident cells never leaving the dermis, while others have the capacity to re-enter the blood circulation. T cells are able to memorize and recognize specific antigens presented by APCs,
hence leading to specific immune responses (Clark 2010). Natural killer (NK) cells are another type of lymphocytes present in the dermis. NK cells are endowed with cytotoxic properties to kill external pathogens, infected or cancer cells without previous need of antigen presentation (Ebert, Meuter et al. 2006). The role of T cells and NK cells in cancer microenvironment will be discussed in chapter III.

1.4. Hypodermis

The hypodermis is the deepest layer of the skin and is mainly composed of adipocytes. It provides insulation and cushioning to the internal organs and store energy in the form of lipids. Within the hypodermis, adipocytes are organized in two distinct tissue subsets: the dermal adipose tissue and the subcutaneous adipose tissue. Studies have shown that murine adipocytes from these two adipose tissues have different developmental origins. Moreover, murine adipocytes from dermal adipose tissue shared the same progenitor with reticular fibroblasts (Driskell, Lichtenberger et al. 2013, Driskell, Jahoda et al. 2014). In addition to the classic metabolic function, adipocytes also play a role in regulation of hair follicle growth, angiogenesis and wound healing (Rivera-Gonzalez, Shook et al. 2014). For a long time, hypodermis was not considered as responsive to UV radiation because UVs can't penetrate the hypodermis. Of note, UVA penetrates deeply into the dermis, while UVB penetrate only up to the superficial dermis (Christensen, Suggs et al. 2017). However, studies from Ji Ho Chung laboratory show that UV indirectly affect adipocytes through keratinocytes and fibroblasts, promoting a pro-inflammatory state that unbalances the homeostasis of the adipose tissue (Kim, Kim et al. 2018).

1.5. Skin appendages

Skin appendages are structures that are essential for skin to fulfil all its functions properly.

1.5.a. Pilosebaceous units

Pilosebaceous units are composed of hair follicles, sebaceous glands and the arrector pili muscle that, when contracted, is responsible for the “raiser” of the hair. Hair follicles derive from epidermal tissue and project into the dermis. The dermal papilla is located at the base of the hair follicle. It is surrounded by a rich extracellular matrix and acts as a mesodermal signaling center. The bulge region, another component of the hair follicle, is a reservoir of stem cells that when activated is responsible for the production of new hair (Alonso and Fuchs 2006, Schneider, Schmidt-Ullrich et al. 2009). Hair growth depends on cycles of growth (anagen), apoptosis-mediated regression (catagen) and relative quiescence (telogen). In each cycle, a
new hair is formed, and the old hair eventually shed. For more information about hair cell cycle please refer to Alonso and Fuchs review (Alonso and Fuchs 2006).

The superficial part of the hair is mainly composed by keratin filaments that derive from heavily keratinized and pigmented cells. Hair length and structure differs in different parts of the body. Hair acts as a mechanoreceptor due to its sensitivity to touch and has other specific functions dependent on the location. For example head hair protects the scalp against UV radiation and eyelashes and eyebrows helps keeping foreign particles out of the eyes (Arda, Göksügür et al. 2014). The complete structure of the hair follicle is represented in figure 1.

1.5.b. Glands

There are two types of skin glands: sebaceous glands and sweat glands. Sebaceous glands are typically associated to hair follicles. They secrete a complex lipid mixture called sebum that allows to maintain skin suppleness and protects the upper part of the epidermis from the external environment. Sebum also presents antimicrobial properties. Sweat glands are subdivided in eccrine and apocrine glands. Eccrine glands are functional after birth and apocrine glands became functional during puberty. Both eccrine and apocrine glands have a thermoregulatory function releasing water to the cell surface when the body temperature rises (Arda, Göksügür et al. 2014, Fuchs 2016).

2. Function

In a 2002 textbook, a large council of biologists and clinicians answered the question “What is the “true” function of the skin?”. The first thoughts come from Albert Kligman, a dermatologist, who allowed himself to reformulate the question to “Is there anything that skin can't do?” (Chuong, Nickoloff et al. 2002). In this section a description of skin functions supports his idea of the skin as a complete, unparalleled, multi-tasking organ.

Skin is frequently described as a barrier, protecting the body from external insults. This barrier is at the same time mechanical, antimicrobial, chemical and hydric.

**Mechanical barrier:** The viscoelastic properties of the skin reflect both its rich fiber composition and the different cell-to-cell interactions. This complex structure confers to the skin resistance against internal and external forces and, at the same time, is capable of providing physiological responses when needed (Tissot, Boulter et al. 2016).

**Antimicrobial barrier and immunesurveillance:** Skin cells like keratinocytes and mast cells, sweat glands and commensal bacteria are able to produce and secrete AMPs that are part of skin defense mechanisms against pathogens and external insults. These AMPs have the
capacity to directly kill pathogens and trigger an immune response via the activation of immune cells. Furthermore it has been shown that skin inflammatory diseases such as atopic dermatitis and psoriasis exhibit a different AMP profile compared to healthy skin (Nakatsuji, Chen et al. 2017, Patra, Mayer et al. 2018). As already mentioned in this manuscript, Langerhans and NK cells play essential roles in the immune system surveillance, permanently screening for the presence of external pathogens in the skin. In presence of microbial invasion, they are able to either initiate immune responses (Langerhans) or directly kill the threat (NK).

**Chemical and hydric barrier:** The structure of the corneocytes together with the specific combination of skin lipids confer at the same time a barrier against passive water loss and chemical penetration. Once again, Langerhans cells can act as sensitizers, triggering immune responses after a chemical assault (Cumberbatch, Dearman et al. 2003, Wickett and Visscher 2006, Jia, Gan et al. 2018). Finally, skin is also an effective barrier against UV radiation through melanocytes and their secreted melanin, and cutaneous appendages such as hair and nails.

Besides its function as a barrier, skin play other essential roles in the homeostasis of the human body.

First, in thermoregulation. There are two mechanisms responsible for the control of body temperature. One involves the ability of the sympathetic nervous system to control vasoconstriction and vasodilatation of blood vessels, therefore regulating heat lost through the skin. The other one involves the capacity of sweat glands to cool the body via the release of water (Charkoudian 2003).

Second and third, in providing insulation and cushion to the internal organs and in storing energy. These two functions are fulfilled by the hypodermis and are related to both structural and cellular characteristics of this layer.

Fourth, as an endocrine organ. Skin specialized cells are able to convert androgens in different activated forms and to produce insulin-like growth factors. After UV exposure, keratinocytes are the ones responsible for the synthesis and metabolism of vitamin D which is essential to regulate the absorption of calcium (Zouboulis 2009).

Fifth, skin is part of the somatosensory system. Through a complex mechanism, involving keratinocytes, Merkel cells, nerve endings and hair, the mechanical information is transformed in the sensation of touch (Tissot, Boulter et al. 2016).

Last, but not least, skin has to be considered as a psychosocial determinant, regarding social interactions. Dermatological patients can easily be stigmatized and stigma often lead to anxiety and depression scenarios. It is important to build strategies to help these patients be more confident, to avoid social reclusion (Dimitrov and Szepietowski 2017).
3. Skin cancers

Cutaneous cancers are the most frequent cancer worldwide and their prevalence has highly increased over the last thirty years. The International Agency for Research on Cancer (IARC) estimate 1.32 million new cases only in 2018 (figure 4 and 5) (Bray, Ferlay et al. 2018). They are sub divided into non melanoma and melanoma skin cancers.

3.1. Non melanoma skin cancers

Non melanoma skin cancers (NMSC) originate from keratinized epithelial cells. There are two main types of NMSC: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC and SCC have different incidence rates, metastatic potential and prognosis.

**BCC** is the most common cancer and its incidence varies around the world. The highest incidence rate is in Australia (>1000/100000 person per year) and the lowest in Africa regions (<1/100000 person per year) (Lomas, Leonardi-Bee et al. 2012). BCCs arise either from interfollicular basal keratinocytes of the interfollicular epidermis or from the hair follicle (Youssef, Van Keymeulen et al. 2010, Wang, Wang et al. 2011). Studies have shown that activation of the Sonic HedgeHog pathway signaling due to mutations of core genes like PTCH and the G-protein coupled receptor smoothened (SMO) promote oncogenic signaling and drive the growth of BCCs (Fan, Oro et al. 1997, Xie, Murone et al. 1998, Aszterbaum, Epstein et al. 1999, Hutchin, Kariapper et al. 2005). BCCs rarely metastasize (incidence rates varies from 0.0028% to 0.55%) and are often associated with good prognosis (von Domarus and Stevens...
There are different clinical subtypes of BCCs all of them occurring essentially in the face and trunk, i.e. sun exposed areas of the skin. Histologically, BCC is characterized by the presence of aggregations of basaloid keratinocytes that typically stay in contact with the epidermis (Stanoszek, Wang et al. 2017).

SCC represents less than 20% of NMSC. Like BCC, the higher incidence rate of SCC is also in Australia with > 380/100000 person per year. The incidence of both BCC and SCC changes between geographic areas. In North America there is a clear distinction between SCC rates in northern (60/100000 person per year) and southern (290/100000 person per year) regions (Lomas, Leonardi-Bee et al. 2012). This difference is most probably related to the UV radiation levels of these two areas. UV radiation is one of the main risk factors in the development of skin cancer and will be discussed later in this chapter. A cohort study from Schmults et al. puts the risk of cutaneous SCC metastasis at 3.7%, which is significantly higher than in BCC and is frequently associated with poor outcomes (Schmults, Karia et al. 2013). SCC commonly arises in areas with existing actinic keratoses (AK) benign lesions. AK lesions are caused by accumulation of UV exposure and clinically manifest as rough, scaly and erythematous patches. Many studies over the years tried to estimate the rate of progression of AK into SCC. A systematic review from 2013 ranged progression rates between 0% and 0.075% per lesion-year, while a previous study puts the rates between 0.025% and 20% per lesion-year (Quaedvlieg, Tirsi et al. 2006, Werner, Sammain et al. 2013). In mice, SCC derives from both hair follicle and interfollicular stem cells however, these observations are highly dependent on the genetic background of the studied mouse as well as their genetically engineered modifications (Lapouge, Youssef et al. 2011, White, Tran et al. 2011). Histologically, SCC presents strands of atypical keratinocytes that infiltrate the dermis and produce high amounts of keratin resulting in the formation of keratin pearls that express terminal markers of epidermal differentiation. They range from well differentiated to poorly differentiated cells showing frequent mitoses (Yanofsky, Mercer et al. 2011, Stratigos, Garbe et al. 2015).

3.2. Melanoma skin cancer

Melanoma is the most threatening skin cancer. The IARC estimations indicated in 2018 that the 5-year prevalence of melanoma worldwide is > 960 000 cases. In 2018, the worldwide incidence was > 280 000 and the number of deaths > 60 000, putting the mortality rates at about 18%. Like with NMSC, Australia is the region with the highest incidence and, UV radiation is the main risk factor (Karimkhani, Green et al. 2017, International Agency for Research on Cancer 2019)
Approximately 50% of melanomas arise from mutations in \textit{BRAF}, a gene that codes for a protein kinase that activates the MAP kinase/ERK-signaling pathway. V600E mutation represents 90% of \textit{BRAF} mutations described in melanoma (Davies, Bignell et al. 2002). Independent of \textit{BRAF} mutations (i) individuals exhibiting a low pigmentation, due to polymorphisms of the melanocortin 1 receptor gene (\textit{MC1R}), are more prone to develop melanoma and (ii) the presence of mutations in microphthalmia-associated transcription factor (\textit{MITF}) predisposes to co-occurring melanoma and renal-cell carcinoma (Bertolotto, Lesueur et al. 2011, Dessinioti, Antoniou et al. 2011, Leonardi, Falzone et al. 2018).

The melanoma-related mortality is due to the ability of melanoma cells to form distant metastases. To avoid a late diagnosis that could lead to poor prognoses, in 1985, an ABCD acronym nomenclature system - coding for Asymmetry, Border, Color and Diameter - was created to help melanoma self-diagnosis. This system was later improved with the notion of lesion evolution (E) and became the ABCDE criteria (table 2) (Friedman, Rigel et al. 1985, Robinson and Turrisi 2006).
### Asymmetry
- One-half of lesion is different than the other half

### Border
- Irregular or poorly defined border

### Color
- Varied from one area to another; different shades of tan, brown, black; sometimes red, white, or blue within the same lesion

### Diameter
- Larger than 6 mm (bigger than the size of a pencil eraser)

### Evolving
- A mole that looks different compared with surrounding moles (“ugly duckling” sign), or the mole is changing in size, shape, or color

(Shenenberger 2012)

**Table 2. The ABCDE criteria allows the self-diagnosis of melanoma.**

3.3. Risk factors

Predisposition to skin cancer include both exogenous and endogenous factors. Although the main environmental and modifiable risk factor is UV exposure, the combination between modifiable factors and non-modifiable ones, like chronological aging, can exacerbate the chances of developing cutaneous cancers.

3.3.a. UV exposure

Over the years, several studies reported a correlation between sun exposure and the development of cutaneous cancer. Even if recent data suggest that different sun exposure profiles are associated with different skin cancer subtypes, both NMSC and melanoma arises in their majority in skin exposed to sunlight areas, most probably due to mutagenic UV radiations (Savoye, Olsen et al. 2018).

The spectrum of solar radiation contains UV, visible light and infrared wavelengths of radiation. UV spectrum ranges from 100 nm to 400 nm and can be further subdivided in UVA (315-400 nm), UVB (280-315 nm) and UVC (315-400 nm). Since UVC is (normally) completely blocked by the ozone layer therefore barely reaching earth surface, UVA and UVB are the sun light UV subtypes that actually penetrate skin (figure 6). 95% of the UV radiation reaching the Earth surface correspond to UVA and the remaining 5% to UVB(Sage 1993). UVA longer wavelength penetrates deeper in skin reaching reticular dermis and is mostly implicated in photo aging due to its capacity to deteriorate dermal fibers. It can also promote oxidative DNA damage through the generation of oxidative base modifications such as 7,8-dihydro-8-oxoguanine (8-oxoG) (Kozmin, Slezak et al. 2005, D’Orazio, Jarrett et al. 2013) UVB radiations (UVBs) are able to penetrate epidermis and superficial dermis and has a strong carcinogenic potential through its direct mutagenic effect in DNA strand (de Gruijl 2000, D’Orazio, Jarrett et al. 2013). UVBs mainly induce the formation of two specific DNA photoproducts -cyclobutane pyrimidine dimers (CPDs) and 6,4 pyrimidine-pyrimidone (6-4 PP) (figure 7). UVA are also capable of inducing
CPDs but not 6-4 PPs, albeit following much higher doses of radiation (Douki, Reynaud-Angelin et al. 2003).

![Figure 6. UV light and the skin.](image)

![Figure 7. Structures of the UV-induced DNA lesions cyclobutane pyrimidine dimer (CPD) and (6-4) photoproducts](image)

(Li, Uchida et al. 2006)

The cell machinery is able to either repair UV-induced DNA lesions or to trigger apoptosis if the amount of DNA damage is too high and considered as “saturating” to the repair
mechanisms. However even with fully functional UV protecting mechanisms, some lesions could persist leading to mutations (Valejo Coelho, Matos et al. 2016). The nucleotide excision repair (NER) is the mechanism responsible for the repair of UV-induced CPDs and 6-4PP and will be further discussed in the next chapter. Base excision repair (BER) is the mechanism responsible for the removal of the damaged bases like 8-oxoG.

3.3.a. Pollution

Atmospheric aerosols containing particles, and biological and gaseous contaminants are considered as the main risk factor towards multiple diseases, especially respiratory and cardiovascular related-diseases. Evidences show that some of the contaminants of these aerosol particles also have a negative impact on skin and are associated with the development of skin cancer (Kim, Cho et al. 2016). Examples are the black carbon, cigarette smoke and polycyclic aromatic hydrocarbons (PHA). Occupational exposure to black carbon increases the incidence of melanoma, and cigarette smoke increases incidence and exacerbation of NMSC through the formation of reactive oxygen species (ROS) that ultimately lead to oxidative DNA damage (Puntoni, Ceppi et al. 2004, Coppe, Boysen et al. 2008). After bio activation, PHA turn into carcinogenic metabolites and are therefore considered as general carcinogens (Siddens, Larkin et al. 2012).

3.3.b. Phototype

Fitzpatrick first proposed a phototype classification (FPC) in 1975 based on the response of different skin colors to the UV radiation, in terms of tanning and tendency to burn. After a modification of the initial Fitzpatrick classification to include people with darker skin colors, individuals falls in six (I to VI) different categories spreading from those who burn easily and never tan and those who never burn and tan profusely (Fitzpatrick 1975, Fitzpatrick 1988). Melanins are considered as skin photoprotectors due to their respective capacity to absorb UV radiations. Epidemiological studies show a correlation between the FPC and the development of skin cancer. High FPCs, (i.e. darker skin phototypes IV -VI) are less prone to develop malignancies while fairly pigmented (i.e. skin phototypes I-III) are more susceptible to skin cancers). Today, FPC values are used by researchers and clinicians as prognostic criteria to assess the risk of developing skin cancer (Sitek, Rosset et al. 2016, Fajuyigbe, Lwin et al. 2018, Holm-Schou, Philipsen et al. 2018).
3.3.c. Aging

Most cancers have high incidence and are more prevalent upon aging. About 95% of the NMSC cases between 2014 and 2018 were reported in people of at least 50 years (International Agency for Research on Cancer 2019).

The accumulation of DNA damage during life is the “primary suspect” in the correlation between skin cancer and aging. Both cancer and aging are processes that share a genomic instability and it can lead to different outcomes from an uncontrolled cellular growth to senescence or apoptosis (Moskalev, Shaposhnikov et al. 2013, Aunan, Cho et al. 2017).

Importantly, senescence process plays a paradoxical role in cancer progression acting both as a protection mechanism against uncontrolled cellular proliferation and a promoter of epithelial growth and tumorigenesis. This notion will be further discussed in chapter III (Krtolica, Parrinello et al. 2001, Coppe, Desprez et al. 2010, Ghosh and Capell 2016).

3.3.d. Immunosupression

The immune system has an important role in controlling cancer progression. Over the years, thousands of studies described the dramatically higher rates of NMSC incidence in organ transplant patients during immunosuppressive periods (Nair, Gongora et al. 2014, Manyam, Gastman et al. 2015, Iannacone, Sinnya et al. 2016, Pinho, Gouveia et al. 2016).

One of the first drug used in post-transplant regimens was the anti-metabolite azathioprine (AZ). It impairs DNA synthesis and was used to lower immune response in post-transplant patients to avoid rejection. However, AZ treatment in monotherapy correlates with occurrence of cancers in post-transplanted patients (Scharf, Nahir et al. 1977, Van Scoik, Johnson et al. 1985). With the discovery of calcineurin as a key signaling enzyme in T-cell activation, new drugs attempting to inhibit this pathway were created. The first one was cyclosporine that, like AZ, is related with higher rates of skin cancer among transplant patients (Dantal, Hourmant et al. 1998). In 2000, the introduction of mTOR (mammalian target of rapamycin) sirolimus inhibitor started a new era in post-transplant therapies. TOR inhibitors prevent cytokine receptors from activating the cell cycle and the use of TOR inhibitors to maintain immunosuppression are associated with a lower risk of post-transplant NMSC comparing to the “first era” treatments (Eng, Sehgal et al. 1984, Bernat-Garcia, Morales Suarez-Varela et al. 2014, Iannacone, Sinnya et al. 2016, Pinho, Gouveia et al. 2016, Okut, Alp et al. 2017).

The role of the immune system in cancer progression is an important part of this manuscript and will be further discussed in chapter III.
3.3.e. Genetic disorders

While a few cancers (such as medulloblastoma) may be observed with a significantly higher prevalence in youth than in the elderly in the general population, cancers such as those affecting skin or hepatogastric epithelia are mostly observed in individuals over 50 (International Agency for Research on Cancer 2019). The diagnosis of skin cancer in young patients could indicate an associated genetic disorder.

**Nevoid Basal Cell Carcinoma Syndrome (NBCCS)** is a rare hereditary autosomal disorder that leads to the development of multiple BCC at early age. The syndrome affects skin, endocrine and central nervous system and is also associated with skeletal abnormalities. It is mostly caused by mutations in genes of the Sonic-Hedgehog pathway responsible for controlling cellular growth (Epstein 2008).

**Epidermolysis bullosa (EB)** is a rare disease that particularly affects children. The main clinical traits are skin fragility, blistering and no healing wounds. The disease is caused by mutations in genes encoding for cohesion molecules at the EDJ like integrin, laminin and collagen and in the basal form of the disease, mutations in K5 and K14 are also present. The recessive dystrophic form of EB (RDEB) caused by mutations in *COL7A1* gene is associated with higher probability of developing SCC in wound, blistered or scar areas (Fine, Bruckner-Tuderman et al. 2014, Mellerio, Robertson et al. 2016).

**Familial melanoma (FM)** is the main cause of melanoma diagnosis in the youth. More than 50% of autosomal dominant mutations transmitted to the offspring are on genes involved in tumor suppression and telomere maintenance. Germline mutations in cyclin-dependent kinase inhibitor 2A (*CDKN2A*) were the first to be associated with familial melanoma susceptibility and account for 20% of the cases (Kamb, Shattuck-Eidens et al. 1994, Goldstein and Tucker 2001). Individuals with familial history of melanoma should be followed by a dermatologist at least once a year to a skin exam and informed of the advantages of the self-evaluation (Aoude, Pritchard et al. 2015, Soura, Eliades et al. 2016).

**Xeroderma pigmentosum (XP)** is a rare autosomal genetic disease caused by mutations on genes involved in the repair of DNA lesions after a UV stress. The accumulation of unrepaired lesions in the DNA strand lead to a propensity to develop cutaneous cancers at an early age. They develop 10,000 more BCC and SCC and 2000 more melanomas than the general population (Bradford, Goldstein et al. 2011). In my thesis work, I used XP as a model to study development and progression of cutaneous cancers. The next chapter will be dedicated to this disease.
3.4. Treatment of skin cancers

Surgical excision is the standard treatment for both BCC, SCC and melanoma. Small and superficial BCC can still be treated with liquid nitrogen or abrasive therapies. Topical treatments with imiquimod, an immunomodulatory agent that induces the release of pro-inflammatory cytokines, or photodynamic therapies are also a possibility. For the rare cases of inoperable BCC, mostly due to its location, the first choice is radiation therapy. Some Hedgehog pathway inhibitors are in clinical testing and seemed to be a good option especially with advanced or metastatic BCCs (Berking, Hauschild et al. 2014).

For SCC, besides the inoperable ones that are treated mostly with radiotherapy, the surgical excision is always the first choice as it allows to definitely confirm the type and extension of tumor invasion and clear the margins. In patients that cannot undergo surgery, radiation therapy is, like with BCC the first choice. In presence of late stages of SCC presenting aggressive and metastatic features, chemotherapy with combinations of the neoplastic alkylating agent cisplatin and the neoplastic anti-metabolite 5-fluoracil could also be considered (Stratigos, Garbe et al. 2015, Strom, Caudell et al. 2016).

In melanoma, as for the NMSC, radiotherapy is only an option when surgery is not possible. When metastases are detected or a high metastatic risk is associated, adjuvant therapies like interferon-α, immunotherapy (further discussed in chapter III), target therapy or chemotherapy (with the neoplastic alkylating agent dacarbazine) can be proposed (Garbe, Peris et al. 2016).
Chapter II: Xeroderma pigmentosum

“The prognosis, [of xeroderma pigmentosum] according to our experience is absolutely unfavorable” - Kaposi 1874 (Hebra and Kaposi 1874)

Xeroderma pigmentosum (XP) is a rare autosomal recessive genetic disease characterized by the patient’s higher susceptibility to develop skin cancers in sun exposed areas. Hebra and Kaposi were the first to describe XP in 1874 pointing out “a remarkable abnormality of pigmentation” and a “parchment-like dryness, thinness, and wrinkling of the epidermis” (Hebra and Kaposi 1874). A century later, James Cleaver classified XP as a genetic disease reporting defects in the DNA repair process in XP patients’ cells after UV radiation (Cleaver 1968). Not long after, De Weerd-Kastelein proved that there is a genetic heterogeneity in XP disease and finally seven groups of genetic complementation were identified (XP-A to XP-G) plus one variant (XP-V) (Cleaver 1972, De Weerd-Kastelein, Keijzer et al. 1972, Keijzer, Jaspers et al. 1979). The clinical and genetic features of XP patients provide a model to study both DNA repair and carcinogenic mechanisms (Kraemer and DiGiovanna 2015).

1. Epidemiology and clinical traits

Estimated incidences of XP vary according to geographical location. The most recent epidemiological study in five Western Europe countries estimates the incidence in autochthonous population in 1 per million births but when considering immigrant population this ratio doubles to 2,3 per million births (Kleijer, Laugel et al. 2008). XP is more prevalent in regions with an history of consanguinity. The prevalence of XP disease is estimated in 1 in 80,000 in Morocco and 1 in 10,000 in Tunisia. In both countries, as in all the Mediterranean region, defects in XPC gene are responsible for more than 80% of cases (Zghal, El-Fekih et al. 2005, Soufir, Ged et al. 2010, Doubaj, Laarabi et al. 2012). In Japan, where XP prevalence is about 1 in 20,000 the main affected gene is XPA (Hirai, Kodama et al. 2006).

XP patients present a sensitivity to sunlight and freckling and pigmentation spots in sun exposed areas as soon the age of two years old. Skin is frequently dry, scaling and atrophic and presents early signs of photoaging. Skin pigmentation is heterogeneous intermixing between hypopigmented and hyperpigmented areas (Kraemer, Lee et al. 1987, Lehmann, McGibbon et al. 2011). Ocular tissue exposed to UV radiation is frequently affected in these patients. Conjunctivitis, corneal neovascularization and eye dryness are the most observed abnormalities (Kraemer, Lee et al. 1987, Brooks, Thompson et al. 2013). About one fourth of XP patients also present neurological symptoms including a progressive mental deterioration.
and hearing loss, deterioration of speech function, ataxia, peripheral neuropathy, abnormal gait, and psychomotor alterations affecting walking (Kraemer, Patronas et al. 2007, Bradford, Goldstein et al. 2011).

The most common cause of death in XP patients is skin cancer. They have a 10,000 fold risk of developing NMSC and a 2000 fold risk of developing melanoma under the age of 20 compared to the general population (Bradford, Goldstein et al. 2011). The most comprehensive XP case-study until now dates from 1987 and puts the median age of the first skin cancer at 8 years old, 50 years earlier than the general population (figure 8) (Kraemer, Lee et al. 1987).

A more recent follow-up of 106 XP patients paid special attention to data related to the median age of the first NMSC and the median age of the first melanoma in XP patients compared to the general population. In the general population, the average age of the first melanoma is 67 years old and the one of the first NMSC is 55 years old. In XP patients, the median age of the first diagnosis of melanoma is 22 years old whereas the one of the first NMSC stays under 10 years old. These data show that not only XP patients develop skin cancers at early age compared to the general population, but also that there is a reversal regarding the type of the first cancer diagnosed. In general population melanomas tend to arise first and the opposite occurs in XP patients with a “preference” for the development of NMSC. This reversal suggests that there are different mechanisms of carcinogenesis between the two types of cancer (Bradford, Goldstein et al. 2011).

In a study from 1984, a 10- to 20-fold increase in the frequency of internal malignancies in XP patients was also reported (Kraemer, Lee et al. 1984).
Immune abnormalities were also identified in XP patients. Several studies from the late 80s / early 90s report that NK from XP patients present a defect in their lytic activity suggesting an implication of the NK cell dysfunction in the cancer proneness of XP (Norris, Limb et al. 1988, Norris, Limb et al. 1990, Mariani, Facchini et al. 1992). Furthermore, activation with polyinosinic cytidylic acid, showed that compared with peripheral blood lymphocytes from the general population (PBL), XP PBL produced reduced levels of interferon gamma and alpha (IFN-γ, IFN-α) that are both potent inductors of NK cell activation, (Gaspari, Fleisher et al. 1993). XP patients also present reduced levels of CD3+ CD4+ circulating lymphocytes (Wysenbeek, Weiss et al. 1986, Mariani, Facchini et al. 1992).

2. Etiology

The “classic” form of XP disease is due to constitutive defects in the mechanism involved in the repair of DNA lesions after UV exposure. James Cleaver was the first researcher identifying the link between XP phenotype and a failure in the DNA repair after UV exposure (Cleaver 1968). Using a technique that was later named “unscheduled DNA synthesis” (UDS), he showed that the majority of XP cells were deficient in UDS after UV irradiation. The principle of UDS is to incorporate labeled nucleotides into the newly DNA patches that are synthetized upon DNA repair. Historically, radionucleotides such as ³H-thymidine or ³H-bromodeoxyuridine were used; quantitation of the incorporation was made by autoradiography, or liquid scintillation counting (Lehmann and Stevens 1980). Today, it is possible to measure UDS with a non-radioactive label such as 5-Ethynyl-2´-deoxyuridine (EdU) and it remains a robust criterion to confirm XP diagnosis (see point 3 for more details) (Limsirichaikul, Niimi et al. 2009).

XP disease can result from mutation in any one of eight genes (XPA to XPG plus XPV i.e. DNA polymerase eta). XPA to XPG are encoding proteins whose expression is altered in the DNA repair mechanism called nucleotide excision repair (NER). Pol eta encodes for a DNA polymerase from the eta DNA polymerases family, and essentially manages the translesion DNA synthesis.

2.1. Nucleotide Excision Repair (NER)

In 1964, Rasmussen and Painter published in Nature the first evidence of a DNA repair mechanism after UV damage in mammalian cells (Rasmussen and Painter 1964). Three decades later, the major steps of the NER mechanism were reported through in vitro
approaches of DNA repair complex reconstruction (Aboussekhra, Biggerstaff et al. 1995, Mu, Park et al. 1995).

There are two converging pathways of the NER distinguished in the initial steps of the recognition of the DNA lesion: Global Genome Repair (GGR) and Transcription coupled-nucleotide excision repair (TC-NER), also called sometimes “preferential repair” (figure 9).

(Stary and Sarasin 2002)

*Figure 9. Nucleotide Excision Repair mechanism.*
The GGR pathway is activated when the lesion stands in a non-transcribed region of the genome while TC-NER is involved in repair lesions in DNA regions that are being transcribed into RNA (including non-transcribed DNA strand). During GGR, DNA distortion led by the lesion is first recognized by a XPC-HR23B-centrine2 protein complex helped by a DDB2/XPE-DDB1 complex when the degree of helix distortion is lower (i.e. in presence of CPD). TC-NER is initiated when RNA polymerase II is thought to stall when encountering a lesion in the transcribed strand. However, the number of blocking DNA transcription following a minimal erythema dose (MED) equivalent (about 2 CPD /1kb) irradiation is far insufficient to explain the global block of transcription, including in active genes with no detectable DNA lesion (Perdiz, Grof et al. 2000). Signaling pathways connecting DNA distortion and/or cellular components to command transcriptional block deserve further investigations. The classical interpretation is based on the fact that the CSA and CSB proteins are recruited to the lesion site due to stalling of RNA polymerase II. After the recognition of the lesion through both GGR and TC-NER sensors, the multiprotein transcription factor TFIIH is recruited to the recognition complex and the NER pathway shared by GGR and TC-NER is turned on (Spivak 2015). TFIIH is a complex composed of ten subunits - XPB, XPD, p62, p52, p44, p34, p8/TTD-A, CDK7, cyclin H, and MAT1. The ATP-dependent helicases XPB and XPD are responsible for the unwinding of the DNA double strand around the lesion. To this end, the P44 protein of TFIIH phosphorylates the carboxyterminal end of RNA pol II (Coin, Auriol et al. 2004). The repair complex is then stabilized by the XPA protein allowing subsequent 3’ a 5’ incisions (Riedl, Hanaoka et al. 2003, Camenisch, Dip et al. 2006). The DNA damaged strand is incised and excised thanks to the intervention of the endonucleases XPF/ERCC1 5’ and XPG 3’. After the dual incisions, DNA polymerases ε/δ/k synthetize a new strand using as matrix the remaining single strand DNA protected by SSB proteins; the newly synthetized strand is covalently linked by DNA ligase I, IIIα and XRCC1 (Moser, Kool et al. 2007, Marteijn, Lans et al. 2014).

NER is only one of the different pathways that can be triggered after induction of DNA damage. The other main DNA repair pathways: BER (Base Excision Repair), HR (Homologous Recombination), MMR (Mismatch Repair) and NHEJ (Non-Homologous End-Joining) are summarized in figure 10.
DNA damage can occur following the “attack” of different endogenous or exogenous factors. Cell machinery is equipped with different repair systems and is able to selectively trigger them depending on the type the DNA damage. NER and BER are the two excision repair pathways. As NER is the only one responsible of the removal of bulky DNA adducts caused by UV radiation (described in the main text), BER is mostly described for its role in recognition and excision of oxidative damaged bases like 8-oxoG. The key enzymes involved in the BER are DNA glycosylases responsible for the removal of damaged bases by cleavage of the N-glycosyl bonds of the nucleotide residues. The DNA glycosylase action is followed by an incision step, DNA synthesis, an excision step, and DNA ligation. The other repair pathways are the HR pathway that uses the homologous DNA strand as a template for re-synthesis when in presence of double strand breaks (DSB); MMR that is implicated in the removal of post-replicative DNA errors like mismatched nucleotides, insertions, deletions and NHEJ that is another pathway able to repair DSB by ligating the two DNA ends (Hoeijmakers 2001).

BER: Base Excision Repair; HR: Homologous Recombination; MMR: Mismatch Repair; NHEJ: Non-Homologous End-Joining

2.1.a. Genotype-phenotype relationships

Products of the seven complementation genes have different roles in the NER and their mutations are associated with variable symptom severities, frequencies and residual levels of DNA repair (as measured by UDS) (table 3). For instance, XP-C and XP-E are less prone to develop acute sunburn and XP-A (De Sanctis-Cacchione syndrome) is related to severe neurological symptoms (Moriwaki, Kanda et al. 2017).
The variant (XP-V) syndrome is not related to mutations in the NER pathway. NER defect can thus not be considered per se as a criteria of diagnostic. Instead XP-V cells present problems during the DNA replication of UV-damaged strands, a process called translesion synthesis (TS). In absence of DNA damage, DNA polymerases (I) is responsible for DNA replication but when in presence of a damage in the DNA template, there is a switch taking place between DNA pol I and other specialized DNA polymerases that are able to process throughout the DNA damage. DNA polymerase eta (pol eta) participates in the replication of DNA strands that present UV related lesions. The gene that encodes pol eta, POLH is mutated in XP-V patients. XP-V patients present a typical XP phenotype but they generally display milder symptoms and some delay in the onset age of skin cancer development (Cordonnier, Lehmann et al. 1999, Gratchev, Strein et al. 2003).

XP is not the only genetic disease associated with defects in NER pathway. There are two other identified genodermatoses - trichothiodystrophy (TTD), and Cockayne’ syndrome (CS) and several related or overlapping disorders that derive from mutations in NER pathway associated genes (figure 11). One disease may be the product of mutations in different genes, like what happens with XP, but the inverse can also occur, meaning that different mutations in the same gene may lead to different diseases (DiGiovanna and Kraemer 2012).

(Lehmann, Seebode et al. 2018)

Table 3. Xeroderma pigmentosum complementation groups: insight in frequency, proneness to skin cancer development, neurological involvement and cellular repair capabilities.
Trichothiodystrophy

Trichothiodystrophy (TTD) was first identified by Pollitt et al. in 1968 (Pollitt, Jenner et al. 1968). Clinically, TTD patients present with brittle hair and normally have an outgoing personality. TTD features range from only hair involvement to massive developmental and neurological abnormalities. Some forms of TTD are related to photosensitivity but patients neither develop skin cancers nor present pigmentary abnormalities like XP patients (Brauns, Schubert et al. 2016). The disease is related to mutations in XPD, XPB or TTDA genes, that all belong or, contribute, to the multifunctional protein complex TFIIH. TFIIH has roles in both DNA repair and basal transcription (Hoeijmakers, Egly et al. 1996, Yew, Giordano et al. 2016). Mutations affecting only the repair function of the complex lead to the XP disease whereas mutations affecting the transcription function lead to TTD (Taylor, Broughton et al. 1997). Patients with XP/TTD overlap disorders were first described by Bernard C. Broughton et al. in 2001. They exhibit features of both diseases but normally present milder symptoms with less prominent hair problems and lower cancer development frequency as compared to XP (Broughton, Berneburg et al. 2001).
Cockayne syndrome

Cockayne syndrome (CS) was first described by Cockayne in 1936 (Cockayne 1936). CS patients present microcephaly, stunted growth, progressive neurologic dysfunction and generally premature aging. Alike TTD patients, they also can present with photosensitivity but neither pigmentation abnormalities nor higher incidence of skin cancer as compared to the general population (Nance and Berry 1992, Yew, Giordano et al. 2016). The disease results typically from mutations in \textit{ERCC8} and \textit{ERCC6} genes, encoding for CSA and CSB proteins, respectively. These proteins are part of the lesion recognition complex formed during the TC-NE branch of the NER (Stefanini, Fawcett et al. 1996). Less frequently, CS can result from mutation in \textit{XPF} gene (Kashiyama, Nakazawa et al. 2013). Overlap syndromes of XP/CS and TTD/CS have been described. XP/CS patients show with the classic symptoms of CS plus a severe photosensitivity and an increased risk of skin cancer development due to additional mutations on \textit{XPB}, \textit{XPD}, \textit{XPG} or \textit{XPF} genes (Natale and Raquer 2017). Ultraviolet sensitive syndrome (UVSS) and Cerebro-oculo-facio-skeletal (COFS) syndrome are CS-associated disorders (Karikkineth, Scheibye-Knudsen et al. 2017). There is a debate whether they can be considered as variant forms of CS presenting different severities or as different diseases. UVSS patients possess mutations in \textit{CSA}, \textit{CSB}, or \textit{UVSSA} genes. Clinical features are mild comparing to classical CS. UVSS patients present acute sunburn, dryness, freckling, and pigmentation anomalies without neurological abnormalities (Itoh, Fujiwara et al. 1995, Karikkineth, Scheibye-Knudsen et al. 2017). COFS can be caused by mutations in \textit{CSB}, \textit{ERCC1}, \textit{XPD}, or \textit{XPG} genes. Clinical features of COFS often overlap with severe forms of CS - microcephaly with cerebral atrophy as well as severe mental and growth retardation. However, microphthalmia and arthrogryposis that are commonly found in COFS are rare in CS patients (Laugel, Dalloz et al. 2008, Yew, Giordano et al. 2016).

3. Diagnosis and treatment

The diagnosis of XP is first based on both clinical symptoms and DNA repair tests. The most frequent DNA repair test is the measurement of the unscheduled DNA synthesis (UDS) after UV irradiation of fibroblasts, already described in point 1. The impairment of UDS synthesis can confirm the XP diagnosis except in the presence of XP-V variant cell. In the latter case, cells should be cultured in presence of caffeine when exposed to UV radiation, and cell viability is checked 2 to 4 days after irradiation. The sensitivity (through survival measurement) of cells to UV in presence of caffeine is a particularity of XP-V cells and together with the normal UDS can confirm the XP-V diagnosis (Broughton, Cordonnier et al. 2002). In fact, caffeine, through
the inhibition of checkpoint kinases ATM and ATR, is able to enhance the inhibition of cell proliferation induced by UV. However this effect is reversed by expression of DNA polymerase η and this is why this is a specific test to confirm an XP-V diagnosis (Kauffman, Cherikh et al. 2005).

After the diagnosis, complementary experiments can determine which gene exactly is altered, by either genetic tests (complementation analysis, DNA sequencing) or, when applicable, by quantification of the suspected defected gene product either by reverse transcriptase-PCR or protein quantification.

Complementation analysis by cell fusion from independent patients were one of the earliest methods to pose the diagnosis of XP. Such approach revealed the existence of the different XP complementation groups. Briefly, cells from different XP patients were fused to form heterokaryons and the levels of UDS were measured after UV irradiation (notably UVC). Recovery of normal UDS levels implied that the two cells belong to two different complementation groups; conversely, absence of UDS improvement meant that cells from the two patients belonged to the same complementation group (Kraemer, Coon et al. 1975, Kraemer, De Weerd-Kastelein et al. 1975, Arase, Kozuka et al. 1979, Keijzer, Jaspers et al. 1979). After the identification of the different groups, and for diagnosis purposes, the cells from the suspected XP patient were fused with cells for which the complementation groups were already known. Because complementation analysis by cell fusion was time-consuming and difficult to perform some alternatives methods were set up. One of the first alternatives to the cell fusion procedure relied in the transfer of vectors (expression plasmid or replication deficient retroviruses expressing the various genes (cDNAs) known to be involved in NER-deficient cells, such as XPD or XPC (Carreau, Eveno et al. 1995).

Although it is not currently used as a diagnostic method, the measurement of UV suspicious cells survival using clonal analysis remains a good practice to determine if we are in presence of the XP-C complementation group. Keratinocytes from XP-C patients shows changes in the representation of the different colony types compared to WT and XP cells from other complementation groups with a preference for abortive ones (Otto, Riou et al. 1999).

One hundred and forty five years have passed since Kaposi first described the prognosis of the XP syndrome as “absolutely unfavorable” (Hebra and Kaposi 1874). Even if many palliative therapeutics have emerged since then, no curative medicine can yet be proposed to these XP patients. Today, treatments are mostly based on cancer prevention or progression. Among those, strict sun avoidance is a crucial preventive measure so long as diagnostic is early enough with age i.e. before 2. Under these circumstances prevention of sun exposure has proven essential to limit progressive alterations of the quality of life of XP patients, at least at
the cutaneous level. Regarding cancer progression, the most frequent treatment to avoid dissemination is the surgical resection of skin lesions.

The potential of gene therapy brings new hope to the possibility of a curative treatment but despite the efforts that have been made, as skin is the largest organ of the human body, its full transplantation following genetic correction remains a challenge. De Luca’s team was until now the only one, to successfully replace almost the entire epidermis - of a patient suffering from junctional epidermolysis bullosa disease - with autologous transgenic epidermal cultures (Hirsch, Rothoeft et al. 2017). However, taking into account the complexity of skin as a multiple embryologic origin, it can be suspected, including in the studies of the laboratory, that full recovery of NER after gene transfer cannot alone normalize all the “side” impacts of XP gene mutations (Arnaudeau-Begard, Brellier et al. 2003, Frechet, Warrick et al. 2008, Warrick, Garcia et al. 2012).

For further details on XP diagnosis and treatment, I invite you to read our review “Genetic therapy of Xeroderma Pigmentosum: analysis of strategies and translation” enclosed in page 192 of this manuscript (Goncalves-Maia and Magnaldo 2016).

4. The XP-C genetic group of complementation

The XP-C genetic group of complementation is the most frequent in USA, Europe and North Africa. XP-C patients present severe skin abnormalities and are particularly hypersensitive to ocular damage (Fassihi, Sethi et al. 2016). Together with group XP-E and XP-V groups, the majority of XP-C patients don’t have abnormal sunburn reactions on minimal sun exposure probably due to the fact that they retain fully efficient TC-NER mechanism (van Steeg and Kraemer 1999, Kleijer, Laugel et al. 2008, Sethi, Lehmann et al. 2013, Jerbi, Ben Rekaya et al. 2016). In the rare cases where XP-C patients develop neurological symptoms, the cause seems to be unrelated with the XPC gene defect but perhaps on other yet undisclosed impacts of ascending consanguinity (Khan, Oh et al. 2009). There is one report of XP-C patients showing an impairment of basal cutaneous cell-mediated immunity response after a sensitization test using the contact allergen diphenylcyclopropenone (DPCP) (Anttinen, Koulu et al. 2008). Furthermore, in early 90-th, some XP-C patients showed defects in NK cell lytic activity in blood samples (Norris, Limb et al. 1990).

The XP-C group is highly predisposed to develop aggressive skin cancers. XP-C patients develop at least 5 times more SCC than BCC whereas the incidence ratio SCC:BCC in the general population under 45 years old is rather 1:4 (Giglia, Dumaz et al. 1998, Chahal, Rieger et al. 2017). Internal afflictions such as pyramidal syndrome, thyroid nodules, as well as internal
cancers (leukemia, non HPV-cervix cancer, kidney cancers and thyroid adenocarcinoma) were also reported in XP-C patients but the physiopathological basis of these “associated diseases” remains poorly understood (Khatri, Bemghazil et al. 1999, Hadj-Rabia, Oriot et al. 2013).

The human XPC gene was (partly) identified in 1992 by Legerski and Peterson (Legerski and Peterson 1992). It is located on chromosome 3p25.1 and has a length of 33 kb with 16 exons and 15 introns (figure 12) (Khan, Muniz-Medina et al. 2002). Of note, Legerski and Peterson published the cDNA sequence, truncated from a part of its NH2-terminal sequence. Thus, the initial XPC protein was supposed to comprise about 100 kDa, instead of the full length 125 kDa actual sequence. The results from Legerski and Peterson were contradicted by Masutani et al. in 1994 (Masutani, Sugasawa et al. 1994). Actually, the misinterpretation of Legerski and Peterson were due to (3) internal codons of initiation of translation contained in the 5’ region of the XPC cDNA (Masutani, Sugasawa et al. 1994, Khan, Oh et al. 2009).

**XPC gene**

chromosome 3p25.1

Figure 12 XPC gene

*Structure of human XPC gene. The 16 exons are numbered and indicated by blue bands.*

The XPC gene encodes a 940-aa DNA-binding protein (figure 13) known for its role in the “lesion” recognition step of the GGR mechanism (Masutani, Sugasawa et al. 1994). The C-terminal region of XPC (residues 492-940) contains the major protein-protein interaction sites - including binding regions to DDB2/XPE, RAD23B, centrin2, p62 and XPB - and the DNA binding domain ; the N-terminal region (residues 1-491) includes the XPA, p62 and 8-Oxoguanine glycosylase 1 (OGG1) binding regions (Yokoi, Masutani et al. 2000, Bunick, Miller et al. 2006, Puomalainen, Ruthemann et al. 2016).
XPC was first found to be complexed with the homologous of RAD23B (RAD23) in yeast in 1994 and a few years later, centrin 2 was identified as the third element of the heterotrimeric complex (Masutani, Sugasawa et al. 1994, Araki, Masutani et al. 2001, Popescu, Miron et al. 2003). The XPC subunit of the complex is the one responsible for the DNA-binding and the recognition of the DNA distortion. Indeed, XPC recognizes bulky distortions in DNA, rather than the lesion itself. It shows higher affinity to 6-4PP probably because 6-4PP are associated with more important helix distortions compared to CPD (Kim, Patel et al. 1995, McAteer, Jing et al. 1998, Sugasawa, Okamoto et al. 2001). RAD23B is a 26S proteasome-interacting factor that protects XPC from proteasomal degradation (Ng, Vermeulen et al. 2003). Centrin 2 is a calcium-binding protein, initially found in centrosomes. It stabilizes the XPC-RAD23B complex and strengthen the DNA-binding activity of XPC (Araki, Masutani et al. 2001, Nishi, Okuda et al. 2005). In the presence of CPD lesions, the XPC-RAD23B-centrin2 complex is assisted by DDB2/XPE DNA-binding protein, whose role is to facilitate the interaction of XPC with CPD sites (Kulaksiz, Reardon et al. 2005, Fei, Kaczmarek et al. 2011). DDB2/XPE together with DDB1 forms the heterodimeric UV-DDB (UV-DNA damage binding protein) complex. After the recognition of the distorted DNA, RAD23B rapidly dissociates from the complex. The remaining heterodimeric XPC-centrin2 complex mediates the recruitment of TFIIH via interaction with p62 and XPB (Evans, Moggs et al. 1997, Bernardes de Jesus, Bjoras et al. 2008, Bergink, Toussaint et al. 2012).

The UV-DDB complex is associated with Cul4A/B-RING ubiquitin ligases essential to the ubiquitination of both XPC and DDB2/XPE upon UV radiation (Sugasawa, Okuda et al. 2005). The p67 segregase, an ATP-driven molecular chaperone, then recognizes the ubiquitinated proteins and is responsible for its proteasomal degradation or recycling. While DDB2/XPE is
rapidly degraded by proteosomal activity, the ubiquitination of XPC is reversible. Lubin et al. proposed that the deubiquitinase OTUD4 was involved in the recycling of XPC after ubiquitination (Lubin, Zhang et al. 2013). The removal of both XPC and DDB2/XPE from DNA upon lesion recognition is crucial since a retention of these proteins in the DNA lesion site impairs further steps of NER (Sugasawa, Okuda et al. 2005, Puumalainen, Lessel et al. 2014).

4.2. Biological roles of XPC beyond NER

4.2.a. Repair pathways

The role of XPC in DNA repair is not restricted to NER. XPC is also recruited during the BER pathway (presented in figure 10). It has a DNA binding affinity to 8-oxoG and methylformamidopyrimidine lesions that are typical substrates of BER. This affinity is consistent with the publications describing that XPC recognizes helix destabilizations rather than specific lesions (Sugasawa, Okamoto et al. 2001, Menoni, Hoeijmakers et al. 2012, Shell, Hawkins et al. 2013) (Sugasawa, Okamoto et al. 2001, D’Errico, Parlanti et al. 2006, Menoni, Hoeijmakers et al. 2012, Shell, Hawkins et al. 2013). XPC is also implicated in the stimulation of at least four DNA glycosylases -methylpurine-DNA glycosylase (MPG), thymine-DNA glycosylase (TDG), 8-oxo-guanine-DNA glycosylase 1 (OGG1) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) - responsible for modified base removal in the BER mechanism (Shimizu, Iwai et al. 2003, D’Errico, Parlanti et al. 2006, Shimizu, Uchimura et al. 2010). Moreover, XP-C keratinocytes and fibroblasts show reduced rates of BER and an accumulation of oxidizing bases in their DNA. D’Errico et al. speculate that the accumulation of oxidative DNA damage in XP-C cells could be related to the reports of internal cancers in these patients. They further support their theory with the results of Giglia et al. showing that p53 mutations found in XP-C primary internal cancers are compatible with oxidative damage mutagenesis (Giglia, Dumaz et al. 1998, D’Errico, Parlanti et al. 2006).

XPC has also been implicated in nonhomologous end joining (NHEJ) pathway (presented in chapter 1). XPC −/− HeLa cells present reduced NHEJ after sensitization with double single breaks (DSB) inducers. Furthermore the XP14BR fibroblast cell line, derived from a XP-C patient, show defects in the repair of DSB after ionizing radiation (Arlett, Plowman et al. 2006, Despras, Pfeiffer et al. 2007). The evidences of the implication of XPC in other DNA repair mechanisms is compatible with the idea that XPC protein may act as a “sensor” of DNA damage across different DNA transaction pathways.
There is evidence of the implication of XPC in gene regulation independently of its role in DNA repair. The XPC-RAD23B-centrin2 complex was described as a co-factor that controls transcriptional activation of OCT4/SOX2. OCT4 and SOX2 are pluripotency factors that together with NANOG are part of the mechanism that controls the embryonic stem cell (ESC) state. The knockdown of the XPC-RAD23B-centrin2 complex in murine ESC lead to a down-regulation of pluripotency genes and up-regulation of differentiation markers (Cattoglio, Zhang et al. 2015). In agreement with these results, Fong et al. showed that the depletion of XPC compromise the induction of pluripotency in mouse embryonic fibroblasts (MEFs) (Fong, Inouye et al. 2011).

The presence of XPC was also detected in the promoter of RARβ2 (retinoic acid receptor β2) in vitro. XPC -/- HeLa cells have an attenuated response to retinoic acid induction corroborating the possible implication of XPC in the RARβ2 transcription. Le May et al. show that XPC not only binds to RARβ2 promoter but also assembles the NER pre incision complex in a transcription-dependent way without the presence of DNA damage. The pre-incision complex proved to be essential for chromatin remodeling via histone modifications and DNA demethylation at the activated gene sites (Le May, Mota-Fernandes et al. 2010, Le May, Fradin et al. 2012).

The implication of XPC in chromatin remodeling has been described during the last decade. Ray et al. showed that XPC associates and co-localizes with SNF5 - a component of the ATP-dependent chromatin remodeling complex SWI/SNF - at sites of UV DNA damage. In SNF5/- fibroblasts, XPC is able to bind DNA lesion but is unable to recruit ATM (ataxia telangiectasia mutated) to the damage site (Ray, Mir et al. 2009). ATM together with ATR (ATM and Rad3 related) are two protein kinases that initiate a phosphorylation cascade leading to cell cycle checkpoint arrest during DNA repair process of SS DNA breaks. Both ATM and ATR were found associated with XPC in UV damage sites. It has been proposed that the association between SWI/SNF and XPC facilitates ATM access to the lesion and allows the downstream pathway to occur (Ray, Milum et al. 2013). Finally, a possible interaction between XPC and a subunit of the chromatin assembly factor 1 (CAF-1) was identified but still needs confirmation. CAF-1 is implicated in the restoration of intact chromatin following DNA replication and repair (Green and Almouzni 2003, Zhu, Wani et al. 2009).

4.3. XP-C patients' mutation profile

Several different XPC mutations have been reported in XP-C patients over the last three decades. The majority are deletions, substitutions, splicing alterations, and insertions mainly
resulting in premature stop codons. In 1993, Li et al. reported six different mutations - c.1001C>A, c.1103_1104delAA, c.1643_1644delTG, c.2092_2093insGTG, c.1643_1644delTG, c.2092_2093insGTG. In 1998, Khan et al. added one more to the list and the list kept growing till today with nearly 50 described mutations (Li, Bales et al. 1993, Khan, Levy et al. 1998, Sheth, Mistri et al. 2015). Until a decade ago, studies reporting mutational analysis in XPC were conducted in small cohort of XP-C patients, which made it difficult to access the possibility of revealing the existence of.hotspots or founder mutations. Exceptions were made with two studies. Gozukara et al. proposed in 2001 the existence of a founder mutation after reporting that XP-C patients from Turkey and Italy were homozygous for the same XPC stop mutation c.1735C>T. They resorted to microsatellite markers, and discarded the possibility of hotspot mutations, suggesting that the founder mutation came from a common ancestor about 300-500 years ago (Gozukara, Khan et al. 2001). In 2007, Cleaver et al. after mutational analysis of patients of an isolated Guatemala village proposed c.940delC as a founder mutation in that area (Cleaver, Feeney et al. 2007).

A major breakthrough for the molecular diagnosis of XP-C in Maghreb came from a large cohort study in 2010 with the discovery that 74% of maghrebian patients shared the same XPC mutation - c.1643_1644delTG(Soufir, Ged et al. 2010). Haplotype microsatellite analysis confirmed that this is a founder mutation and suggested that the original mutation occurred 1,260 years ago, right in the middle of the Muslim conquests of the Maghreb and Iberia. In light of these data, it is not surprising that the same founder mutation was found in patients from Italy, Middle East and Sudan (Chavanne, Broughton et al. 2000, El-Harith, Pahl et al. 2012). The geographical situation of Maghreb together with the history of frequent consanguineous marriages are important factors that contribute to the higher prevalence of XP-C in this region. Since early diagnosis is essential to minimize the symptoms of XP disease, the discovery of this founder mutation opens the possibility of prenatal newborn molecular diagnosis in the at-risk populations or families.

Other founder mutations have been reported or suggested in different geographical areas. A study in a tertiary center in Nepal reported that all the fifteen XP-C patients in the center shared the same c.1243C>T mutation (Esip, Parajuli et al. 2018). Recently, Ijaz et al. reported a prevalent founder mutation in XP-C patients from Pakistan and correlated the findings with a study reporting the same mutation in Comorian patients. The founder effect have an estimated age of about 800 years (Cartault, Nava et al. 2011, Ijaz, Basit et al. 2019) a period corresponding to the migration of Muslims in this region. Therefore, authors suggest that the ancestor was actually from the Middle East region.
Although there is a line of work suggesting that it could be a correlation between XP-C patients’ phenotype and XPC mutation profile, until now there is not a major cohort study that can validate the results obtained in small groups of XP-C patients. For instance, the proposition that the c.2033+2A>C splice mutation could be associated with hyperglycinemia comes from a case report of only one XP-C patient (Khan, Levy et al. 1998). Besides, XP-C patients are often described as a homogeneous group regarding their phenotypic characteristics (Fassihi, Sethi et al. 2016). Moreover, Schafer et al. proposed that extrinsic factors like the individual sun-exposure behavior are the greatest contributors to XP-C variable symptoms severity (Schafer, Hofmann et al. 2013).

4.4 XP-C group as a model to study both non melanoma skin cancer and aging

XP-C patients are prone to develop aggressive cancers. One of the first evidences of this aggressive phenotype was published in 1998, after the study of the mutational spectra of the tumor supressor gene TP53 in XP-C patients. The molecular analysis of mutations TP53 from 58 XP-C skin biopsies showed two important things: first, SCC are more frequent than BCC (ratio 5:1); second, the majority of XP-C skin cancers present TP53 mutations. These mutations are located at dipyrimidine sites and predominantly correspond to transitions C to T or tandem CC to TT mutation that both, constitute a signature of UV-induced lesions (Giglia, Dumaz et al. 1998). The “preference” for the CC to TT tandem mutations seems to be specific from XP-C group. In the general population, TP53 mutations are found in at least 50% of skin cancers, and about 60% correspond to CG to TA transitions (Giglia-Mari and Sarasin 2003). The P53 protein is implicated in cell-cycle regulation, apoptosis and DNA repair (Lane 1992, Balint and Vousden 2001). Interestingly, p53 seems to control the UV inducible expression of XPC through the binding to XPC promoter (Adimoolam and Ford 2002). Why XP-C patients develop more SCCs than BCCs is still not yet understood. There is evidence showing that in the general population, XPC is well expressed in BCCs and conversely is frequently inactivated in SCCs (de Feraudy, Ridd et al. 2010, Yang, Hsiao et al. 2015). To my knowledge, there are no studies that directly correlates TP53 mutations in BCC and SCC with expression of XPC in the general population. However, the difference in XPC expression in BCC vs SCC together with findings showing different hotspot mutations in TP53 from BCCs and SCCs could let us hypothesize that TP53 mutations associated with BCC, somehow, are not detrimental to the role of p53 in XPC induction (Giglia-Mari and Sarasin 2003).

Although SCCs are naturally more aggressive than BCCs, tissue invasion and metastasis also depends on cancer cell microenvironment composed mainly by fibroblasts, dendritic cells, B lymphocytes, T lymphocytes and NK cells. Briefly, fibroblasts that are associated with cancer cells become “activated” (CAFs) and the crosstalk between CAFs and the other cells within
the tumoral microenvironment are preponderant to the fate of cancer progression (Kalluri 2016). Fibroblasts from XP-C patients from non-photo exposed areas promote keratinocyte and SCC invasion in organotypic cultures and express other similarities with CAFs. Most notably, studies from the laboratory have indicated that the overexpression of the hepatocyte growth factor (HGF) and the Matrix Metalloproteinase 1 (MMP1) are implicated in ECM remodeling (Alqaraghuli, in preparation) (Bernerd, Asselineau et al. 2005, Frechet, Warrick et al. 2008, Cui, Wang et al. 2018). Unlike CAFs, XP-C fibroblasts show a pro-invasive phenotype at the steady state, i.e. even in the absence of activation by cancer cells. The identification of the molecular deregulations underlying the XP-C fibroblasts phenotype could help understand the very first steps of cancer cell invasion and the typology of cancer that XP-C patients preferentially develop, i.e. SCCs.

XP-C fibroblasts also show similarities with senescent cells. They have photo-aged like features such as elongated dendritic shape and high levels of reactive oxygen species (ROS) accumulation (Frechet, Warrick et al. 2008). XPC −/− mice show increase in skin cancer after massive and chronic UV exposure, reproducing in part the human XP-C phenotype (Sands, Abuin et al. 1995). Surprisingly, these mice also present high levels of internal cancers, particularly in lung - a tissue that is constantly exposed to oxygen. Mutational spectra of the rare internal cancers found in XP-C patients and Xpc defective mice shows an accumulation of oxidative DNA damage (i.e compatible with 8oxoG mutagenesis) (Giiglia, Dumaz et al. 1998, Wijnhoven, Kool et al. 2000, Melis, Kuiper et al. 2013, Nemzow, Lubin et al. 2015). The implication of XPC in BER could be directly linked to these phenomena, and the accumulation of oxidative lesions explain both the incidence of internal cancers and the aged phenotype of XP-C cells. Furthermore, young XPC−/− mice present features of premature skin aging including overexpression of p16INK4a, senescence-associated beta-galactosidase (SA-β-gal) activity and NADPH oxidase 1 (NOX). The activation of NOX triggers a disturbed redox homeostasis and its inhibition is sufficient to rescue premature skin aging in XPC −/− mice (Hosseini, Mahfouf et al. 2015). These results strongly strengthen the idea that XP-C is not only a cancer-prone but also and age-related disease.

The importance of the tumor microenvironment (TME) and aging in cancer progression will be further discussed in the next chapter.
Chapter III: Tumor microenvironment

“When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil” – Paget, 1889

Over the last decades, the study of cancer cell mutagenesis allowed the identification of oncogenes and tumor-suppressor genes that actively participate in the tumorigenesis process. Although cancer cells suffer modifications of their own that favor their growth and spread, their environment has also an influence in cancer progression and metastasis (Fidler 2003). In 1889, Paget, inspired by some previous notes from Fuchs, published the “seed and soil” hypothesis to explain different frequencies of metastasis in different organs. Making an analogy to what happened with plants, he proposed that cancer cells, alike seeds, are numerous and capable of development but they are only able to disseminate if they fall in fertile microenvironment (soil) (Paget 1989). This delicate theory set the basis for the study of the tumor microenvironment as an active part in the fate of cancer cells.

Hanahan and Weinberg were able to determine processes shared by many types of cancers. They established these similarities as the “hallmarks of cancer” (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). Of the ten hallmarks of cancer, sustainment of proliferative signals, evasion of growth suppressors, resistance to cell death, neo-angiogenesis, activation of invasion and metastasis, escape from immune destruction, and tumor-promoting inflammation, all involve the various contributions from TME cells (figure 14) (Hanahan and Coussens 2012).
1. Constituents of tumor microenvironment

Besides cancer cells, TME is composed of an ECM, and non-malignant cells embedded in blood vessels. The non-malignant cells are mainly fibroblasts, vascular and immune cells. In some cases, adipocytes can also be present (Junttila and de Sauvage 2013).

1.1. Fibroblasts associated to cancer

Fibroblasts account for the majority of non-tumor cells within the TME (Kalluri 2016). As described in chapter I, normal fibroblasts have an important role in skin homeostasis, primarily via ECM upon development, maintenance, and remodeling. Fibroblasts are able to interact with various different cell types. Under conditions that require tissue remodeling, normal fibroblasts become “activated” and acquire morphological and functional specificities compared to non-activated, usually quiescent fibroblasts. Giulio Gabbiani was the first to identify this particular type of activated fibroblasts upon tissue wound healing. He showed that fibroblasts in wounds present a developed fibrillar system and surface similarities with smooth muscle cells (Gabbiani, Ryan et al. 1971). These so-called myofibroblasts present de novo expression of α-smooth muscle actin (α-SMA), contractile stress fibers, and splice variants of fibronectin (ED-A). Myofibroblasts are able to highly contract ECM through acto-myosin activation to bring closer the marges of the wound; myofibroblasts secrete matrix proteins. 

Adapted from (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011) 

*Figure 14. The hallmarks of cancer.*
facilitating migration (and proliferation) of epithelial cells to complete the healing process (Castor, Wilson et al. 1979, Serini, Bochaton-Piallat et al. 1998, Hinz, Celetta et al. 2001, Tomasek, Gabbiani et al. 2002). As tumors have been considered as wounds that never heal, it became conceivable that fibroblasts associated to tumors were showing similarities to activated fibroblasts (Dvorak 1986). They both share expression of α-SMA and ED-A fibronectin but differ in the duration and the reversibility of the “activation”. While in wounds, myofibroblasts are removed from the site after the healing process. In TME, fibroblasts stay in place and gain enhanced proliferative properties, becoming the irreversibly CAFs (Desmouliere, Redard et al. 1995, Bizik, Kankuri et al. 2004, Bechtel, McGooohan et al. 2010).

CAFs are defined as a fibroblastic heterogeneous cell population. They can be identified by a panel of activation markers such as expression of α-SMA, FSP-1, fibroblast-activation protein α (FAP-α) and platelet-derived growth factor (PDGF) receptors-β but none of these markers seem exclusive of CAFs (Garin-Chesa, Old et al. 1990, Desmouliere, Guyot et al. 2004, Zhang, Chen et al. 2013, Ostman 2017). Although fibroblasts and usually cells from mesodermal, are the main direct source of CAFs, stellate cells, bone marrow–derived cells, adipocytes, endothelial cells (from mesodermal origin) and epithelial cells (from ectodermal origin) have also been reported as possible source of CAFs (Iwano, Plieth et al. 2002, Ishii, Sangai et al. 2003, Zeisberg, Potenta et al. 2007, Kordes, Sawitza et al. 2009, Kidd, Spaeth et al. 2012).

In the early stages of carcinogenesis, fibroblasts are able to inhibit cancer progression. It is presumed that the ability of fibroblasts to maintain the homeostasis of tissue architecture is essential to avoid cancer dissemination. Of note, the results presented in this manuscript also suggest a possible role of fibroblasts in the early immune response against cancer cell invasion. Once fibroblasts start changing their phenotype, the architecture and composition of the ECM change, and they become active actors in cancer cell growth and fate (Trimboli, Cantemir-Stone et al. 2009, Bissell and Hines 2011).

1.2. Vascular cells

Vascular cells are key players in the formation of new blood vessels in a process called angiogenesis. Angiogenesis is beneficial to physiological processes like wound healing but when in a TME context, it contributes to tumor growth and metastasis. Briefly, the formation of new blood vessels within tumors allows the oxygenation of the tissue, supplies important metabolites and removes unimportant or detrimental ones (Carmeliet and Jain 2011, Marcola and Rodrigues 2015). This process is reminiscent of the vascularization process that happens during embryonic development. Endothelial progenitor cells (EPC) are recruited or already reside in the TME and, after maturation, start the formation of de novo vascular structures. Asahara et al., were the first to identify these progenitor cells and determined that they
originated from the bone marrow (Asahara, Masuda et al. 1999). Pericytes are the other vascular cell type important in angiogenesis, particularly during its initial stages. Activated pericytes lose their attachment to microvessels and starts developing cytoplasmic extensions into the tumor allowing endothelial cells to migrate into the perivascular space and create the migration tube (Bergers and Benjamin 2003, Raza, Franklin et al. 2010). Both cancer cells and CAFs are able to recruit and modulate endothelial precursor cells (EPC) in the TME (Marcola and Rodrigues 2015).

1.3. Immune cells

Different types of immune cells from both lymphoid and myeloid lineages can be found in TME. They can interact with cancer cells both by direct contact and through chemokine and cytokine secretion and subsequent signaling. More importantly, they play dual roles in cancer progression, acting against or in favor of tumor growth and progression (Zamarron and Chen 2011, Gajewski, Schreiber et al. 2013). Below are listed most of the lead actors in cancer immune microenvironment.

1.3.a. T cells

The different types of T cells found within the tumors can be divided in those associated with anti-tumor properties and those associated with tumor progression. The presence of CD8+ T cells and CD4+ T helper 1 (Th1) cells in TME correlates with good prognosis in many types of solid tumors (Pages, Berger et al. 2005, Fridman, Pages et al. 2012). T cells are thought to become activated through a process requiring antigen presentation by APCs including macrophages, B cells and conventional dendritic cells (cDC). APCs will uptake antigens in peripheral locations and then process and present antigens to naïve T cells in the lymphoid organs. In tumor context, cDC1, a specific type of cDC is of major importance as the most efficient APC that cross-present major histocompatibility complex class I (MHC-I) restricted antigens to CD8+ T cells. After priming, T cells will migrate and infiltrate TME (Boon, Cerottini et al. 1994, Hildner, Edelson et al. 2008, Bottcher, Bonavita et al. 2018). This process is summarized in figure 15.
(1) Antigen presenting dendritic cells acquire cancer cell antigens and (2) present the phagocytosed antigens to naïve T cells. Activated T cells (3) proliferate and (4) migrate into tumor site where they are able to (5) recognize and kill cancer cells.

Activated CD8+ cytolytic T cells, helped by Th1, are able to kill cancer cells (Shankaran, Ikeda et al. 2001, Dunn, Bruce et al. 2005, Galon, Costes et al. 2006, Gajewski 2007). Moreover, both CD8+ T cells and Th1 cells express IFN-γ. IFN-γ is known for its role as protector against tumor development. IFN-γ can be secreted by a large panel of cells and targets both tumor cells and hematopoietic cells. Multiple different tumor protective effects of IFN-γ have been published over the last few years. IFN-γ prevents primary tumor development and activates APCs needed for the cytolytic functions of T cells and cytotoxic macrophages (Shankaran, Ikeda et al. 2001, Mocikat, Braumuller et al. 2003). IFN-γ can also prevent angiogenesis and is necessary for the induction of Th1 responses (Qin and Blankenstein 2000, Martin-Fontecha, Thomsen et al. 2004).

Not all T cells are classically considered as potent anti-tumor effectors. Regulatory T cells (Tregs) (a subpopulation of CD4+ T cells) are able to impair DC maturation therefore contributing to suppress the activation of effector T cells. They can also induce apoptosis and cytolysis of effector T cells (Fallarino, Grohmann et al. 2003, Vignali, Collison et al. 2008). The switch from Th1 to Th2 phenotype mediated by DC and CAFs also contributes to tumor
evasion. A lower Th1/Th2 ratio correlates directly with poor prognosis in pancreatic cancer. Th2 cells participate in the remodeling of ECM and in the modulation of macrophages and NK phenotype, all important processes to tumor growth and dissemination (De Monte, Reni et al. 2011). Finally, the presence of CD4⁺ T helper 17 (Th17), secreting IL-17 cytokine, still remains the subject of debate as to whether their presence is of good or bad prognosis (Woo, Chu et al. 2001, Curiel, Coukos et al. 2004). Th17 T cells are associated with poor prognosis in colorectal, lung and hepatocellular cancers. The presence of Th17 T cells, however, seems of good prognosis in ovarian, gastric and esophageal cancers (Wilke, Kryczek et al. 2011, Fridman, Pages et al. 2012).

1.3.b. B cells

Like with T cells, subpopulations of B cells have been associated with anti-tumor functions and others associated with tumor progression. The presence of CD20⁺ B cells in TME correlates with favorable outcomes in ovarian, cervical cancers and non-small lung carcinomas, possibly related to its expression of IFN-γ (Al-Shibli, Donnem et al. 2008, Nedergaard, Ladekarl et al. 2008, Nielsen, Sahota et al. 2012). In contrast, regulatory B cells (Bregs), a subtype of B cells, have been associated with pro-tumoral activities, like suppression of T cell subpopulations or attenuation of their activity (Schioppa, Moore et al. 2011, Sarvaria, Madrigal et al. 2017). Furthermore, Andreu et al. show that the accumulation of antibodies, produced by B cells, in premalignant skin favors the entrance of macrophages and Myeloid-derived suppressor cells (MDSC) into the tissue and contribute to chronic inflammation that promote de novo carcinogenesis (Andreu, Johansson et al. 2010).

1.3.c. NK cells

High numbers of infiltrating NK cells in tumors are associated with good prognosis and individuals with low NK cell cytotoxic activity have a higher incidence of cancer (Imai, Matsuyama et al. 2000, Ishigami, Natsugoe et al. 2000, Villegas, Coca et al. 2002, Remark, Alifano et al. 2013). Studies show that NK cells (i) have a particular important role in immune surveillance of hematological malignancies and metastatic processes and (ii) in vitro they have a preference for cancer cells with stem cell features (Street, Hayakawa et al. 2004, Castriconi, Daga et al. 2009, Ames, Canter et al. 2015, Ilander, Olsson-Stromberg et al. 2017). The ability of NK cells to kill “foreign” cells depends on a balance between the engagement level of activating and inhibitory receptors on NK cell surface. Briefly, cancer cells can either lack ligands to NK inhibitory receptors or overexpress ligands recognized by NK cell activating receptors (figure 16).
(Morvan and Lanier 2016)

**Figure 16. NK cell killing.**

(a) Balanced signals delivered by activating and inhibitory NK cells receptors are recognized as healthy and spared from NK cell-mediated lysis. NK cells recognize and kill tumour or damaged cells that (b) downregulate MHC class I molecules - 'missing self' lysis; (c) upregulate stress-or damage related ligands that are recognized by activating NK cell receptors - 'induced-self' lysis or (d) through antigen-specific antibodies that bind CD16 on NK cells resulting in antibody-dependent cell-mediated cytotoxicity (ADCC).

Whatever the process, this leads to the balance between the different receptor engagements and consequently to the secretion of cytotoxic molecules – perforin and granzymes - responsible for the activity and targeting of NK-cell mediated killing. Perforins are capable of disrupting membrane proteins and, in turn, granzymes are proteolytic enzymes. (Voskoboinik, Whisstock et al. 2015, Morvan and Lanier 2016). Antibody-dependent cell-mediated cytotoxicity is another mechanism allowing the elimination of cancer cells by NK cells. In the latter case, the Fc gamma receptor III CD16 on NK cells, recognizes antibodies that bound to specific antigens expressed at the surface of cancer cells, allowing the killing of the “antibody coated” malignant cells (figure 16) (Morvan and Lanier 2016). Lastly, through an autocrine signaling cascade involving tumor necrosis factor (TNF) receptors family, NK cells induce the expression of “death ligands” FasL and TRAIL that promote apoptosis when bound to their
receptors expressed in cancer cells (Cretney, Takeda et al. 2002, Wallin, Screpanti et al. 2003).

Besides their killing capacities, NK cells can also contribute to the protection against cancer progression through secretion of IFN-γ and (TNF-α) (Muller-Hermelink, Braumuller et al. 2008). The anti-tumor properties of IFN-γ has already been described. As for TNF-α most properties overlap with those from IFN-γ like inhibition of angiogenesis and tumor proliferation and induction of Th1 cell responses (Stoelcker, Ruhland et al. 2000, Muller-Hermelink, Braumuller et al. 2008).

1.3.d. Macrophages

Macrophages are classified in two functional polarized states – M1 and M2. While M1 macrophages contribute to anti-tumor immunity for instance through triggering apoptosis in tumor cells via TNF receptors, M2 macrophages promote angiogenesis, tumor invasion and metastasis (Mantovani, Sozzani et al. 2002). Tumor associated macrophages (TAMs) usually switch from an M1 to an M2 phenotype. In spite of the secretion of different cytokines, both M1 and M2 recruit pro-tumoral immune cells and contribute to the impairment of T cell priming (Sica and Bronte 2007).

1.3.e. Neutrophils

Similarly to what happens with TAMs, tumor associated neutrophils (TANs) also switch from an anti-tumoral to a pro-tumoral phenotype. Their anti-tumoral activity is based on their ability to trigger cancer cell death and stimulate adaptive immune responses (Stockmeyer, Beyer et al. 2003, Mantovani, Cassatella et al. 2011, Martin, Seignez et al. 2018). Conversely, protumoral TANs normally associated to a N2 polarization are implicated in dissemination and metastases, facilitating the bridge between cancer cells and the metastatic sites; these cells sustain the ECM remolding through the secretion of MMPs, in particular MMP9, and inhibit CD8+ T cell function (Fridlender and Albelda 2012).

1.3.f. Myeloid-derived suppressor cells

MDSC are generally defined as a heterogeneous population of immature myeloid cells. Contrary to TAMs and TANs, they only have immunosuppressive properties. Among these properties, the most relevant are the activation, expansion and recruitment of Treg cells and the impairment of T cell trafficking to- and from-lymph nodes (Serafini, Mgebroff et al. 2008, Gabrilovich, Ostrand-Rosenberg et al. 2012, Schlecker, Stojanovic et al. 2012). They are subdivided in two populations: monocytic MDSCs (M-MDSCs) and granulocytic or
polymorphonuclear MDSCs (PMN-MDSCs). M-MDSC are phenotypically and morphologically similar to monocytes and PMN-MDSC are phenotypically and morphologically similar to neutrophils. HLA-DR expression patterns allows the distinction between M-MDSC (HLA-DR low) and monocytes (HLA-DR+) (Bronte, Brandau et al. 2016). As for PMN-MDSC and neutrophils, there is no really distinction between the two populations besides of their different densities (Sagiv, Michaeli et al. 2015). PMN-MDSC share the same phenotype with TAN and there is a debate whether they belong in fact to the same population (Groth, Hu et al. 2019).

1.4. Adipocytes

Adipocytes are one of cell types that can be at the origin of CAFs but infiltrating adipocytes can also be modulated by cancer cells into cancer associated adipocytes (CAA). CAA could support cancer growth and dissemination through different mechanisms. They (i) provide tumors with fatty acids and other adipocyte-related metabolites that fuel tumor growth, (ii) promote invasive properties of tumor cells and (iii) protects cancer against some cytotoxic therapies through the induction of pro-survival signals in cancer cells (Iyengar, Combs et al. 2003, Nieman, Kenny et al. 2011, Duong, Geneste et al. 2017).

2. The prison break of cancer cells

A complex crosstalk between the cellular components of the TME is needed for tumor progression. The way CAFs, cancer and immune cells communicate, activate with one another and play their role in cancer progression is the subject of this section.

2.1 Crosstalk between fibroblasts and cancer cells

Cancer cells are able to recruit nonmalignant cells and create conditions that promote the switch of ECM fibroblast to CAFs. One of the most known and well-studied tumor-cell derived factor affecting CAF activation is TGF-β. In a TGF-β rich microenvironment, fibroblasts start expressing factors involved in ECM production and remodeling and through a paracrine signaling cascade, they downregulate the gap junctions that are important to their intercellular communication (Lieubeau, Garrigue et al. 1994, Lohr, Schmidt et al. 2001, Stuhlmann, Steinbrenner et al. 2004, Hadj-Rabia, Oriot et al. 2013). TGF-β alone is enough to induce the expression of α-SMA in fibroblasts (Ronnov-Jessen and Petersen 1993). However, TGF-β is not required for the invasive properties of activated fibroblasts (Albrengues, Bourget et al. 2014). PDGF and basic fibroblast growth factor (bFGF) are two other growth factors highly expressed in cancer cells that stimulate proliferation and activation of CAFs (Shao, Nguyen et
al. 2000, Strutz, Zeisberg et al. 2000). After the switch, a positive feedback is established between CAFs and cancer cells (figure 17). While cancer cells maintain fibroblast activation, fibroblasts respond with secretion of growth factors, cytokines and proteases that sustain tumor progression.

Without being exhaustive, among CAFs’ secreted factors, CXCL12 /SDF1α, CXCL14 and HGF have been proven to have a major role in cancer cell growth. CXCL12 binds to its CXCR4 receptor in cancer cells and promote both proliferation of cancer cells in vitro and tumor growth in vivo (Orimo, Gupta et al. 2005). Furthermore, Wang et al. reported that the inactivation of CXCL12/CXCR4 signaling in pancreatic cancer cells led to a partial inactivation of the Wnt signaling pathway which is known for its role in proliferation, invasion, metastasis, and angiogenesis (Taketo 2006, Wang, Ma et al. 2008). CXCL14 autocrine signaling is activated in CAF following nitric oxide synthase 1 (NOS1) signaling. Induction of NOS1 is associated with oxidative stress and its inhibition reduces the ability of CXCL14-expressing fibroblasts to promote tumor formation in presence of breast and pancreatic cells (Augsten, Sjoberg et al. 2011)
Finally, the overexpression of HGF by CAFs has been shown to promote proliferation of cancer cells and increases their invasive properties in 3D cultures through the paracrine activation of HGF/c-Met signaling pathway (Jedeszko, Victor et al. 2009, Ding, Ji et al. 2018). CAFs are also key players in the modulation of adhesive and proteolytic activities that are essential in cancer invasion and dissemination. Through the overexpression of matrix metalloproteinases (MMPs), CAFs are able to disrupt ECM by proteolysis, making it more prone to tumor expansion and invasion (Chenard, Lutz et al. 1999, Foley, Fanjul-Fernandez et al. 2014). Furthermore, in pancreatic cancer cells, MMP-2 and MMP-9 are able to elicit an epithelial-mesenchymal transition (EMT) which promotes motility and migratory abilities (Giannoni, Bianchini et al. 2010). IL-6 and CXCL12/SDF-1α also trigger cancer cell EMT through activation of JAK2-STAT3 and Hedgehog pathways (Li, Ma et al. 2012), respectively. In a mechanical process independent of MMPs activity, CAFs are able to disrupt the basement membrane and induce the formation of gaps through which cancer cells can migrate (Glentis, Oertle et al. 2017).

One of the hallmarks of cancer is the acquisition/re-expression of stem-cell like plasticity by cancer cells. Numerous factors secreted by CAFs have been shown to be involved in the increase of the cancer stem cell (CSC) population. For instance, connective tissue growth factor (CTGF) increases the expression of multiple pluripotency genes in head and neck cancer cells. HGF seems to regulate CSC population through both activation of Wnt and c-Met signaling pathways (Vermeulen, De Sousa et al. 2010, Chang, Hsu et al. 2013, Li, Wang et al. 2019).

2.2. Tumor immune microenvironment

Different types of immune cells exhibiting opposite functions in cancer progression are recruited to the TME and can be modulated by CAFs and cancer cells. The dual role of the immune system in tumor progression is the basis of the “immunoediting theory” first proposed by Dunn et al in 2002.

The “immunoediting theory” (figure 18) characterizes three different phases of interactions between immune and cancer cells i.e., i-) elimination, ii-) equilibrium and iii-) escape (Dunn, Bruce et al. 2002). During these three phases, distinct subsets of immune cells are found in the TME. Here, I will pay more attention on the contribution of cancer cells and CAF’s to the evolution of tumoral immune microenvironment upon the different phases of the immunoediting process.
2.2.a. Elimination

The elimination phase is characterized by a major infiltration of T cells and NK cells in an attempt to mount an efficient anti-tumor immune response aiming at the elimination/eradication of cells recognized as "abnormal". Through the secretion of CXCL9 and CXCL10, cancer cells drive T-cell tumor infiltration (Mikucki, Fisher et al. 2015). In such a way, T cells can also secrete type I interferon (IFN-α and IFN-β) which stimulates accumulation of DC in TME. Accumulation of DC contributes to the presentation the “foreign” antigen to T cells and subsequently to T cell priming and tumor cell elimination (Diamond, Kinder et al. 2011).

Regarding NK cells, the signaling that drives their migration into solid tumors is not yet completely understood. If we stick to the theory that cancer is a wound that never heals, we...
may assume that the classic chemoattractant molecules that drive NK cells into the wound – namely IL-2, IL-12 and IFN-α, - are already present in the early steps of tumorigenesis (Hodge, Schill et al. 2002, Hu, Jiang et al. 2014, Doersch, DelloStritto et al. 2017). However, some reports have also suggested that specific signals expressed by cancer cells may also trigger NK cell infiltration. Among them, CXCL9 and CXCL10 promote triggering of NK cell migration to the TME (Przewoznik, Homberg et al. 2012). These two cytokines have also been identified as expressed in CAF’s (Ziani, Chouaib et al. 2018).

Effector T cells and NK cells exhibit complementary roles in the killing process of foreign cells. The cytotoxic capacities of effector T cells are dependent on the expression of MHC-I at the surface of cancer cells. MHC-I molecules are required for detection and subsequent destruction of malignant cells by T cells (Atkins, Breuckmann et al. 2004). NK cells, however, are able to kill cells that lack MHC-I molecules when they overexpress NK activator receptors at their surface. Besides the canonical function of NK cells as cell killers, a recent paper from Böttcher et al. has shown that they can also have a role in the recruitment of antigen-presenting dendritic cells through the expression of XCL1 and CCL5 which leads to the infiltration of T cells (Bottcher, Bonavita et al. 2018).

Other immune cells that can infiltrate the neoplastic structure and kill cancer cells are two specific subsets of T cells (NKT cells and γδ T cells), TAMs and TANs.

2.2.b. Equilibrium and Escape

After a first phase where cancer cells become highly immunogenic and can easily be eliminated, in the “equilibrium phase” some of them start eluding the immune system and escape killing. They may live for a quite long time while in an equilibrium with the infiltrated immune cells that still continue to prevent tumor growth and at the same time sculpt cancer cells immunogenicity. (Koebel, Vermi et al. 2007). However, the immunosuppression of anti-tumor properties of immune cells, the infiltration of pro-tumoral immune cells and the newly acquired phenotypes that allow cancer cells to protect themselves against immune responses prompt cancer cells into the escape phase.

Modulation of effector T cell function

To dribble the ability of CD8⁺ cytotoxic T cells to recognize them, cancer cells acquired the capacity to downregulate MHC-I expression at their cell surface (Atkins, Breuckmann et al. 2004, Garrido, Romero et al. 2016). This is the first step of the “tumor editing” as it starts to shape cancer cells into a less immunogenic phenotype which allows them to escape the control of immune surveillance (figure 18). In addition, some cancer cells upregulate the non-classical
MHC-I molecule HLA-E that interacts with the inhibitory receptor CD94/NKG2A induced on effector T cells and expressed by NK cells, leading to the impairment of their anti-tumor activities (Braud, Allan et al. 1998, Haanen and Cerundolo 2018). More generally, a number of inhibitory receptors are induced on tumor-reactive T cells that lead to “exhausted” T cells that no longer exert efficient anti-tumor activities (Pardoll 2012). These interactions, so-called “immune checkpoints” are targeted in the newly developed immunotherapies for the treatment of cancer (see point 4.2 of this chapter).

TGF-β is expressed by both cancer cells and surrounding fibroblasts. TGF-β has also an important role in inhibiting T cell proliferation and cytotoxic properties. TGF-β mediates conversion into Tregs (T-Regulator lymphocytes) already describe as inhibitors of CD8+ effector T cells. TGF-β promotes cell death and alteration of cytotoxic properties of CD8+ T cells by inhibiting the expression of the pro-survival protein Bcl-2 and inhibiting the expression of key genes involved in their cytotoxic activity, respectively (Ahmadzadeh and Rosenberg 2005, Sanjabi, Mosaheb et al. 2009, Hargadon 2016).

Modulation of NK phenotype

Besides downregulation of MHC-I molecules, cancer cells have other ways to shuffle immune system through the modulation of their membrane receptors. They can protect themselves from NK cell killing by integrating in their membrane, vesicles containing MHC-I molecules previously released by platelets. This mechanism confers to cancer cells a “pseudo normal phenotype” allowing them to impair NK cytotoxicity (Placke, Orgel et al. 2012). Similarly to HLA-E discussed previously, another efficient way to negatively affects immune cell functions is the expression of the non-canonical MHC molecule, namely the human leukocyte antigen G (HLA-G). HLA-G is expressed in embryos and has an important role in protecting placenta from rejection (Rizzo, Vercammen et al. 2011). The neo expression of HLA-G engages receptors from both NK cells and CD8+ effector T cells generating inhibitory signals that impair their activation. It also triggers pro-tumoral effects in DC, neutrophils and MDSCs (Lin and Yan 2015).

Alike what happens with T cells, TGF-β is also implicated in the modulation of NK phenotype. TGF-β is able to decrease NK cell activation and cytotoxic activity by reducing the expression of some NK-activating receptors through both its soluble or membrane-bound form (Castriconi, Cantoni et al. 2003, Park, Choi et al. 2011). In fact, besides of the secretion of soluble TGF-β by cancer cells and CAFs, another important source of TGF-β are Treg. Treg cells express membrane-bound TGF-β at their surface which is directly responsible for the down regulation of the NK activating receptor NKG2D and activity restriction of the NK cell effector functions in vitro (Ghiringhelli, Menard et al. 2005).
The secretion of prostaglandin 2 (PGE2) again by both CAFs, cancer cells and even by some infiltrated immune cells, is also able to impair NK cell (and T cell) infiltration, as well as their cytotoxic activity (Li, Yang et al. 2012, Zelenay, van der Veen et al. 2015). PGE2 is the prostaglandin most commonly found in human cancer and its expression is associated with poor life span prognosis (Wang and Dubois 2010). Although the mechanisms through which PGE2 modulates NK and T cell phenotypes are not yet fully understood, at least one of the signaling pathways was identified. Indeed, PGE2 leads to the downregulation of IFN-α levels within the TME (Zelenay, van der Veen et al. 2015). Type I IFN (α/β) increases NK cell mediated killing, promotes the differentiation of CD8+ T cells and enhance the dendritic cell cross-presentation of tumor antigens to CD8+ T cells (Nguyen, Salazar-Mather et al. 2002, Swann, Hayakawa et al. 2007, Diamond, Kinder et al. 2011, Bhat, Leggatt et al. 2017).

Recently, a study from Ziani et al. reported that CAFs associated with melanoma could also modulate the activation of NK cells through the secretion of MMPs. MMPs are able to cleave MHC-I-related chain (MIC)-A and MIC-B, two ligands of the NK activating receptor NKG2D and, consequently, decrease the NKG2D-dependent cytotoxic activity of NK cells against melanoma tumor cells and their secretion of IFN-γ (Ziani, Safta-Saadoun et al. 2017). Finally, the hypoxic tumor microenvironment leads to secretion of lactate by cancer cells. This phenomenon has been shown to reduce the numbers and activity of NK cells and CD8+ T cells both in vitro and in vivo (Brand, Singer et al. 2016).

Modulation of other immune cells

Finally, the modulation of dendritic cell activity and the transition of macrophages and neutrophils into pro-tumoral TAMs and TANs are also processes that contribute to the escape of cancer cells and are mostly regulated by cancer cells secreted factors.

The production of vascular endothelial growth factor (VEGF), IL-6, IL-10, TGF-β, macrophage colony-stimulating factor (M-CSF) and cyclooxygenase-2 (COX-2) by cancer cells is implicated in the impairment of differentiation, maturation and function of dendritic cells with direct consequences on the activation of effector T cells (Zou 2005).

In TAMs, the activated M2 phenotype may arise due to the hypoxic conditions of the TME and to the presence of M-CSF, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and TGF-β secreted by cancer cells or IL-4 secreted by CD4+ T cells (Lin, Gouon-Evans et al. 2002, Gocheva, Wang et al. 2010, Su, Liu et al. 2014, Zhang, Wang et al. 2016). TANs’ polarization into pro-tumoral N2 phenotype can be induced by TGF-β, G-CSF and tumor-derived exomes (Coffelt, Wellenstein et al. 2016, Zou, Qin et al. 2017, Zhang, Shi et al. 2018).
To conclude, as a consequence of the immunoediting process, the TME is no longer efficiently anti-tumoral and allows tumor dissemination.

3. Senescent microenvironment drives tumorigenesis

As mentioned in chapter 1, aging is a risk factor for the development of cancer. Hayflick first described cellular senescence in 1965. He showed that primary cells have finite replicative lifespan and when they reach it, they are no longer able to reenter cell cycle yet remaining metabolically active (Hayflick 1965). Senescent cells express different cell cycle inhibitors - like p16, p21 and p53 – a specific senescence associated β-galactosidase (SA-βGal) and exhibit a particular alteration of their secretory profile known as the senescent-associated secretory phenotype (SASP) (Dimri, Lee et al. 1995, Alcorta, Xiong et al. 1996, Coppe, Desprez et al. 2010). In a tumor context, senescence plays a paradoxical role being both tumor promoter and tumor suppressor. This can partially depend on the cell in which senescence occurs. For instance, cancer cells presenting a senescent phenotype are able to trigger immune responses that lead to tumor clearance in mice (Xue, Zender et al. 2007, Kang, Yevsa et al. 2011). On the other hand, senescent stromal cells within TME promote tumor growth (Krtolica, Parrinello et al. 2001).

The possible implication of a senescent microenvironment in tumor progression was extensively studied in immune deficient or immune compromised systems. Senescent stromal cells are able to stimulate both epithelial and cancer cell proliferation through the expression of tumor promoting factors like HGF, FGF7 and CXCL12 (Bavik, Coleman et al. 2006). Furthermore, through the secretion of MMPs, they are able to promote cancer cell EMT and ECM remodeling (Begley, Monteleon et al. 2005, Parrinello, Coppe et al. 2005). This line of work points out the high similarity between SASP and CAFs associated phenotype. However, in contrast with the anti-tumor processes that can be triggered by senescent carcinogenic cells, a recent study shows that senescent stromal cells drive a tumor immunosuppressive microenvironment independently of tumor presence. Through the secretion of IL-6, senescent stromal cells recruit and accumulate MDSCs in the tissue which will suppress CD8+ T cells and in a tumor context, will promote tumor growth (Ruhland, Loza et al. 2016). This paper was the first to demonstrate that a senescent healthy microenvironment can be considered as a protumoral immunosuppressive ME. After that, other studies have confirmed that MDSCs are in fact more present in a senescent tissue and can be considered as inducers of immunosenescence state (Salminen, Kaarniranta et al. 2019).
The at least partial impairment of the “elimination phase” by a MDSC rich senescent ME can have two paradoxical consequences: (i) it will more easily allow the progression of cancer from a pre-malignant lesion but (ii) it will also partially impair the immunoediting process and as a consequence, cancer cells stay more immunogenic which could translate into less aggressive and more easily treatable cancer cell phenotypes. Although until now no study prove these speculations, there are correlation studies that show that in elderly, tumors are more present but are less aggressive compared to younger population (Berger, Savvides et al. 2006).

4. Microenvironment as a target for cancer therapy

Chemotherapy often combined with surgery and radiotherapy are normally the first line of treatment for cancer. Despite the effectiveness of chemotherapy treatments, cancer cells are becoming multidrug resistant (MDR) which drives relapse and compromises the prognosis of the patients (Wu, Yang et al. 2014). For a long time, cancer research has been focused on cancer cell itself but with all the body of evidence confirming the important role of TME in cancer progression and even in the MDR, new perspectives of therapies based on TME start to emerge (Shain and Dalton 2009). Here, I will focus on the TME perspective and/or based therapies targeting fibroblasts and immune cells.

4.1 Targeting CAFs in cancer therapy

Many molecules are currently in different phases of clinical trials to prove their efficacy in targeting CAFs or CAFs’ related functions. Based on solid in vitro evidence one of the candidates to target CAFs is the membrane protease FAP-α. FAP-α is one of CAFs well-known markers and in vitro, its depletion from fibroblast membranes impairs FAP-α-mediated migratory and invasive capacity (Teichgraber, Monasterio et al. 2015). However, the first trials using the sibrotuzumab, an antibody targeting FAP-α, or small molecule FAP-α inhibitors did not reveal any improvement in patient outcome (Hofheinz, al-Batran et al. 2003, Narra, Mullins et al. 2007). Today, there are new ongoing clinical trials that use different methods to target FAP-α (U.S. National Library of Medicine 2019). Another recent approach aiming to deplete CAFs from the microenvironment is the targeting of NAD(P)H Oxidase-4 (NOX4). NOX4 is essential for the regulation of fibroblast to myofibroblast differentiation and its pharmacological inhibition on CAFs can abrogate their tumor-promoting function in vitro and in vivo (Hanley, Mellone et al. 2018). Until now no clinical trial is reported.

Besides depleting CAFs from the TME, other possible therapeutic approaches are the impairment of CAFs’ recruitment, activation and functions. PDGF expressed by cancer cells can recruit CAFs and promote growth and metastasis (Tejada, Yu et al. 2006, Anderberg, Li et al. 2009). There are many available PDGFR inhibitors but none of them is specific for
PDGFR inhibition as they also target other tyrosine kinases like VEGFR. However, studies proved that PDGFR inhibition is the one responsible for the positive effects of imatinib, sunitinib and nilotinib – three PDGFR inhibitors - in the treatment of gastrointestinal stromal tumors and dermatofibrosarcoma protuberance (Heldin 2013). To overcome the difficulty of the selectivity of the target, new specific antibodies were made but until now no clinical trial show a significant antitumor activity. However, there are clinical trials still ongoing (U.S. National Library of Medicine 2019).

TGF-β as one of the main factors that can drive a normal cell into a CAF is also a good target for cancer therapy. There are many different types of TGF-β targeting agents and some of them show good results in clinical trials. This is the case of galunisertib, a TGB-β receptor kinase inhibitor that shows anti-tumor responses in glioblastoma patients and in combination with cytotoxic gemcitabine, improves overall survival in pancreatic cancer patients (Rodon, Carducci et al. 2015, Colak and Ten Dijke 2017, Melisi, Garcia-Carbonero et al. 2018). Because of the pleiotropic effects of TGF-β, it is difficult to say which mechanism is responsible for the therapeutic effect. Combining TGF-β targeting with other cancer therapies could help to determine the leading mechanism in TGF-β action in favor of cancer development.

Targeting the ECM composition is also a valid alternative in cancer therapy targeting microenvironment phenomena. One example is targeting ECM fibrosis with a Pirfenidone (PFD) treatment. PFD is a small molecule with anti-fibrotic properties that inhibit tumor fibrosis in vivo and when associated with the classic cytotoxic antineoplastic drug doxorubicin inhibits tumor growth and metastasis (Takai, Le et al. 2016). Phase I clinical trials are ongoing to approve the combination of pirfenidone with standard first line chemotherapy to treat non small lung cancer (NCT03177291)(U.S. National Library of Medicine 2019).

If at least some of all these clinical trials succeed, we will definitely enter in a new era of cancer treatment that will acknowledge the independent and important role of CAFs in cancer progression.

4.2 Targeting immune system in cancer therapy

The important role of the immune system in cancer progression pushed for the development of cancer immune based therapies far before what happened with fibroblasts. Besides the multiple clinical trials that are ongoing, today, different approaches regarding cancer immunotherapy are already used in clinic.

To maintain immune tolerance and avoid excessive systemic inflammation in the body, there are inhibitory pathways that act as regulators of the immune system called immune checkpoints. Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and programmed cell
death protein 1 (PD-1) are two receptors expressed in T cells that triggers inhibitory cascades leading to impairment of T cell effector functions and immunosuppression. CTLA-4 is expressed on both CD4+ and CD8+ T cells upon activation (Perkins, Wang et al. 1996). CTLA-4 competes with the co-stimulatory CD28 receptor for the same ligands – CD80 and CD86. However, CTLA-4 has higher affinity for both ligands and outcompete CD28. The engagement of CTLA-4 leads to T cells that are more resistant to activation, which reduces immune responses towards weak antigens such as self- and tumor antigens (Rudd, Taylor et al. 2009, Seidel, Otsuka et al. 2018). PD-1 is expressed in T cells, also upon activation. In T cells, the expression of PD-1 is induced upon antigen recognition via T cell antigen receptor (TCR). The engagement of PD-1 with its ligands PD-L1 and PD-L2 leads to exhaustion and apoptosis of effector T cells (Keir, Butte et al. 2008). Cancer cells are able to activate immune inhibitory checkpoints to avoid anti-tumor immune responses.

Approaches based on blocking ligand access to CTLA-4 and PD-1 are already used in the treatment of cancer. Ipilimumab and tremelimumab are two therapeutic anti-CTLA-4 antibodies that impair the binding of CTLA-4 ligands and show good efficacy in the treatment of numerous solid tumors (Rowshanravan, Halliday et al. 2018). In clinic they are used for the treatment of metastatic melanoma and renal cell carcinomas (U.S. Food & Drug Administration 2019). The engagement of PD-1 by PD-L1 expressed in cancer cells makes them resistant to CD8+ T direct lysis and to apoptosis (Azuma, Yao et al. 2008, Kinter, Godbout et al. 2008). Pembrolizumab, nivolumab and cemiplimab are three anti-PD-1 antibodies approved and currently used for the treatment of solid tumors. In addition, anti-PD-L1 antibodies were also designed and atelolizumab, avelumab and durvalumab are also suitable for clinical use (U.S. Food & Drug Administration 2019). Similar efforts are being made regarding the blocking of inhibitory receptors in NK cells. Lirilumab and monalizumab, two antagonist antibodies of NK inhibitory receptors are presently in clinical trials and first results show an enhancement of NK activity against tumor cell lines (Romagne, Andre et al. 2009, Vey, Bourhis et al. 2012, Andre, Denis et al. 2018). Monalizumab, a humanized anti-NKG2A antibody also shows efficacy in early clinical trials, mostly by restoring anti-tumor activity of T cells and also NK cells (Andre, Denis et al. 2018, van Montfoort, Borst et al. 2018).

Besides from therapies targeting inhibitory checkpoints like the ones mentioned before, diverse strategies are used to enhance endogenous antitumor immune responses. As antigen presentation is one of the first step in generating immune response, therapeutic vaccines could augment adaptive immune responses. There are several therapeutic vaccines in clinical trials but until now, only Sipuleucel-T passed the cut off of FDA. Sipuleucel-T consists in autologous APC incubated ex vivo with a prostate antigen. It is an alternative treatment for prostate cancer (Kantoff, Higano et al. 2010, Gardner, Elzey et al. 2012).
Therapeutic modulation of the cytokine environment is also a way to enhance anti-tumor immune responses. IL-2 was the first cytokine to be considered and later approved for its efficacy in cancer immunotherapy (Jiang, Zhou et al. 2016). It induces proliferation and enhances cytotoxicity of NK cells, activates T cells and promotes the differentiation of B cells (Miyawaki, Suzuki et al. 1987, Sharma and Das 2018, Tognarelli, Wirsching et al. 2018). In metastatic melanoma and renal cell carcinoma, IL-2 therapy mediates tumor regression and provides clinical benefits to patients but at least monotherapy is not enough to improve patient’s survival. Because (i) it also activates pro-tumoral Tregs expansion and (ii) it is associated with toxicity at high doses, its use in clinic remains restricted (Fisher, Rosenberg et al. 2000, Saraceni, Agostino et al. 2015). Another cytokine currently used as an adjuvant in cancer treatment is IFN-α. During clinical trials it proved to have a positive impact in recurrence free survival of metastatic melanoma (Eggermont, Suciu et al. 2012). It is also approved for first line treatment of renal cell carcinoma - where its administration correlates with increased in cellular immune responses - and some forms of lymphoma and leukemia (Kosmidis, Baxevanis et al. 1992, Floros and Tarhini 2015).

Finally, similarly to the approach of targeting inhibitory signaling in T cells, targeting costimulatory receptors of effector cells are currently under development. Agonist antibodies for OX40 and CD40 - two costimulatory receptors expressed in T cells - and CD137 - an activating receptor of both T cells and NK cells - are currently being tested. In different phases of trials, the treatment with such antibodies promotes anti-tumor immune responses and induces tumor regression (Vonderheide, Flaherty et al. 2007, Redmond, Ruby et al. 2009, Kohrt, Houot et al. 2012).
Chapter IV: NK cells and their receptors

*It has been a long and astonishing journey for a cell that started its life as a background noise in a laboratory assay – Rolf Kiessling (Kiessling 2010)*

One of the first description of the existence of cells that are able to produce cytotoxicity only by contact with cancer-associated antigens was made by Rosenberg et al. in 1972 (Rosenberg, Herberman et al. 1972). At that time, research efforts were focused on the study of T-cell immune reactivity to tumor antigens and on determining the best methods to evaluate this reactivity. First attempts to standardize the $^{51}$Chromium release assay to measure T cell reactivity against murine Moloney Virus induced leukemia failed because of the “background lysis” of YAC-1 mouse lymphoma cell line in presence of cells from mouse spleen (Kiessling, Petranyi et al. 1975). Researchers quickly understood that in fact this was not background noise but the effect of a different population of lymphocytes - the NK cells. The first characterizations of the NK cell phenotype were published in 1975. NK cells were described as a population with antitumor effector cells functions from non-tumor bearing mice that differed from T- or B-lymphocytes on the cell-surface markers (Herberman, Nunn et al. 1975). Authors suggested at the time that the lytic NK cell activity were triggered by murine endogenous type C viruses associated antigens (Herberman, Nunn et al. 1975).

However, despite this early discoveries, the mechanisms underlying this unique capacity to distinguish between healthy and infected or cancer cells remained unknown until the discovery in 1990 that NK cells were able to kill cells that fail to express the MHC class I molecules (Ljunggren and Karre 1990).

After that, the puzzle was rapidly solved with the identification of different NK inhibitory and activating receptors that clarified how NK cell functions are regulated. The unbalance between these two classes of receptors is at the basis of the regulation of NK cell cytotoxic activity.

1. NK receptors

The interaction between inhibitory receptors and MHC-I molecules is essential for NK cell development and maturation in the bone marrow. This is a process called NK cell education and will determine the threshold for activation in mature NK cells. Depending on the level of MHC-I inhibitory signals that they receive, NK cells are able to balance their activating receptors to adapt their phenotype to those of the host (Raulet and Vance 2006). This dance of acquiring properties of self and missing-self recognition gives rise to a large NK
heterogeneous population with 6000 to 30,000 different phenotypes within an individual (Husain, Alper et al. 2002, Brodin, Karre et al. 2009, Horowitz, Strauss-Albee et al. 2013).

The genes that encode for NK cell receptors are mostly clustered in two complexes at different sites of human genome: the natural killer complex (NKC) located on chromosome 12p12-p13 encoding for the C-type lectin-like molecules, and the leukocyte receptor complex (LRC), located on chromosome 19q13.4 encoding genes of the immunoglobulin superfamily (IgSF) receptors (Trowsdale 2001, Kelley, Walter et al. 2005). Importantly both activating and inhibitory receptors could be MHC-I dependent or independent.

LRC encodes for killer immunoglobulin-like receptors (KIR) that are NK receptors for MHC-I molecules and for one member of the natural cytotoxicity receptors (NCR), the NKp46, which is an activating receptor (Wende, Colonna et al. 1999, Hadad, Thauland et al. 2015). Nkp30 and Nkp44 are two other NCR activating receptors that are not encoded in none of the two complexes but instead on chromosome 6 (Enk and Mandelboim 2014). Another activating receptor, DNAX accessory molecule-1 (DNAM-1) is also a member of IgSF encoded on chromosome 18 (de Andrade, Smyth et al. 2014).

The C-type lectin like receptors (CTLR) encoded in the NKC complex are classified according to the cell type in which they are expressed: killer cell lectin like receptors (KLR) if they are expressed on NK cells and C type lectin receptors (CLEC) if they are expressed on non NK cells (Bartel, Bauer et al. 2013). CTLR are structurally type II membrane-bound surface receptors with one extracellular C-type lectin-like domain (CTLD). The ones encoded in NKC complex belong to the C type lectin subfamily V that is characterized by the loss of a functional Ca$^{2+}$-dependent ability to bind carbohydrates. They instead bind to protein ligands (Iizuka, Naidenko et al. 2003, Zelensky and Gready 2005). KLR can be further divided in two subclasses: the ones that bind MHC-I like and non-classical MHC-I molecules, and the ones that do not bind MHC-I ligands. To the first class belong the well-studied NKG2D/KLRK1 activating receptor (their ligands MICA/B et ULBPs are not MHC-I but MHC-I-like) and the subfamily CD94/NKG2 complex binding the non-classical MHC-I molecule HLA-E (Braud, Allan et al. 1998, Bauer, Groh et al. 1999, Borrego, Masilamani et al. 2006). To the second class belong the members of the NK receptor protein subfamily 1 (NKRP1). NKRP1 subfamily is the topic of next section.

1.1 NKRP1 subfamily

In human there are three well described receptor/ligand couples among the NKRP1 subfamily: KLRB1/CLEC2D, KLRF1/CLEC2B and KLRF2/CLEC2A.
1.1.a. KLRB1/CLEC2D

KLRB1 encodes for the NKRP1A/CD161 receptor that is expressed on both NK cells and T cells (Lanier, Chang et al. 1994). Its ligand is the lectin-like transcript-1 (LLT1) encoded by CLEC2D (Aldemir, Prod'homme et al. 2005, Rosen, Bettadapura et al. 2005). LLT1 is expressed on activated hematopoietic cells including dendritic cells (DC), plasmacytoid DC, B cells, T cells and NK cells (Rosen, Cao et al. 2008, Germain, Bihl et al. 2010, Germain, Meier et al. 2011). In addition, IFN-$\gamma$ is a key signal amplifying LLT1 induction by TLR or viral infections on DC and B cells (Germain, Meier et al. 2011).

The interaction between CD161 and LLT1 inhibits NK cell-mediated cytotoxicity and cytokine production. LLT1/CD161 interaction is therefore important in the crosstalk of antigen presenting cells with NK cells by conferring them protection against NK-cell mediated killing, independently of MHC-I molecules (Aldemir, Prod'homme et al. 2005, Rosen, Bettadapura et al. 2005, Germain, Meier et al. 2011). In addition, CD161 is a costimulatory receptor on T cells and is mainly expressed by Th17, Th1-like and MAIT cells (Aldemir, Prod'honne et al. 2005, Fergusson, Fleming et al. 2011, Germain, Meier et al. 2011).

1.1.b. KLRF1/CLEC2B

Nkp80 is an activating receptor expressed by NK cells and some subsets of T cells that is encoded by KLRF1. Its ligand is the activation-induced C-type lectin (AICL) encoded by CLEC2B and expressed on myeloid cell lines and on tumor cell lines of hematopoietic and non-hematopoietic origins (Vitale, Falco et al. 2001, Welte, Kuttruff et al. 2006, Kuttruff, Koch et al. 2009, Akatsuka, Ito et al. 2010). The engagement of Nkp80 receptor by myeloid cells activate both myeloid and NK cells into releasing pro-inflammatory cytokines. Moreover, when the AICL-Nkp80 interaction is promoted by malignant myeloid cells, it triggers direct NK cell mediated cytolysis (Welte, Kuttruff et al. 2006). In an unexpected way, NK cells contain intracellular stores of AICL that through specific stimulation of IL-12 and IL-18 can become extracellular, whereas at the same time expression of NKP80 is downregulated. This now AICL$^+$ NK cells can be recognized and killed by autologous NK cells. The intra circuit Nkp80/AICL could be important to control NK cell responses during inflammation (Klimosch, Bartel et al. 2013).

1.1.c. KLRF2/CLEC2A

The couple KLRF2/CLEC2A was the last to be identified (Spreu, Kienle et al. 2007). KLRF2 encodes for the Nkp65 receptor that until now, was only described in NK cell lines. There are however suspicions about its presence also in innate lymphoid cells (ILC) subsets (Steinle,
Bauer et al. 2016). Its ligand is encoded by CLEC2A and was described in keratinocytes (KACL), U937 monocyctic cell line and more recently, our laboratory identified its expression on skin healthy fibroblasts (unpublished) (Spreu, Kienle et al. 2007, Spreu, Kuttruff et al. 2010). Differently from other members of the CLEC2 family, CLEC2A is almost exclusively expressed on skin (Spreu, Kienle et al. 2007). CLEC2A is composed by five exons and four introns with a total length of 33.7kb. There are four CLEC2A variants described: CLEC2A1, CLEC2A2 and its spliced alternative variants that lack the exon coding for the transmembrane domain (figure 19). Only CLEC2A1 was identified as expressed at the cell surface. Interestingly the alignment of amino acid sequences of human CLEC2A1 with putative CLEC2A1 amino acid sequences from chimpanzee, rhesus macaque and cow revealed homologous conserved regions. Besides the already mentioned species, CLEC2A gene is also homologous to murine Clec2f. This indicates that CLEC2A is part of a stable conserved mechanism of the mammalian immune system (Spreu, Kienle et al. 2007, Zhang, Rahim et al. 2012, National Center for Biotechnology Information 2019).

Figure 19. CLEC2A transcript variants.
CLEC2A is a glycoprotein of approximately 32 kDa prior to the removal of glycosylated units (Spreu, Kienle et al. 2007, Spreu, Kuttruff et al. 2010). The engagement of Nkp65 by CLEC2A-transfected cells enhances the cytotoxic response of NK cells that effectively kill target cells and secrete IFN-γ. The cytosis of NK cells towards U937 cells is also at least partially dependent on Nkp65 engagement. Furthermore, in co-culture experiments, freshly isolated keratinocytes partially mediate the degranulation of NK cells via CLEC2A-NKp65 binding (Spreu, Kuttruff et al. 2010).

The functional interaction between Nkp65 and CLEC2A is independent of CLEC2A glycosylation status (Bauer, Spreu et al. 2015). Structurally, 20 residues from loops L0, L3, L5, and L6 and strands β3 and β4 of Nkp65 contact with 16 residues, also from loops L0, L3, L5, and L6 and strands β3 and β4 of CLEC2A (figure 20) (Li, Wang et al. 2013).

![Figure 20. The NKp65–KACL binding structural interface. 
β-strands and loops are cyan for CLEC2A (upper) and purple for NKp65 (lower); α-helices are red for both structures; carbohydrate chains (GluNAc-GluNAc-Man) attached to CLEC2A are yellow. Loops and strands involved in the contact are labeled.](image)

2. The diversity of NK cell subsets in human skin

In healthy skin, the most prevalent NK cell population is part of the CD56+CD16- subset which contrasts to the circulating NK cell population (cNK) that is mainly CD56+CD16+. A CD16+ phenotype correlates with high cytolytic activity against infected or transformed cells whereas
a CD16- phenotype has been associated with cytokine production and modulation of dendritic cell and T cell priming (Moretta, Marcenaro et al. 2008). The CD16- subset is characterized by the absence of perforin expression which is also a characteristic of skin-related NK cells (srNK). However, differently from cNK, in skin, NK cells lack the expression of CCR7, important chemoattractant receptor which will interfere with migration properties (Ebert, Meuter et al. 2006).

Another difference between cNK and srNK cells is the expression of NKG2D. Although this activating receptor is widely expressed in cNK cells, it is absent in freshly isolated cutaneous NK cells from healthy patients. However, its ligands MICA, MICB and ULBP are present in skin cells and NKG2D can be induced in srNK upon in vitro culture, suggesting it is downregulated in the skin upon binding with its ligands (Zwirner, Dole et al. 1999, Chalupny, Sutherland et al. 2003, Ebert, Meuter et al. 2006, Knight, Spain et al. 2012). Both MICA and MICB are upregulated in skin inflammatory diseases which could indicate that skin cells are able to modulate their crosstalk with NK cells depending on the health status of the tissue (Molinero, Gruber et al. 2003, Knight, Spain et al. 2012).

Most studies of NK cell functions in skin are related to their implication in autoimmune and inflammatory diseases like psoriasis and dermatitis. Psoriasis is described as a chronic inflammation of the skin. NK cells isolated from psoriatic skin show significant differences from srNK of healthy individuals. They express high levels of CXCR3 and CCR5, two chemokine receptors that contribute to the NK cell homing when engaged with their ligands CXCL10 and CCL5 overexpressed by psoriatic keratinocytes (Ottaviani, Nasorri et al. 2006, Nestle, Kaplan et al. 2009). CD16- population is increased in NK cells associated with psoriatic lesions compared to srNK from healthy individuals. Furthermore, these alterations correlate with higher expression of NKG2A receptor and IFN-γ production upon IL-2 stimulation (Batista, Ho et al. 2013). IFN-γ is able to induce in keratinocytes the appearance of ICAM-1 and HLA-DR, the overexpression of MHC-I molecules and the secretion of CXCL10, CCL5 (Ottaviani, Nasorri et al. 2006). This finding show that a modulation of NK phenotype occurs in psoriasis and suggest a role of NK-keratinocytes crosstalk in the chronic inflammatory process in psoriasis.

A specific NK cell subset was also identified in allergic contact dermatitis (ACD). This CD16- NK cell population lacks CCR7 and like the one associated with psoriasis, highly expresses CXCR3. In ACD, NK cells play a role in the apoptosis-induced keratinocytes through the secretion of cytokines secreted upon T cell stimulation. This interaction between innate and adaptive immune responses is at least partially responsible for the exacerbation of allergic responses in ACD (Carbone, Nasorri et al. 2010).
Modulation of NK receptors during cancer was already described in chapter 3. In the skin context, the interaction between melanoma cells and NK cells leads to the downregulation of activating receptors NKG2D, NKp44, and DNAM-1 which render melanoma cells resistant to NK mediated killing (Balsamo, Vermi et al. 2012).

Together, all these data strongly suggest that the important modulation of NK receptors expression in skin can contribute to the active participation of NK cells in both immunosurveillance and physiopathology of skin diseases.
Thesis objectives

The susceptibility of XP patients to the development of skin cancers, and the propensity for SCC development in XP-C group set the bases for the different research axes of the laboratory. In skin cancer, together with UV radiations that induce mutations in the keratinocytes, the microenvironment created by skin fibroblasts and immune cells is an important factor that leads to promotion of mutated cell invasion. When I arrived in the laboratory, the identification of genetic alterations induced by UV radiation and the role of XP-C dermal fibroblasts in XP carcinogenesis was extensively analyzed. However, another aspect concerning the implication of possible alterations in the immunosuppressive microenvironment of XP patients in cancer progression was not explored.

The main objective of my PhD work was therefore to assess the role of the skin microenvironment in XP-C patients on the development of squamous cell carcinomas (SCC) and to explore the cross-talk between fibroblasts and immune cells in immunosurveillance of the skin.

Preliminary comparative transcriptomic analysis of WT and XP-C fibroblasts cultured in both two dimensional and three dimensional cultures, revealed that transcripts of CLEC2A, a ligand of the activating NK cell receptor NKp65, were present in WT fibroblasts and absent in XP-C fibroblasts. I therefore focused my research project on the study the role of CLEC2A expression in a healthy skin and the implications of its absence in XP-C dermis, more specifically in a context of SCC development.

Towards this goal, I structured the research in two main axes:

1) Aiming to get hints on the function roles of CLEC2A in the skin, I (i) analyzed the expression of CLEC2A in skin cells of healthy individuals, XP-C patients, and from SCC areas and (ii) explored the regulation of CLEC2A expression in aging and in pathological conditions such as skin cancer and infection.

2) Using the expertise of the laboratory in reconstructing skin models in vitro, I evaluated the outcome of the cross-talk between fibroblasts and NK cells in the presence and absence of functional CLEC2A/NKp65 interaction. To do so, I developed a 3D organotypic NK immunocompetent model to measure SCC invasion in the presence and absence of NK cells and blocking anti-CLEC2A antibody. As the interaction between fibroblasts and NK cells in skin is relatively unexplored, I could therefore evaluate the impact of CLEC2A expression on SCC invasion in the dermis.
Altogether, these studies serve to assess whether CLEC2A could serve as a diagnostic tool and a therapeutic target in both XP patients and more generally in patients developing SCC.
Scientific article
CLEC2A regulates natural killer cell and fibroblast-mediated control of cutaneous squamous cell carcinoma invasion.

Xeroderma pigmentosum patients from the complementation group C are prone to develop aggressive skin cancers. Dermal XP-C fibroblasts show pro-invasive properties in 3D organotypic cultures, suggesting that they have an active role in cancer progression. To identify deregulations in gene expression of XP-C fibroblasts that could favor the dissemination of cancer cells, we performed a comparative transcriptomic analysis of healthy and XP-C dermal fibroblasts cultured in three dimensional (3D) collagen matrices.

Our results revealed that CLEC2A, a ligand of the activating NK cell receptor NKp65, is present in healthy fibroblasts and absent in XP-C fibroblasts. Until now, CLEC2A had only been identified in differentiated keratinocytes and in U-937 monocytic cell line. The crosstalk between dermal fibroblasts and NK cells is largely unexplored.

In this scientific paper, we dissected the role that the dermal expression of CLEC2A in cancer progression. We first showed that the expression of CLEC2A is also absent in CAFs and that SCC cells are able to downregulate CLEC2A expression in healthy fibroblasts through secreted factors. We then developed 3D organotypic NK immunocompetent models, to measure SCC invasion in the presence and absence of NK cells and blocking anti-CLEC2A antibody. We measured the invasion rate of SCC cells in WT, XP-C and CAF collagen matrices. In presence of NK cells, we only saw a decrease in the invasion rate in the WT condition, with fibroblasts expressing CLEC2A. When this interaction was blocked by anti-CLEC2A or did not occur because of loss of CLEC2A expression by XP-C fibroblasts or CAFs, SCC invasion rate was increased. Thus, we showed that CLEC2A/NKp65 interaction regulates NK cell-mediated control of SCC invasion by fibroblasts. The results of this article unveil a role for CLEC2A in skin immunosurveillance.
CLEC2A regulates natural killer cell and fibroblast-mediated control of cutaneous squamous cell carcinoma invasion

Maria Gonçalves-Maia¹, Miguel Basante¹a, Yannick Gache¹, Estelle Cosson², Emie Salavagione³, Margot Muller¹, Alexander Steinle³ François Bernerd⁴, Sébastien Schaub⁵, Véronique M. Braud², Thierry Magnaldo¹

¹Université Côte d'Azur, INSERM, CNRS, Institute for Research on Cancer and Aging, Nice, U1081, UMR7284, Nice, France
²Université Côte d'Azur, Centre National de la Recherche Scientifique, Institut de Pharmacologie Moléculaire et Cellulaire, UMR7275, Valbonne, Sophia Antipolis, France
³Institute for Molecular Medicine, Goethe-University Frankfurt am Main, Frankfurt am Main, Germany
⁴L'Oréal Research and Innovation, Aulnay sous Bois, France
⁵Université Côte d'Azur, CNRS, Inserm, Institut de Biologie Valrose, Nice, France

Present address:
¹aUniversité Côte d'Azur, CNRS, Inserm, Institut de Biologie Valrose, Nice, France

Corresponding author:
Thierry Magnaldo, DR CNRS
UMR CNRS 7284 INSERM U1081 UNS
Institute for Research on Cancer and Aging, Nice
Faculté de médecine, 28, Avenue de Valombrose
06107 Nice Cedex 2, FRANCE
Phone: 33 (4) 93 37 76 70 (office)
Email: thierry.magnaldo@univ-cotedazur.fr

The authors declare no conflict of interest.
This article is a PNAS Direct Submission
Abstract

The ability of cancer cells to invade and disseminate can be affected by components of the surrounded microenvironment particularly fibroblasts and immune cells. Xeroderma pigmentosum (XP) disease is a good model to study the impact of the ME in cancer progression. XP patients are more prone to develop skin cancers compared to the general population. XP-C fibroblasts, by themselves, do not develop skin cancers, but show pro-invasive phenotype similar to cancer associated fibroblasts (CAFs) from the general population. To identify dermal components regulating the growth of skin cancers, we performed transcriptomic analysis of 3D-dermal cultures of fibroblasts from healthy and XP-C patients. Interestingly, we identified the expression of CLEC2A, the ligand of the activating Natural Killer (NK) cell receptor NKp65, in healthy but not in XP-C fibroblasts. CLEC2A was also absent in dermal CAFs and was downregulated by secreted factors of squamous cell carcinomas (SCC), suggesting that loss of CLEC2A is part of the transitioning process that WT fibroblasts undergo to become CAFs. Furthermore, the development of 3D organotypic models containing NK cells showed for the first time that CLEC2A/NKp65 interaction regulates NK cell-mediated control of SCC invasion by fibroblasts. Taken together, these data strongly suggest a major role of CLEC2A in skin immunosurveillance against cancer cells.

Significance statement

The influence of the interaction between fibroblasts and Natural Killer (NK) cells in cancer progression is poorly explored. In this report, we show that CLEC2A, a ligand of a NK activating receptor, is expressed in dermal fibroblasts from healthy patients and absent in fibroblasts from patients suffering from skin cancer-prone xeroderma pigmentosum disease and in sporadic cancer associated fibroblasts (CAFs). These findings indicate that CLEC2A expression decreases during the transition of fibroblasts to CAFs. Furthermore, using 3D NK immune competent organotypic cultures, we unveil a key regulation of SCC invasion by NK cells through a cross-talk with fibroblasts and a major role of CLEC2A in skin immunosurveillance against cancer cells.
**Introduction**

Skin cancer is the most frequent cancer worldwide and its prevalence has highly increased over the last thirty years (Bray, Ferlay et al. 2018). Patients suffering from xeroderma pigmentosum (XP), a rare genetic autosomal recessive disease caused by mutations on complementation genes involved in the nucleotide repair of DNA lesions after a UV stress, exhibit a very high propensity to develop cutaneous cancers (Kraemer, Lee et al. 1987). They develop 10,000 more basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) and 2000 more melanomas than the general population. In addition, XP-C patients, the most frequent complementation group in Europe and US, are highly susceptible to aggressive (SCC) skin cancers; however, mechanisms underlying the typology of XP-C skin cancer are not yet understood (Giglia, Dumaz et al. 1998).

Cancer progression depends on both mutations in cancer cells that favor their growth and spread and the characteristics of the surrounding environment and its cellular components (Fidler 2003). Within the tumor microenvironment (TME), fibroblasts represent the majority of non-tumor cells. There, they convert into cancer-associated fibroblasts (CAFs) (Kalluri and Zeisberg 2006). CAFs are irreversibly activated fibroblasts that change the architecture and composition of TME and are active stimulators of cancer growth and progression leading poor survival. Immune cells also infiltrate tumors and can have anti- and pro-tumoral functions. Natural Killer (NK) cells have been shown to interact with cancer cells both by direct contact and through chemokine and cytokine signaling. Their presence in the TME correlates with good prognosis in many types of cancers including cutaneous ones (Li, Simmons et al. 1998, Imai, Matsuyama et al. 2000, Ishigami, Natsugoe et al. 2000).

Interestingly, XP patients show specific phenotypes for both fibroblasts and immune cells that resemble those of CAFs and immunocompromised immune cells respectively (Norris, Limb et al. 1988, Mariani, Facchini et al. 1992, Bernerd, Asselineau et al. 2001). XP-C fibroblasts, isolated from healthy non-photo exposed areas promote keratinocyte invasion in organotypic cultures. They express other similarities with CAFs, like the overexpression of the hepatocyte growth factor (HGF) *(in preparation)* and Matrix Metalloproteinase 1 (MMP1) implicated in ECM remodeling (Bernerd, Asselineau et al. 2001, Frechet, Warrick et al. 2008, Cui, Wang et al. 2018). Immune abnormalities have also been identified in XP patients. Several studies from the late 80s / early 90s reported that NK cells from XP patients present a deficiency in their lytic activity, suggesting that NK cell dysfunction may contribute to cancer progression in these patients (Norris, Limb et al. 1988, Norris, Limb et al. 1990, Mariani, Facchini et al. 1992).

These observations suggest that XP disease can be a good model to study development and progression of skin cancers. To address this question, we investigated the role of XP-C fibroblasts, first by performing a transcriptomic analysis of three dimensional (3D)-dermal
cultures of fibroblasts from healthy (WT) and XP-C patients. Surprisingly, among the few differentially expressed genes, we identified the presence of \textit{CLEC2A}, the ligand of the activating NK receptor NKp65, in healthy fibroblasts and its absence in XP-C fibroblasts. The modulation of CLEC2A expression in fibroblasts unveil an important crosstalk between fibroblasts and NK cells in healthy and cancerous tissues.

\textbf{Results}

\textit{CLEC2A} differential gene expression in WT and XP-C fibroblasts from 3D-dermal cultures. In an attempt to identify dermal components regulating the growth of skin cancers in XP-C patients, we first examined the genome wide expression profile of six primary independent fibroblast cell lines (3 WT, 3 XP-C) cultured in 3D-collagen matrices. Principal component analysis (PCA) showed that XP-C fibroblasts displayed a distinct transcriptomic profile and higher heterogeneity compared to WT fibroblasts (Fig. 1A). Twelve genes were found upregulated and ten downregulated in WT fibroblasts compared to XP-C fibroblasts (Fig. 1B and Table S1). Among these genes, we identified the C-type lectin domain family 2 member A (\textit{CLEC2A}) as one of the genes that were the most significantly differentially expressed (Table S1). Two \textit{CLEC2A} transcripts variants were found expressed in WT but not in XP-C fibroblasts (Fig. 1C) (Spreu, Kienle et al. 2007). \textit{CLEC2A} variant 1 encodes for a cell surface molecule that interacts with the activating receptor, NKp65, expressed by NK cells (Spreu, Kuttruff et al. 2010). This finding suggested a putative cross-talk between fibroblasts and NK cells in the skin of healthy individuals which may dysfunction in XP-C patients.

Selective expression of CLEC2A at the cell surface of cutaneous primary fibroblasts from healthy individuals. To further document CLEC2A mRNA and protein expression in the skin, we analyzed a panel of WT and XP-C fibroblasts and keratinocytes that we isolated from healthy and XP-C skin biopsies (Otto, Riou et al. 1999). Using a set of primers that detects both \textit{CLEC2A1} together with \textit{CLEC2A2}, we confirmed that \textit{CLEC2A} transcripts only accumulated in WT fibroblasts and not in XP-C fibroblasts (Fig. 2A). In addition, when using primers specific for each variant, \textit{CLEC2A1} and \textit{CLEC2A2} mRNA were detected in WT fibroblasts and CLEC2A-expressing cell line U937 but not in XP-C fibroblasts (Fig. S1A) (Spreu, Kienle et al. 2007). Surprisingly, we could not detect \textit{CLEC2A} transcripts in basal keratinocytes from both WT and XP-C patients (Fig. 2A). Likewise, when we monitored CLEC2A protein expression at the cell surface by flow cytometry, we found CLEC2A solely expressed on WT fibroblasts (Fig. 2B and C). Using imaging flow cytometry, we found that CLEC2A was predominantly expressed at the cell surface (Fig. S1B). This suggested the accumulation of \textit{CLEC2A1} membrane related variant is predominant over the intracellular...
related variant CLEC2A2. Because XP-C patients bear deleterious mutation in the XPC gene, we considered the possibility of a direct implication of XPC protein in the regulation of CLEC2A expression. To test this hypothesis, we efficiently restored XPC expression in XP-C fibroblasts by retroviral transduction of a cassette containing XPC (Fig. S2A). We confirmed that transduced XP-C fibroblasts were able to perform DNA repair after UV radiation, using the measurement of unscheduled DNA synthesis (UDS) by incorporation of 5-ethynyl-2'-deoxyuridine (EdU) labeled nucleotides into the newly DNA patches that are synthetized upon DNA repair (Fig. S2B). Under these circumstances, however, expression of CLEC2A was not restored in XP-C reverted fibroblasts (Fig. S2C). Altogether, these data highlight a default of CLEC2A expression in XP-C patients that is not directly reverted after XPC re-expression.

**Downregulation of CLEC2A expression within SCC tumor microenvironment.** Because phenotypical similarities are observed between XP-C fibroblasts and CAFs, we investigated whether CLEC2A could be modulated in SCC microenvironment. We successfully isolated two different dermal CAF cell lines from human skin SCC that positively expressed α-SMA, consistent with a CAF phenotype (Fig.3A). Contrary to control (WT) fibroblasts, CLEC2A transcripts and protein could not be detected in these two dermal CAFs (Fig. 3B and C). These data suggested that the expression of CLEC2A in skin fibroblasts could be downregulated during tumor development upon transformation into CAFs. To test this hypothesis, we generated conditioned-medium from an SSC12 tumor cell line that is able to convert WT fibroblasts into CAFs in vitro, as shown by the moderate induction of α-SMA on SCC12-conditioned medium (SSC12_CM)-treated WT fibroblast (Fig. S3A). We then studied the impact of factors secreted by SCC12 tumor cell line on CLEC2A expression. SCC12_CM was added to the culture medium of WT fibroblasts. After 5 days of incubation with SCC12_CM, the expression of CLEC2A in WT fibroblasts was found downregulated both at mRNA and protein level (Fig.4 A and B). By contrast, no significant modulation in CLEC2A expression was seen after incubation of WT fibroblasts with conditioned-media from WT keratinocytes and fibroblasts (Fig. S3B and C). Furthermore, as TGF-β is one major driver of the transition of WT fibroblasts into CAFs, we checked the possible role of TGF-β in the modulation of CLEC2A. Supplementation of the culture medium with TGF-β did not decrease CLEC2A mRNA transcripts while it induced α-SMA expression (Fig. S4A and B). Moreover, neutralizing TGF-β in SCC12_CM by addition of an anti-TGF-β did not block CLEC2A downregulation triggered by SCC12_CM (Fig. S4A). We conclude that TGF-β is not the secreted factor responsible for the downregulation of CLEC2A expression in WT fibroblasts.
CLEC2A regulates SCC invasion through a cross-talk between fibroblasts and NK cells.

To determine whether the loss of CLEC2A has a real impact on cancer progression, we developed a 3D-immunocompetent organotypic culture model to measure SCC12 invasion in the presence or absence of NK cells. We generated a NK cell line that killed SCC12 and not the WT and XP-C fibroblasts that we used in the 3D-dermal cultures (Fig. S5A and B). Interestingly, SCC12 cells, like WT and XP-C fibroblasts, express MHC-I molecules. Killing of SCC12 may therefore result from abundant expression of ligands of activating NK receptors, however, they do not express CLEC2A (Fig. S6B). Thanks to our newly developed software Epidepth, we were able to analyze SCC12 invasion rates in different conditions (Fig 5C). Our results showed that the presence of NK cells significantly reduced the levels of SCC12 invasion in a WT 3D-dermal culture but failed to limit SCC12 invasion rates in a XP-C 3D-dermal culture (Fig. 5 A and C). Furthermore, when we added the anti-CLEC2A blocking antibody OMA1 to the WT 3D-dermal immunocompetent culture, the levels of SCC12 invasion returned to those of the condition without NK cells (Fig 5 A and C). This was not the case when the isotype Ig was added (Fig 5 A and C). This revealed a direct and positive impact of CLEC2A expression on WT fibroblasts in controlling SCC12 invasion via NK cells. Additionally, the same experiment was performed with CAF 3D-dermal culture and results showed that, like what happened with XP-C dermal equivalents, the presence of NK cells had no impact in SCC12 invasion rates. These results indicate that the cross-talk between fibroblasts and NK via CLEC2A interaction with NKp65 plays a role in the regulation of SCC invasion and progression.

Discussion

Our findings uncover a key role for CLEC2A in immunosurveillance of the skin through a cross-talk between fibroblasts and NK cells. We show that CLEC2A/NKp65 interaction regulates NK cell-mediated control of SCC cancer invasion by WT fibroblasts. This regulation is disrupted in XP-C fibroblasts and sporadic CAFs. We propose loss of CLEC2A by CAFs as a novel tumor evasion mechanism.

The data described here show for the first time that CLEC2A is expressed in skin primary dermal fibroblasts of healthy individuals but is absent in skin primary dermal fibroblasts of cancer-prone XP-C patients. Previous RNA-seq analysis comparing skin WT and XP-C fibroblasts isolated by enzymatic digestion and cultured in classic two-dimension cultures didn’t reveal a difference in CLEC2A expression (Chacon-Solano, Leon et al. 2019). This suggests that spatial structure of fibroblasts interfere with the expression of genes as pointed by other studies (Duval, Grover et al. 2017, Tolle, Gaggioli et al. 2018).

We also did not find expression of CLEC2A in basal keratinocytes from both WT and XPC patients. Others, previously reported expression of CLEC2A in primary keratinocytes isolated
from skin (Spreu, Kuttruff et al. 2010). Whether CLEC2A is specifically expressed at some stages of keratinocytes’ differentiation deserves further studies.

Understanding the mechanisms through which CLEC2A is modulated in WT dermis is a key issue to decipher the consequences of its absence in XP-C dermis. Mutations in XPC gene are mostly deleterious (Stary and Sarasin 2002) and, beyond NER, XPC has been suggested to have a role in the regulation of transcription in absence of genetic attack (Le May, Mota-Fernandes et al. 2010). We checked whether the lack of XPC could directly lead to loss of CLEC2A. Our results demonstrate that the absence of CLEC2A expression in XP-C fibroblasts is not directly (nor immediately) dependent on the expression of XPC. The evidences that XP-C fibroblasts behave like CAFs, enhancing epidermal invasion, even in absence of cancerous cells (Bernerd, Asselineau et al. 2001) prompted us to look into the expression of CLEC2A in human dermal skin CAFs. The absence of CLEC2A expression in dermal CAFs, similarly to XP-C fibroblasts strengthens relationships between the two and suggests that loss of CLEC2A facilitates somehow, the evasion of cancer cells. In agreement with this hypothesis, we were able to demonstrate that SCC cells can downregulate CLEC2A expression through still unidentified secreted factors. This data strongly supports that CLEC2A downregulation is part of the transitioning process that WT fibroblasts undergo to become CAFs.

Since the identification of CLEC2A expression in skin (Spreu, Kienle et al. 2007), studies have shown that CLEC2A is able to trigger NK cell cytotoxicity in vitro through the engagement of NKp65 receptor (Spreu, Kuttruff et al. 2010, Li, Wang et al. 2013, Bauer, Spreu et al. 2015). However, no study until now explored the role of CLEC2A in a more general context of immunosurveillance in the skin. Our results suggest that CLEC2A could facilitate the elimination of cancer cells by NK cells at early tumorigenesis stages, when fibroblasts are still expressing CLEC2A and have not shifted their phenotype to a CAF phenotype.

Materials and Methods

Tissues and cells. Normal human skins were collected from mammary or abdominal plastic resection surgery. XP skin biopsies were collected from non-sun-exposed and normally pigmented sites. Skin SCC biopsies were collected from patients undergoing surgical excision. All samples were obtained with the patient's or parents' informed consent according to the Declaration of Helsinki under local French ethic committee (CCPRPB: CSET935) or under institutional review board (Hôpital Pasteur, Centre Hospitalier Universitaire de Nice, France) approval. Human keratinocytes and fibroblasts from healthy, XP-C or cancer patients were obtained and cultured as described (Rheinwald and Green 1975, Otto, Riou et al. 1999). NK
cell lines were generated from human blood purchased from the Etablissement Français du Sang (Marseille, France), as previously described (Fassy, Immunology, 2017).

Cells are described in detail in SI Table S2.

**3D-skin cultures.** For transcriptomic analysis, fibroblasts \((1 \times 10^6)\) were cultured in a tridimensional type I collagen matrix at a concentration of \(2.5 \times 10^5 \text{ cells/mL}\) for 4 days. After that, the matrix was cultured 7 days in immersion and then 7 days at the air-liquid interface as described in (Bernerd, Asselineau et al. 2001). For 3D immunocompetent models, fibroblasts \(\left(5 \times 10^5\right)\) were cultured for 24 h in a tridimensional type I collagen matrix at a concentration of \(2.5 \times 10^5 \text{ cells/mL}\). Then SCC12 cells \(\left(2.5 \times 10^5\right)\) were dissociated and then seeded on the top of the matrix and the organotypic construct were cultured 48 h in immersion and then 5 days at air-liquid interface. To incorporate NK cells into the model, we cultured NK cells \(\left(5 \times 10^5\right)\) in a type I collagen matrix at \(5 \times 10^5 \text{ cell/mL}\) for 24 h in a transwell insert (Corning) and then added the fibroblasts-SCC12 construct on the top at air-liquid interface allowing NK cells to migrate up to “dermal” compartment for 48 hours (Bernerd, Asselineau et al. 2001, Valin, Barnay-Verdier et al. 2009).

**RNA extraction.** 3D-dermal cultures were frozen in liquid nitrogen, and then disrupted and solubilized in Qiazol reagent (Qiagen) using a gentleMACS™ Dissociator (Miltenyi Biotech) with M tubes and the RNA_02 program recommended by the manufacturer (Miltenyi Biotech). Total RNAs were extracted using miRNeasy Mini kit (Qiagen) (3D-dermal cultures) or mRNeasy Mini kit (Qiagen) (cells) and then purified using the RNA cleanup and concentration kit (Qiagen).

**Whole genome microarray analysis.** The integrity of the RNA was assessed using an Agilent BioAnalyzer 2100 (Agilent Technologies). Amplification and labeling was performed with the LowInput QuickAmp Labeling Kit (Agilent Technologies). cRNA were then hybridized on \(8 \times 60\text{K}\) high density SurePrint G3 human gene expression Agilent microarrays according to manufacturer's instructions (Agilent Technologies). The microarray experimental data were deposited in the NCBI GEO under the serial record number (to do). The data were quantile normalized using the Bioconductor package limma. Means of ratios from all comparisons were calculated and the moderated t-statistic of the limma package provided the per gene P values.

**Quantitative real-time PCR.** mRNA levels were determined by RT-PCR after DNase I digestion and reverse transcription using SuperScript II (Thermo Fisher Scientific). For quantitative RT-PCR, cDNA was amplified with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOne RT-PCR thermal cycler (Applied Biosystems) following the manufacturer’s instructions and data analysis was performed by \(\Delta\Delta CT\) method for relative quantification. SB34 and 36B4 were both used for gene-expression data normalization. Each
The experimental condition was performed in triplicate. The following primers were used: CLEC2A (5′-ataccagaaattgacacc-3′/5′-gatgtggtgccatttctc-3′), 36B4 (5′-tgcatgatccccattc-3′/5′-aggcagatggatcagccaaga-3′), SB34 (5′-gcatgatccccattctatcat-3′/5′-aggttaatccgtctccacaga-3′).

**Tissue histology.** 3D organotypic tissues were fixed in Antigen-Fix reagent (Diapath) for 1 h at 4°C and incubated in 34% sucrose for 16 h at 4°C prior being embedded in OCT (Tissue-Tek) and immediately frozen in liquid nitrogen. Hematoxylin and eosin staining were performed by standard protocol in 5 µm thickness OCT sections.

**Immunofluorescence staining.** Cells were fixed in 3% paraformaldehyde for 20 min at RT and permeabilized with 0.5% Triton X-100 for 5 min. Following a blocking step, cells were incubated with purified Mo anti-α-SMA (1/400) (Sigma Aldrich) for 1 h at RT. After washing, antibody binding was detected with A488-conjugated anti-Mo IgG secondary antibody (1/400) (Thermo Fisher Scientific). The coverslips were mounted onto glass slides using mounting media containing DAPI (Southern Biotech) and images were acquired using a Zeiss Axio Observer.Z1 fluorescent microscope.

**Flow Cytometry.** Cells were first incubated in a blocking solution to saturate Fc receptors and then incubated with 10µg/mL purified Mo anti-CLEC2A (OMA1) kindly provided Prof. Dr. Alexander Steinle (Frankfurt, Germany) or Mo IgG2a control isotype (BD Biosciences) for 30 min at 4°C. After washing, antibody binding was detected using A488-conjugated goat anti-Mo mAb (1/800) (BD Biosciences). Fluorescence staining was analyzed on a FACScalibur (BD Biosciences) unit using CellQuest Pro Software (BD Biosciences).

**Conditioned media preparation.** Cells were grown to 70% confluence, washed twice with PBS and then incubated in serum-free medium at 37°C. After 48 h, culture medium was collected, centrifuged at 5000g for 5 min to remove cell debris and the supernatant stored at -80°C. These conditioned media or control serum free medium were used to stimulate fibroblasts for five days. In some experiments, blocking antibodies were added in the last 48 h.

**Epidepth software.** We analyzed the images with Epidepth2.1, a program dedicated to skin sample analysis. Written in Matlab (The MathWorks, Inc.) with a user-friendly interface, it automatically segments each image between the dermis, the epidermis and the invasions (in contact or not with the epidermis). Then the program automatically extracts statistical parameters to quantify the image (see Sup Data for details) (to do). More specifically, we propose an “Invasion Rate” as:
\[ IR = \frac{1}{L_{epi}} \sum_{j \in inv} dist_{epi}(j) \]

where \( L_{epi} \) is the epiderm length, \( dist_{epi}(j) \) is the distance from the epiderm for all pixel "j" in an invasion. Measured in \( \mu m^2 \), this index has the advantage to take into account both the invasion sizes and how deep they migrate in the dermis. The program also manages the image library, providing statistics per experiment and per conditions. For this project we analyzed 343 images and 4390 invasions (~1s per image). On request, this program is freely available (as an executable).

**Statistical Analysis.** Statistical analyses were performed using GraphPad Prism 8.0 software. Each statistical test is specified in the text. A \( P \) value <0.05 was considered as evidence for statistical significance.

**Acknowledgements**

We thank Dr. O. Camuzard (Hôpital Pasteur, Centre Hospitalier Universitaire de Nice, France) for providing biopsies for CAF skin extractions, and F. Tessier (supported by Canceropôle PACA) for bioinformatics assistance. The authors acknowledge the IPCM’s Genomic core facility and IRCAN’s Cytometry (Cytomed), Microscopy (PICMI) Histology and Genomic core facilities.

This work was supported by the French Government (National Research Agency, ANR; CNRS; INSERM) through the ‘Investments for the Future’ LABEX SIGNALIFE: program reference # ANR-11-LABX-0028-01; UCA (Université Côte d’Azur) and Association René Tourraine. MGM was supported by the Fondation de l’Avenir (PhD fellowship 2015) and LABEX SIGNALIFE; TM was supported by the Fondation ARC (SFI201212055859), the Fondation de l’Avenir, Société Française de Dermatologie, and The Institut National du Cancer. VMB was supported by Centre National de la Recherche Scientifique, Cancéropole PACA, Région Provence-Alpes-Côte d’Azur, Institut National du Cancer and Fondation ARC pour la recherche sur le Cancer.
References


Figures

Fig. 1. Transcriptomic analysis of WT and XP-C fibroblasts cultured in 3D collagen matrices. (A) Principal component analysis (PCA) showing the clustering of WT and XP-C fibroblasts in two groups. (B) Volcano plot highlighting the 22 differentially expressed genes (DEG) between WT and XP-C fibroblasts. On the upper left quadrant, upregulated mRNAs in WT fibroblasts and on the upper right quadrant, downregulated mRNAs in WT fibroblasts are shown. (C) Boxplot of log2 intensity CLEC2A variant 1 and mRNA levels in WT (n=3) and XP-C (n=3) fibroblasts.

Fig. 2. CLEC2A expression in primary fibroblasts isolated from healthy and XP-C individuals. (A) Relative CLEC2A mRNA transcript levels in WT and XP-C fibroblasts (F-WT, F-XP-C), and in WT and XP-C basal keratinocytes (K-WT, K-XP-C) determined by quantitative RT-PCR. Data represent mean ± SD of five cell lines per group. ***p < 0.001 Mann-Whitney U-test. (B) Representative histograms of CLEC2A expression in WT and XP-C fibroblasts and in WT and XP-C keratinocytes determined by flow cytometry. (C) Frequency of CLEC2A positive cells in WT and XP-C fibroblasts and keratinocytes as determined by flow cytometry. Data are representative of three independent experiments with five cell lines per group. ***p < 0.001 Mann-Whitney U-test.

Fig. 3. CLEC2A expression in dermal CAFs. (A) α-SMA and DAPI immunofluorescence staining of WT and XP-C fibroblasts and primary isolated dermal CAFs. Data are
representative of three independent experiments. (B) Relative CLEC2A mRNA transcript levels in WT fibroblasts and dermal CAFs determined by quantitative RT-PCR. Data represent mean ± SD of three independent experiments. ***\( p \leq 0.001 \) Mann-Whitney \( U \)-test. (C) Representative histograms of CLEC2A expression in WT fibroblasts and dermal CAFs determined by flow cytometry. Three independent experiments were performed.

**Fig. 4.** CLEC2A expression in WT fibroblasts stimulated by SCC12-conditioned medium (SCC12_CM) (A) Relative CLEC2A mRNA transcripts levels in WT fibroblasts stimulated by SCC12_CM for 5 days. Data represent mean ± SD of five different SCC12_CM. **\( p \leq 0.01 \) Mann-Whitney \( U \)-test. (B) Representative histograms of CLEC2A expression on WT fibroblasts stimulated or not by SCC12_CM as determined by flow cytometry. Three independent experiments were performed.

**Fig. 5.** 3D immunocompetent model that assess the impact of CLEC2A on the ability of NK cells to control invasion of SCC12 cells in WT, XP-C and CAF 3D-dermal cultures. (A) Representative images of hematoxylin and eosin (H&E) staining of SCC12 cell invasion in WT and XP-C 3D-dermal cultures in absence or presence of NK cells (first and second line), anti-CLEC2A blocking antibody (OMA1) (third line), and isotype Ig (fourth line). (B) Representative images of H&E staining of SCC12 cell invasion in CAF 3D-dermal culture in absence or presence of NK cells. (C) Quantification of SCC12 invasion rate in the aforementioned conditions in box plots. ***\( p \leq 0.001 \) Tukey’s test in one-way ANOVA. Two independent experiments were performed.
Figure 1

Individuals PCA

Groups

WT
XP

PC1: 20.9%
PC2: 31.9%

Figure 2

A

Relative CLEC2A mRNA transcript levels (fold)

F-WT F-XP-C K-WT K-XP-C

B

Counts

FTM1_WT AS798_XP-C KTM1_WT KAS798_XP-C

CLEC2A

IgG2a isotype CLEC2A

C

% of CLEC2A+ cells

Figure 3

A

α-SMA+ DAPI

FH122_WT AS798_XP-C FTM23_CAF FTM24_CAF

B

Relative CLEC2A mRNA transcript levels (fold)

FTM5_WT FTM23_CAF FTM24_CAF

C

Counts

CLEC2A

IgG2a isotype CLEC2A
Figure 4

![Graph showing relative CLEC2A mRNA levels.](image)

Figure 5

A

<table>
<thead>
<tr>
<th>SCC12 FH84_WT</th>
<th>SCC12 AS148_WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK +</td>
<td>NK +</td>
</tr>
<tr>
<td>NK + OMA1 +</td>
<td>NK + OMA1 +</td>
</tr>
<tr>
<td>NK + IgG2a +</td>
<td>NK + IgG2a +</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>SCC12 FTM23_CAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK +</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>FH84_WT</th>
<th>FH84_WT + NK</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS148_XP-C</td>
<td>AS148_XP-C + NK</td>
</tr>
<tr>
<td>FTM23_CAF</td>
<td>FTM23_CAF + NK</td>
</tr>
</tbody>
</table>

**Note:** Relative CLEC2A mRNA transcript levels (fold)
Supplementary Information for

CLEC2A regulates natural killer cell and fibroblast-mediated control
of cutaneous squamous cell carcinoma invasion

Maria Gonçalves-Maia¹, Miguel Basante¹a, Yannick Gache¹, Estelle Cosson², Emie Salavagione², Margot Muller¹, Alexander Steinle³, Françoise Bernerd⁴, Sébastien Schaub⁵, Véronique M. Braud⁶, Thierry Magnaldo¹

Dr. Thierry Magnaldo

Email: Thierry.magnaldo@univ-cotedazur.fr

This PDF file includes:

- Supplementary text
- Figs. S1 to S6
- Tables S1 to S2
- References for SI reference citations
Supplementary Information Text

Epidepth. to do

Supplementary Materials and Methods

Cell lines. U937 (ATCC; CRL-1593.2) and K562 (ATCC; CCL-243).

Quantitative RT-PCR. Quantitative RT-PCR were performed using the following primers: CLEC2A1 (5'-tccatcggttagcgccttg-3'/5' acagaatagtgagaagccact-3), CLEC2A2 (5'-atgattaatccagagctgcgg-3'/5'-tctaaggggtcccagcag-3').

Flow cytometry and imaging flow cytometry. Cells were stained with purified Mo anti-MHCI (DX17, kindly provided by L. Lanier, UCSF, USA, and W6/32, BD Biosciences) followed by PE-conjugated goat anti-Mo mAb (1/50) (BD Biosciences). Fluorescence staining was analyzed on a FACScalibur (BD Biosciences) unit using CellQuest Pro Software (BD Biosciences). Imaging flow cytometry analysis were performed on an ImageStreamX MKII operated by INSPIRE software (Amnis Corporation). Cells were permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences) and stained with purified Mo anti-CLEC2A (OMA1) followed by A488-conjugated goat anti-Mo (BD Biosciences). Data analysis was performed using the IDEAS software (Amnis Corporation).

Retroviral transduction and purification. Fibroblasts were seeded at a density of 8,000 cells/cm² and were transduced with concentrated RV supernatants in serum-free medium for 16 h. As CD24 gene was present in the retroviral vector cassette, transduced cells were purified by flow cytometry cell sorting using FACSaria III (BD Biosciences) following staining with Mo anti-CD24-PE (1/10) (Beckman Coulter) (Warrick, Garcia et al. 2012).

Western blot analysis. Total proteins were extracted from fibroblasts in 8 mol/l urea buffer and protein concentrations were determined by Bradford assay (Bio-Rad). 30µg of protein for each condition were separated on 8% SDS–polyacrylamide gels and transferred onto a polyvinyl difluoride membrane (GE Healthcare). Membranes were blocked and incubated with rabbit polyclonal anti-XPC (dilution 1/1,000; Bethyl), or anti-GAPDH (dilution 1/2,000; Abcam). After washing, membranes were stained with goat anti-rabbit HRP (1/5000; Bethyl) or rabbit anti-mouse (1/5000; Dako) HRP respectively. Blots were revealed using electrochemiluminescence reagents (GE Healthcare).

EdU incorporation. To access EdU incorporation 2 h after 500 J/cm² of UVB irradiation the EdU staining the Click-IT Alexa Fluor 488 Imaging Kit (Molecular Probes Invitrogen) was used following the manufacturer's instructions. EdU-stained cells were mounted in standard mounting media and images were acquired a Zeiss Axio Observer.Z1 fluorescent microscope.
**TGF-β1 and α-TGF-β1,2,3 stimulations.** Fibroblasts were stimulated with 2ng/ml TGFβ1 (Peprotech) for 48 h in serum-free medium or with 10µg/mL α-TGF-β1,2,3 neutralizing antibody (R&D) for 48 h in SCC12_CM.

**Real-time NK cell cytotoxic assay.** A real-time cytotoxic assay was performed as previously described (Fassy, Tsalkitzi et al. 2017, Fassy, Tsalkitzi et al. 2017). Briefly, target cells were labelled with 0.5 µM Calcein-AM (Molecular Probes) for 15 min at room temperature. Fibroblasts FH84_WT and AS148_XP-C were additionally treated with 100 µM Indomethacin (Sigma Aldrich) to block multidrug-resistance transporters that expulse calcein. The inhibitor was maintained in the medium during the assay. Calcein-labelled targets were incubated with NK cell lines for 4 h at 37°C, 5% CO₂ and real-time monitoring of NK cell killing was performed on a Cytation™ 5 (Biotek). Cell images were processed using GEN5 software (Biotek). The percentage of lysis from triplicates was calculated as follow: % lysis = \( 1 - \left( \frac{(\text{experimental well at } t/\text{experimental well at } t_0)}{(\text{control well at } t/\text{control well at } t_0)} \right) \) x 100.
Fig. S1. CLEC2A expression in fibroblasts. (A) CLEC2A transcript variants were amplified by RT-PCR in FH122_WT fibroblast (lane 1), U937 cell line (lane 2), AS433_XP-C fibroblast (lane 3), RT negative control (lane 4), PCR buffer negative control (lane 5). (B) CLEC2A expression in FH84_WT fibroblasts, determined by imaging flow cytometry. Data is representative of two independent experiments.
**Fig. S2.** Expression of CLEC2A in XPC-reverted fibroblasts. (A) Expression of XPC protein in WT, XP-C and transduced XP-C fibroblasts determined by Western blot: FTM1_WT fibroblast (lane 1), FH84_WT fibroblast (lane 2), AS433_XP-C fibroblast (lane 3), AS433+XPC reverted fibroblast (lane 4), AS798_XP-C fibroblast (lane 5), AS798+XPC reverted fibroblast (lane 6). Anti-GAPDH antibody was used as a loading control. (B) Measurement of UDS by EdU incorporation 2 h after 500 J/cm² UVB in WT, XP-C and transduced XP-C fibroblasts shows that genetically corrected XP-C fibroblasts recovered full NER capacity. (C) Relative CLEC2A mRNA transcripts levels in WT, XP-C, and XPC-reverted fibroblasts determined by quantitative RT-PCR. Data represent mean ± SD of two independent experiments. ***p <0.001 Mann-Whitney U-test.
**Fig. S3.** Modulation of CLEC2A expression in WT fibroblasts stimulated by WT fibroblasts or keratinocytes-conditioned medium for five days. (A) α-SMA immunofluorescence staining of WT fibroblasts after stimulation with SCC12_CM. (B and C) Relative CLEC2A mRNA transcript levels in WT fibroblasts stimulated or not with (B) WT keratinocytes_CM and (C) WT fibroblasts_CM, as determined by quantitative RT-PCR.
Fig. S4. CLEC2A expression in WT fibroblasts after TGF-β1 stimulation. (A) Relative levels of CLEC2A mRNA transcripts in WT fibroblasts after stimulation with TGF-β1 or SCC12_CM with or without TGF-β neutralizing antibody determined by quantitative RT-PCR. ns, Mann-Whitney U-test. (B) α-SMA immunofluorescence staining of WT fibroblasts after stimulation with TGF-β1. Data are representative of two independent experiments.
Fig. S5. NK cell-mediated cytotoxicity assay. (A) Time-course of NK cell-mediated lysis of SCC12 cells and K562 erythroleukemic target cells. (B) Time-course of NK cell-mediated lysis of WT and XP-C fibroblasts and K562 target cells in presence of indomethacin (100µM).
Fig. S6. MHC-I and CLEC2A expression in WT, XP-C fibroblasts and SCC12 cells determined by flow cytometry.  (A) MHC I cell surface expression measured using anti-MHC I antibodies, clone DX17 and W6/32 compared to isotype Ig controls.  (B) CLEC2A expression on SCC12 cells using OMA1 antibody compared to isotype Ig control.
<table>
<thead>
<tr>
<th>GeneName</th>
<th>Systematic Name</th>
<th>accs</th>
<th>accs</th>
<th>accs</th>
<th>wt fmr5</th>
<th>wt fmr1</th>
<th>wt fmr12</th>
<th>baseMean xpc</th>
<th>baseMean wt</th>
<th>logFC xpc</th>
<th>Adj P val Xpc wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSP07</td>
<td>NM_0027974</td>
<td>8.2560</td>
<td>7.8527</td>
<td>8.3432</td>
<td>11.5624</td>
<td>10.7654</td>
<td>11.4308</td>
<td>9.9650</td>
<td>11.2530</td>
<td>2.5866</td>
<td>0.0210</td>
</tr>
<tr>
<td>TRX5</td>
<td>NM_000192</td>
<td>5.8061</td>
<td>5.4688</td>
<td>5.8386</td>
<td>8.1331</td>
<td>8.4030</td>
<td>8.5422</td>
<td>5.7662</td>
<td>8.3585</td>
<td>2.8533</td>
<td>0.0159</td>
</tr>
<tr>
<td>GALT14</td>
<td>NM_0024572</td>
<td>7.9420</td>
<td>7.4639</td>
<td>7.8685</td>
<td>10.2728</td>
<td>9.9064</td>
<td>10.7702</td>
<td>7.7649</td>
<td>10.3175</td>
<td>2.5827</td>
<td>0.0362</td>
</tr>
<tr>
<td>TRX5A51</td>
<td>NR_034440</td>
<td>6.6025</td>
<td>8.1613</td>
<td>5.2728</td>
<td>7.1414</td>
<td>7.3742</td>
<td>7.4771</td>
<td>3.5495</td>
<td>7.3709</td>
<td>2.0459</td>
<td>0.0159</td>
</tr>
<tr>
<td>ENST00000369474</td>
<td>ENST00000369474</td>
<td>6.5157</td>
<td>6.6269</td>
<td>8.8031</td>
<td>8.2659</td>
<td>7.3931</td>
<td>7.7566</td>
<td>6.6486</td>
<td>7.9882</td>
<td>1.3217</td>
<td>0.0614</td>
</tr>
<tr>
<td>SGN1B</td>
<td>NM_196037</td>
<td>5.9619</td>
<td>6.2493</td>
<td>6.0716</td>
<td>7.2621</td>
<td>7.3750</td>
<td>7.5031</td>
<td>6.0443</td>
<td>7.3856</td>
<td>1.4795</td>
<td>0.0566</td>
</tr>
<tr>
<td>GREB1L</td>
<td>NM_00142888</td>
<td>7.1860</td>
<td>7.2188</td>
<td>7.2980</td>
<td>8.1826</td>
<td>8.2817</td>
<td>8.2392</td>
<td>7.2585</td>
<td>8.2345</td>
<td>0.9990</td>
<td>0.0532</td>
</tr>
<tr>
<td>PRDM16</td>
<td>NM_0021114</td>
<td>9.9021</td>
<td>8.8009</td>
<td>8.6777</td>
<td>5.5648</td>
<td>5.5949</td>
<td>5.6949</td>
<td>9.1410</td>
<td>5.5849</td>
<td>3.6597</td>
<td>0.0157</td>
</tr>
<tr>
<td>ENST00000424943</td>
<td>ENST00000424943</td>
<td>7.8891</td>
<td>7.7313</td>
<td>8.0557</td>
<td>5.2095</td>
<td>5.7835</td>
<td>5.7027</td>
<td>7.8923</td>
<td>5.5652</td>
<td>3.2702</td>
<td>0.0241</td>
</tr>
<tr>
<td>HOTAR</td>
<td>NR_047518</td>
<td>7.9124</td>
<td>7.4639</td>
<td>7.6399</td>
<td>5.3487</td>
<td>5.6073</td>
<td>5.3866</td>
<td>7.6811</td>
<td>5.4475</td>
<td>2.2335</td>
<td>0.0159</td>
</tr>
<tr>
<td>UNC00892</td>
<td>NR_015540</td>
<td>8.6455</td>
<td>6.7627</td>
<td>8.0999</td>
<td>5.4068</td>
<td>5.1531</td>
<td>5.4068</td>
<td>8.0332</td>
<td>5.3221</td>
<td>1.5173</td>
<td>0.0241</td>
</tr>
<tr>
<td>LOC80246</td>
<td>NR_020564</td>
<td>7.8328</td>
<td>7.6730</td>
<td>7.7894</td>
<td>6.4094</td>
<td>5.6825</td>
<td>6.7358</td>
<td>7.7554</td>
<td>6.5759</td>
<td>1.1796</td>
<td>0.0726</td>
</tr>
<tr>
<td>LOC100130372</td>
<td>AK127532</td>
<td>6.8857</td>
<td>6.8678</td>
<td>6.9327</td>
<td>6.0094</td>
<td>6.0715</td>
<td>5.8559</td>
<td>6.0504</td>
<td>5.9789</td>
<td>0.9265</td>
<td>0.0651</td>
</tr>
<tr>
<td>PDLIM7</td>
<td>NM_005451</td>
<td>12.1877</td>
<td>12.0746</td>
<td>12.2514</td>
<td>11.2963</td>
<td>11.2035</td>
<td>11.2083</td>
<td>12.1712</td>
<td>11.2054</td>
<td>0.9158</td>
<td>0.0614</td>
</tr>
<tr>
<td>PDLIM7</td>
<td>NM_005451</td>
<td>10.6617</td>
<td>10.6504</td>
<td>10.7061</td>
<td>8.9433</td>
<td>9.8206</td>
<td>9.9146</td>
<td>10.7024</td>
<td>9.8614</td>
<td>0.8480</td>
<td>0.0553</td>
</tr>
</tbody>
</table>

For RSP03, GREB1L, PDLIM7 two probe sets for the same gene sit side by side.
CLEC2A NM_001130711 corresponds to CLEC2A transcript variant 1; CLEC2A NM_207375 corresponds to CLEC2A transcript variant 2.
<table>
<thead>
<tr>
<th>Patients cells</th>
<th>Origin</th>
<th>Fibroblasts</th>
<th>Keratinocytes</th>
<th>Phenotype</th>
<th>XP-C mutation</th>
<th>Age at biopsy</th>
<th>Clinical characteristics</th>
<th>Onset of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM1</td>
<td>Caucasian</td>
<td>FTM1</td>
<td>KTM1</td>
<td>NA</td>
<td>NA</td>
<td>40 years</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FH29</td>
<td>Caucasian</td>
<td>FH29</td>
<td>X</td>
<td>NA</td>
<td>NA</td>
<td>30 years</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FH84</td>
<td>Black</td>
<td>FH84</td>
<td>X</td>
<td>NA</td>
<td>NA</td>
<td>28 years</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FMD</td>
<td>Caucasian</td>
<td>FMD</td>
<td>X</td>
<td>NA</td>
<td>NA</td>
<td>16 years</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>TM5</td>
<td>Caucasian</td>
<td>FTM5</td>
<td>KTM5</td>
<td>NA</td>
<td>NA</td>
<td>25 years</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FH122</td>
<td>Caucasian</td>
<td>FH122</td>
<td>X</td>
<td>NA</td>
<td>NA</td>
<td>19 years</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SK</td>
<td>Caucasian</td>
<td>X</td>
<td>SK</td>
<td>NA</td>
<td>NA</td>
<td>7 years</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>YF29</td>
<td>ND</td>
<td>X</td>
<td>YF29</td>
<td>NA</td>
<td>NA</td>
<td>newborn</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AS148</td>
<td>Caucasian</td>
<td>AS148</td>
<td>KAS148</td>
<td>XP-C</td>
<td>c.1643_1644delTG; p.Val548Alafs25</td>
<td>12 years</td>
<td>Multiple BCCs and SCCs; face and exposed areas. Several SCC and 1 BCC on the face</td>
<td>9 years</td>
</tr>
<tr>
<td>AS188</td>
<td>Caucasian</td>
<td>AS188</td>
<td>KAS188</td>
<td>XP-C</td>
<td>c.1643_1644delTG; p.Val548Alafs25</td>
<td>7 years</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AS373</td>
<td>Caucasian</td>
<td>X</td>
<td>KAS373</td>
<td>XP-C</td>
<td>c.1643_1644delTG; p.Val548Alafs25</td>
<td>4 years</td>
<td>1 facial BCC</td>
<td>2 years</td>
</tr>
<tr>
<td>AS399</td>
<td>Caucasian</td>
<td>X</td>
<td>KAS399</td>
<td>XP-C</td>
<td>ND</td>
<td>9 years</td>
<td>ND</td>
<td>5 years</td>
</tr>
<tr>
<td>AS433</td>
<td>Caucasian</td>
<td>AS433</td>
<td>X</td>
<td>XP-C</td>
<td>c.1643_1644delTG; p.Val548Alafs25</td>
<td>2 months</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AS629</td>
<td>Caucasian</td>
<td>X</td>
<td>KAS629</td>
<td>XP-C</td>
<td>c.1643_1644delTG; p.Val548Alafs25</td>
<td>8 years</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AS728</td>
<td>Caucasian</td>
<td>X</td>
<td>KAS728</td>
<td>NA</td>
<td>NA</td>
<td>37 years</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>AS798</td>
<td>Caucasian</td>
<td>AS798</td>
<td>KAS798</td>
<td>XP-C</td>
<td>c.1643_1644delTG; p.Val548Alafs25</td>
<td>2 years</td>
<td>Photoaging (hands), ephelides, Actinic keratosis</td>
<td>NA</td>
</tr>
<tr>
<td>AS875</td>
<td>Caucasian</td>
<td>AS875</td>
<td>X</td>
<td>XP-C</td>
<td>ND</td>
<td>20 years</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TM23</td>
<td>Caucasian</td>
<td>FTM23</td>
<td>X</td>
<td>NA</td>
<td>NA</td>
<td>82 years</td>
<td>SCC in left ankle</td>
<td>ND</td>
</tr>
<tr>
<td>TM24</td>
<td>Caucasian</td>
<td>FTM24</td>
<td>X</td>
<td>NA</td>
<td>NA</td>
<td>96 years</td>
<td>SCC</td>
<td>ND</td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not determined
References


Materials and Methods
This page was intentionally left blank
Otherwise indicated, for cell culture conditions, cell descriptions, characteristics, Flow Cytometry, quantitative real-time RT-PCR, Western blot and statistical analysis, please refer to Material and Methods of the “Scientific Article” on page 90.

**Table 4. Patients and cell descriptions**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patients</th>
<th>Age at Biopsy (years)</th>
<th>Ethnic origin</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP-D</td>
<td>AS204</td>
<td>15</td>
<td>Caucasian</td>
<td>XPD R683W</td>
</tr>
<tr>
<td>NBCCS</td>
<td>AS537</td>
<td>42</td>
<td>Caucasian</td>
<td>PATCHED1 c.1762supG; p.Val588GlyfsX39</td>
</tr>
<tr>
<td></td>
<td>AS587</td>
<td>43</td>
<td>Caucasian</td>
<td>PATCHED1 c.1366del; p.Thr456ProfsX35</td>
</tr>
</tbody>
</table>

**Lipopolysaccharide (LPS) stimulation.** Fibroblasts cultured at 70% of confluence were treated with 200ng/mL of LPS (# 0111:B4, Sigma-Aldrich, Lyon, France) in 0.5% fibroblast DMEM 10% FBS cell culture medium for different periods of time.

**Indirect immunofluorescence analysis.** Cells were grown on glass coverslips, fixed in 4% paraformaldehyde / 0.25% glutaraldehyde for 15 min at room temperature, rinsed twice in PBS and treated in PBS, 0.25% Triton X-100 for 5 min. Cells were next blocked in PBS, 3% BSA for 30 min and incubated for 1 h with mAb anti-CLEC2A OMA6 in blocking buffer (PBS / 0.5% BSA). After two washes in PBS, cells were incubated in the presence of Alexa Fluor 488-conjugated goat α-mouse secondary antibody for 1 h. Coverslips were then rinsed twice with PBS and mounted (Fluoromount-G, 0100-20, Southern Biotech). Images were acquired using a Zeiss Axio Observer Z1 fluorescent microscope.

**Ultrastructural analysis.** Cells were fixed in 1.6% glutaraldehyde in 0.1 M PBS, rinsed in 0.1 M cacodylate buffer and post-fixed for 1 h in 1% osmium tetroxide and 1% potassium ferrocyanide in 0.1 M cacodylate buffer. Cells were rinsed in distilled water, dehydrated in in increasing concentrations of alcohol and lastly embedded in epoxy resin. Contrasted ultrathin sections (70 nm) were analyzed under a JEOL 1400 transmission electron microscope mounted with a Morada Olympus CCD camera.

**Population doubling (PD) assay.** Fibroblasts were seeded at a density of 6600 cells/cm2 in DMEM, 10% SVF. At 70-80 % confluence, cells were treated with trypsin / EDTA and the
number of cells counted before pursuing with flow cytometry analysis. PD was calculated using the formula PD=1/2log2 (A / B), where A is the number of harvested cells and B is the number of plated cells.

**Senescence-associated β-galactosidase staining.** Senescence-associated β-galactosidase staining was conducted to detect cellular senescence using a Senescence beta–Galactosidase Staining Kit (# 9860, Cell Signaling) according to the manufacturer’s protocol.

**Antibodies.** Mouse monoclonal (mAb) anti-CLEC2A antibody OMA6 was kindly provided by Prof. Dr. Alexander Steinle (Frankfurt, Germany) and was used at 10µg/mL for flow cytometry and indirect immunofluorescent analysis; Alexa Fluor 488-conjugated goat α-mouse antibody (#A-11029, Thermo Fisher Scientific) was used at 1/1000 for flow cytometry analysis and at 1/400 for indirect immunofluorescent analysis; mAb anti-p16 (# 554079, BD Bioscience) was used at 1/1000; mAb anti-p21 (clone EA10, #33-7000, Thermo Fisher Scientific) was used at 1/1000; mAb anti β-tubulin (# 86298, Cell Signaling) was used at 1/1000.
Results
This page was intentionally left blank
A. Modulation of CLEC2A expression in aging and infection

As infection and aging are two “conditions” associated with changes in the immune system we decided to evaluate their impact in the expression of CLEC2A in healthy WT fibroblasts.

We started with infection. Because skin is an organ that is particularly exposed to microorganisms, we stimulated WT fibroblasts with lipopolysaccharide (LPS), a component present in the cell walls of Gram-negative bacteria. CLEC2A mRNA transcripts levels were transiently upregulated followed the LPS stimulation. However, by immunofluorescent analysis, we only detected intracellular levels of CLEC2A that transiently accumulate in small spots and disappeared. To determine the nature of these spots we performed electron microscopy analysis in WT fibroblasts with or without LPS stimulation and identified these spots as lysosomes. In parallel, we also performed the same electron microscopy experiment with XP-C fibroblasts. Our results reveal that XP-C fibroblasts do not accumulate lysosome after a LPS stimulation and, the presence of lipid droplets that we saw in their cytoplasm unveil a possible deregulation in XP-C lipid metabolism.

To investigate a possible modulation of CLEC2A during aging we looked into the expression of CLEC2A in three primary cell lines of WT fibroblasts during replicative senescence. Our results show a progressive decrease in the surface levels of CLEC2A during the consecutive propagation of fibroblasts. We stopped our experiment when saw decline in cellular replication of our cell lines and we confirmed the senescent state of the fibroblasts that expressed low levels of surface CLEC2A by analyzing the expression of senescent markers.

B. Expression of CLEC2A in other cancer-prone skin related diseases

Finally, because our previous experiments showed that XPC is not directly implicated in the absence of CLEC2A in XP-C fibroblasts we looked into the levels of CLEC2A expression first in fibroblasts from a different XP complementation group – XP-D, and after in fibroblasts from a different cancer-prone skin related disease – NBSCC.

Our results show that CLEC2A is also absent in fibroblasts from XP-C patients and CLEC2A mRNA transcripts levels are dramatically decreased in fibroblasts from NBSCC patients. These results suggest that the absence of CLEC2A could be a characteristic of cancer-prone skin.
A.I. CLEC2A modulation during infection

The skin being the first barrier to microorganisms, we evaluated whether CLEC2A expression could be modulated in the context of infection. We did not perform an extensive analysis but restrained our study to a stimulation of fibroblasts expressing CLEC2A with lipopolysaccharide (LPS). LPS is a component present in the cell walls of Gram-negative bacteria that binds to the Toll-like receptor 4 (TLR4). The activation of TLR4 triggers the production of pro-inflammatory cytokines and chemokines, upregulation of costimulatory molecules and activation of antigen presentation by DC (Chow, Young et al. 1999, Akira and Takeda 2004, Wang, Hori et al. 2011).

Expression of CLEC2A mRNA was transiently upregulated in presence of LPS in WT dermal fibroblasts (figure 21A). We found that CLEC2A expression is enhanced 2/3 hours after LPS incubation and declines back to a baseline level 6 hours after treatment. However, the increase in CLEC2A mRNA levels didn’t correlate with the increase in the levels of membrane associated CLEC2A that stayed unchanged with or without LPS stimulation (figure 21B).

We then looked into cytoplasmic levels of CLEC2A by immunofluorescence analysis and observed that in presence of LPS, WT fibroblasts overexpressed intracellular CLEC2A that transiently accumulates with time, thus correlating with mRNA data (figure 22). Moreover, the staining shows an accumulation of the signal in small spots that disappear between 8 and 24 hours after LPS stimulation.
**Figure 21.** Kinetics of CLEC2A expression in WT fibroblasts (F-WT) after incubation with LPS.

(A) Relative CLEC2A mRNA transcripts levels in WT fibroblasts after incubation with 200 ng/mL LPS measured by quantitative RT-PCR. Data represent mean ± SD of three cell lines (FTM1, FTM5, FH122). ***p ≤0.001, *p≤0.05 Mann-Whitney U-test. (B) Representative histograms of CLEC2A expression in FTM1_WT fibroblasts after incubation with LPS for 3, 6 and 12 hours as determined by flow cytometry. Two independent cell lines were analyzed.

To determine the nature of these spots, we performed electron microscopy analysis in WT and XP-C fibroblasts after 6 hours of LPS incubation (figure 23 A and B). In WT fibroblasts, we identified a 5-fold increase in lysosome organelles in presence of LPS that could correlate with the spots that we saw in immunofluorescence assays (figure 23B). However, XP-C fibroblasts showed no change in lysosome numbers and we saw instead lipid droplets in XP-C fibroblasts cytoplasm that were present even in the absence of LPS stimulation (figure 23 A and B).
Figure 23. Lysosome quantification in WT and XP-C fibroblasts after stimulation with LPS for 6 hours.

(A) Representative images of ultrastructural micrograph of FTM1_WT (upper panels) and AS798_XP-C (lower panels) fibroblasts with or without LPS incubation. Ly: lysosome, N: nucleus, LD: lipid droplets. Scale bar = 5µm. (B) Normalized numbers of lysosomes per 50µm².

Together, these data suggest that upon infection and stimulation with LPS, CLEC2A expression is stimulated but the protein remains intracellular and is degraded. This constitutes
a pool that may be induced on the cell surface upon unknown mechanisms. In addition, our electron microscopy analysis has unveiled a possible deregulation in XP-C lipid metabolism.
A. II. CLEC2A modulation upon replicative senescence

Because XP-C fibroblasts share phenotypical characteristics with senescent cells, we analyzed whether the expression of CLEC2A was modulated in WT fibroblasts during population doublings (PD). WT fibroblasts were propagated in culture until we saw a decline in cellular replication. Expression levels of CLEC2A during PDs were determined by flow cytometry. We observed a progressive decrease of CLEC2A expression in three different cell lines of WT fibroblasts over PD (figure 24 A and B).

![Figure 24. CLEC2A expression in WT fibroblasts over population doublings determined by flow cytometry.](image)

(A) Representative histograms of CLEC2A expression in WT fibroblasts at low (PD15) and high (PD31) PD. (B) Frequency of CLEC2A positive cells in three primary cell lines of WT fibroblasts over PD.

To assess the senescent state of the fibroblasts that expressed low levels of surface CLEC2A at late cumulative PD, expression of p21 and p16, two senescence markers, was analyzed in WT-fibroblasts at early and late cumulative PDs. Our results show that at late PD, fibroblasts overexpress p16 and p21 which is consistent with a typical phenotype of senescent cells (figure 25). Furthermore, we performed a senescence associated (SA) β-galactosidase staining that reveals the presence of high SA-β-gal activity in late PD fibroblasts (figure 26). These results confirmed the senescent state of WT fibroblasts at late PD.

In conclusion, our results show a decrease in CLEC2A expression during replicative senescence. This is in correlation with the loss of CLEC2A expression in CAFs (detailed in Scientific article, page 90).
Figure 25. Expression of p16 and p21 in three primary cell lines of WT fibroblasts at low and high PD as determined by Western blot. Anti-tubulin antibody was used as a loading control.

Figure 26. Senescence associated β-galactosidase staining in two primary cell lines of WT fibroblasts at low and high PD.
B. CLEC2A expression in other skin related genetic diseases

We successfully transduced XP-C fibroblasts with a retroviral vector expressing the WT XPC and showed that the reintroduction of XPC in XP-C fibroblasts do not allow the novo expression of CLEC2A (detailed in Scientific article, page 90). This suggests that lack of CLEC2A in XP-C fibroblasts is not a direct consequence of the absence of XPC and prompted us to hypothesize that the absence of CLEC2A is not specific to XP-C fibroblasts. To prove our hypothesis, we first analyzed the expression of CLEC2A in fibroblasts from XP patients of another complementation group – XP-D. Using quantitative RT-PCR and flow cytometry analysis, we found that fibroblasts from XP-D patients present, like XP-C fibroblasts, a total absence of CLEC2A expression, both at the transcript and protein levels (figure 27 A and B).

We then analyzed the expression of CLEC2A by quantitative RT-PCR in two primary cell lines of fibroblasts from patients suffering from Nevoid Basal Cell Carcinoma Syndrome (NBCCS), an autosomal dominant disorder caused mainly by mutations in PTCH1 gene. This pathology is unrelated with changes in DNA repair mechanisms but is characterized by the development of multiple BCCs. Figure 28 shows a drastic and statistically significant decrease in the expression of CLEC2A mRNA in NBCCS fibroblasts compared to WT fibroblasts.

![Figure 27. CLEC2A expression in primary WT and XP-D fibroblasts.](image)

(A) Relative CLEC2A mRNA transcripts levels in FTM1_WT and AS204_XP-D fibroblasts. Data represent mean ± SD of two independent experiments. ***p≤0.001 Mann-Whitney U-test.

(B) Representative histograms of CLEC2A expression in FTM1_WT and AS204_XP-D fibroblasts determined by flow cytometry. Three independent experiments were performed.
Figure 28. Relative CLEC2A mRNA transcripts levels in FH84_WT, AS537_NBCCS and AS_587_NBCCS fibroblasts determined by quantitative RT-PCR.

Data represent mean ± SD of two independent experiments. ***p <0.001 Mann-Whitney U-test.

Although they need clearly more investigations, these results are interesting because we show that CLEC2A expression is deregulated in the skin fibroblasts of different cutaneous cancer prone pathologies (i.e. XP and NBCCS). They suggest that the deregulation of CLEC2A may be a common feature in cancer-prone skin related diseases.
Discussion and Perspectives
Challenges in rare genetic diseases research

“Rare diseases are diseases which affect a small number of people compared to the general population. In Europe, a disease is considered to be rare when it affects 1 person per 2000” – from (Orphanet).

Doing research in rare genetic diseases is a challenge for various reasons. First, “rare”, means that there are small numbers of patients suffering from the disease and this can introduce serious difficulties to produce statistically robust results, at least as expected from conventional, large cohort studies. Xeroderma pigmentosum (XP) affects less than 3 people per million births in Europe (Kleijer, Laugel et al. 2008). Because, along the past 20 years, our laboratory has accumulated a large collection of skin primary cells from XP patients, I wasn’t directly confronted with the difficulty of not having enough samples from different patients. Nevertheless, I have to acknowledge that the need for a consistent collection of cells limits the development of new research projects from laboratories that didn’t have this resource. Second, research on rare diseases, particularly on curative therapies, tends to attract less investment compared to more common diseases due to the limited financial benefits for pharmaceutical companies and priorities of social security services. In spite of these pragmatic and disappointing considerations, genetic diseases can also be used as models to study physiological and mechanistic processes in human body. In fact, there are many examples of scientific breakthrough that were made thanks to the study of genetic diseases. A famous one is the discovery of Goldstein and Brown showing that mutations in the cell surface receptor of low density lipoprotein (LDL) lead to the familiar hypercholesterolemia disease (Goldstein and Brown 1973). LDL receptors are now indirect targets of the most used drugs (the statins) in the control of high levels of cholesterol and in the prevention of atherosclerosis (Sirtori 2014).

In the laboratory, research projects focus in (i) the development of safe curative approaches for xeroderma pigmentosum patients (Warrick, Garcia et al. 2012) (ii) the use of XP-C keratinocytes to study cancer development under UV solar radiations; (iii) the use of XP-C fibroblasts as scaffold for the progression of cutaneous cancers. My thesis project derives from this third axe and is particularly focused on the interactions between skin fibroblasts and the immune system.

Fibroblasts cultures – 2D vs 3D

Transcriptomic analysis of the mRNA expression profiles of WT and XP-C fibroblasts in conventional 2D cultures performed by the laboratory showed that almost three hundred genes were deregulated in XP-C fibroblasts compared to WT fibroblasts (unpublished results, please refer to page 182 for data). These data allowed the team to detail the role of HGF, surexpressed by XP-C fibroblasts, in cutaneous SCC invasion (Alqaraghuli et al., in
The high amount of deregulated genes observed in 2D cell culture are implicated in numerous different physiologic pathways. We then were unable to clearly identify other important alterations that may explain the propensity of XP-C dermis to cancer cell invasion.

Aware that culture conditions of primary cells can change the expression of genes, we decided to perform transcriptomic analysis of WT and XP-C fibroblasts cultured in dermal equivalents (3D). This was for us a more relevant condition to study dermal fibroblasts in their dermal context, as suggested by several groups (Ghosh, Spagnoli et al. 2005, Griffith and Swartz 2006, Duval, Grover et al. 2017). Two things immediately drove our attention after comparison of our transcriptomic analysis under 2D or 3D culture conditions: (i) only 22 genes came out as differentially expressed in 3D cultured cells compared to almost 300 in 2D cultures and (ii) only five genes, CLEC2A, PITX1, PRDM16, TBX5 and HOXC10 presented significant deregulation both in 2D and 3D approaches.

The paper of Tolle et al., 2018 compared protein expression in normal fibroblasts versus CAFs both in 2D and 3D cultures (Tolle, Gaggioli et al. 2018). This paper shows that in 2D culture, proteins involved in adherent junctions, cell–cell adhesion and wound healing are found differentially expressed between the two populations. In 3D cultures, a different protein expression profile emerges with an enrichment for differential expression in proteins involved in vacuolar and lysosomal transport, regulation of response to stress, multicellular organismal process and cellular development and differentiation. They furthermore compare the two sets of data with analysis of breast cancer biopsies and conclude that the 3D approach better mimic what happens in vivo. These recent data support the idea that 3D cultures better mimic fibroblasts behaviors in vivo in general.

What we found at mRNA level in our comparative transcriptomic analysis of WT and XP-C fibroblasts in 2D and 3D cultures, however, goes beyond the notion that culture conditions can change protein expression. Indeed, our results not only validate the notion that the expression of proteins/genes changes between 2D and 3D culture conditions, but they also unveil the dramatic decrease in the number of differentially expressed genes between two populations of dermal fibroblasts when cultured in 3D dermal equivalents. Our results indicate that at steady-state, fibroblasts from WT and XP-C patients cultured in 3D did not change drastically the expression of their genes as a 2D culture could prompt us to assume.

We can hypothesize that the genes that show differential expression profiles both in 2D and 3D cultures could be major actors in the phenotypic differences observed between WT and XP-C fibroblasts. Based on these results, we recommend that further transcription analysis comparing fibroblasts from two different populations should be performed using 3D systems.
3D cultures could lead to a more precise and pertinent identification of potentially relevant genes.

**Fibroblasts and NK cells**

The importance of fibroblasts on skin homeostasis and cancer progression has been well described over the years and is summarized in the introductory chapters of this thesis. However, the way they interact with immune cells and their potential role as an immune system component are much less explored.

**A. CLEC2A role in cancer invasion**

The discovery of CLEC2A, a NK ligand, as a differentially expressed gene in WT and XP-C fibroblasts following the 2D and 3D transcriptomic analysis immediately caught our attention. It reminded us of work from the late 80s / early 90s that described modifications of the NK cell phenotype in XP patients and it suggests an implication of a deregulated immune system in the cancer invasion patterns associated with XP disease (Norris, Limb et al. 1988, Norris, Limb et al. 1990, Mariani, Facchini et al. 1992). Until our discovery, CLEC2A was only described as expressed in differentiated skin keratinocytes and its physiological role remains unclear (Spreu, Kuttruff et al. 2010). Our study shows for the first time that CLEC2A is also expressed in skin WT fibroblasts. We did not find expression of CLEC2A on basal WT keratinocytes suggesting that not all the different differentiation stages of keratinocytes are associated with CLEC2A expression. The total absence of CLEC2A in XP fibroblasts and CAFs and the downregulation of CLEC2A expression in WT fibroblasts by factor secreted by SCC cells prompted us to look into a possible protective role of CLEC2A against cancer invasion. Our results clearly show that the expression of CLEC2A in WT fibroblasts negatively affects the invasion rate of SCC cells in a 3D NK immunocompetent model. These results raise several questions. How fibroblasts interact with NK cells? Are fibroblasts able to activate NK cells via direct contact mechanisms? Do fibroblasts have a role in the immunosurveillance of the skin?

In the search for possible mechanisms that could answer some of these questions, we searched the literature for descriptions of NK cell regulation when NK cells are activated by cells that are not NK’s direct targets. Indeed, NK cells can be activated by either target cells or accessory cells like DC. After stimulation by stress signals, DC undergo maturation and overexpress cytokines and NK ligands responsible for NK cell activation. However, they are not targets of NK cells, probably because they express high levels of MHC-I molecules (Monteiro, Harvey et al. 1998, Fernandez, Lozier et al. 1999, Pollara, Jones et al. 2004, Andrews, Andoniou et al. 2005, Barreiros da Silva and Munz 2011). Although it has been shown that DC activate NK cells mostly via cytokine expression, the direct contact alone is able to
control NK cytotoxicity and in some cases mediate NK cell survival (Poggi, Carosio et al. 2002, Jinushi, Takehara et al. 2003, Brilot, Strowig et al. 2007).

In our experimental system, we show that NK cells are unable to kill WT and XP-C fibroblasts but contrary to what happens with DC, the interaction between NK and fibroblasts didn’t reveal an increase in their expression of IFN-γ (data not shown). These data suggest that fibroblasts alone are not able to activate, nor change NK cell phenotype like DC. However, our cytotoxic experiments with fibroblasts were done at steady state and we cannot exclude that stimulated fibroblasts could at least partly, behave like mature DC, contributing therefore to the modulation of NK cell phenotype in the skin. Finally, the use of allogeneic IL-2 stimulated NK cells in our system skews the results as the use of already activated NK cells can hide the true role of fibroblasts in NK activation.

With our results, we propose that in a cancer system, NK cells are indeed mostly activated by cancer cells, but a direct contact with fibroblasts in early steps of cancer development may contribute to “drag” NK cells into the tissue and further contributes to their activation. We can hypothesize that in skin, CLEC2A has an important role in the regulation of the interaction between NK cells and fibroblasts. When this interaction is compromised, it may provide a less immunosuppressive microenvironment that will favor cancer cell invasion (figure 29). The absence of CLEC2A in other cancer-prone related skin diseases reinforce this hypothesis.

**Figure 29. Crosstalk between fibroblasts and NK cells.**

Left panel. (1) In early steps of cancer development, the presence of CLEC2A in skin WT fibroblasts is able to trigger NK cell cytotoxicity in vitro through the engagement of NKp65 receptor. (2) SCC cell secreted factors downregulate CLEC2A expression, possibly as part of the transitioning process that WT fibroblasts undergo to become CAFs. The loss of CLEC2A facilitates the evasion of cancer cells. Wright panel. In an XP-C skin, the absence of CLEC2A; weakens the skin immunosurveillance against cancer cells.
Considerations on CLEC2A expression in human skin cells

At first sight, our result showing the absence of CLEC2A on primary keratinocytes is contradictory to the previously published data that identify CLEC2A as expressed in keratinocytes (Spreu, Kienle et al. 2007). While Spreu et al. performed their analysis with freshly isolated keratinocytes from skin biopsies, we did our study using primary keratinocytes in culture. This means that while they work with a heterogeneous pool of keratinocytes coming from all the differentiation layers of the epidermis, we work with a homogenous population of basal keratinocytes. We can then hypothesize that CLEC2A can be expressed in keratinocytes upon the differentiation process. Their immunofluorescence results of skin tissue samples, showing that the expression of CLEC2A is restricted to the stratum corneum, corroborate our hypothesis (Bauer, Spreu et al. 2015). To validate this hypothesis, cytometric analysis experiments in cells freshly isolated from skin are ongoing.

Another note to add is that in their experiments, both Spreu et al. and Bauer et al. first separate the epidermis from the dermis of skin biopsies and concentrate their attention only in the epidermal portion of the skin. This method prevented them to conclude on the expression of CLEC2A in fibroblasts as we did.

B. Modulation of CLEC2A expression in fibroblasts in a non-cancerous microenvironment

The increase of CLEC2A expression during infection and its decrease during replicative senescence experiments support our belief that the crosstalk between fibroblasts and NK cells has an important role in innate immune system besides the one played during cancer invasion. Many studies before ours tested the effects of infection on fibroblast phenotype. During inflammation processes, fibroblasts undergo phenotypical changes including mechanisms involved in ECM remodeling. Besides this architectural capacity, some studies also showed that fibroblasts can regulate inflammatory processes through CD40–CD40 ligand (L) interactions or engagement of TLR receptors. The engagement between CD40 receptors expressed by fibroblasts and CD40L expressed by T lymphocytes enhances the inflammatory process by inducing the synthesis of cytokine mediators IL-1, IL-8, adhesion molecules ICAM-1 and VCAM and PGE2 by fibroblasts (Yellin, Winikoff et al. 1995, Zhang, Cao et al. 1998, Buckley, Pilling et al. 2001). More importantly, the engagement of fibroblasts’ TLR4 receptors by LPS induces expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, and TNF-α (Chakravortty and Kumar 1999). Our results add the information that LPS stimulation also induces the expression of a gene coding for a ligand of NK cell receptor (CLEC2A) and implies for the first time that fibroblasts should be considered as actors in NK cell activation during infection. The fact that we were unable to detect changes in surface expression of
CLEC2A prompt us to think that other inflammatory signals are needed to translocate the newly synthetized CLEC2A to the surface membrane of fibroblasts.

Senescent stromal cells can drive a pro-tumoral immunosuppressive microenvironment, the recruitment of MDSC cells (Ruhland, Loza et al. 2016). As for NK cells, several reports have shown lower infiltration of NK cells in murine senescent tissues and defective migration to lymph nodes upon viral infection (Fang, Roscoe et al. 2010, Beli, Clinthorne et al. 2011). In young organisms, NK cells are able to kill senescent cells that overexpress activating ligands, and this is an important process that allows the regeneration of the tissues (Krizhanovsky, Yon et al. 2008, Sagiv, Burton et al. 2016). In old tissues, senescent cells do accumulate contributing to aging and age-associated pathologies (Baker, Childs et al. 2016). A recent study proved that the accumulation of senescent cells in old tissues is caused by the lack of cytotoxic activity from both NK and T cells. However, the mechanisms underlying these defaults of cytotoxic activity are not fully understood (Ovadya, Landsberger et al. 2018). Even if our results are only preliminary ones we can suppose that the loss of CLEC2A upon senescence could contribute to a decrease of the accumulation of functional NKs in skin. We tried to validate this hypothesis by including replicative senescent fibroblasts in our 3D immunocompetent model. However, senescent fibroblasts did not contract the collagen matrix in the same way that non senescent fibroblasts do, and we were unable to perform the experiment. As an alternative approach, it would be interesting to test “natural” senescent fibroblasts from old people in our system.

The low levels of NKp65 (receptor of CLEC2A) in NK cells extracted from human peripheral blood of different donors intrigued the authors that first described it. However, tissue-resident and recruited NK cells differ in the expression profile of their receptors from those that remain in circulation (Bjorkstrom, Ljunggren et al. 2016, Shahradi, Zayeri et al. 2019). As CLEC2A is almost exclusively expressed in skin, we cannot exclude that only NK cells present in skin tissue will acquire the capacity to enhance the expression of NKp65 (Spreu, Kienle et al. 2007). If we take into account these considerations, we can imagine that in our 3D system the contact between fibroblasts and NK cells, or the different culture conditions (culture in suspension or in collagen matrix) may lead to an overexpression of Nkp65. Alternatively, the level of Nkp65 at cell surface may not need to be high as it has been reported a high affinity of CLEC2A-Nkp65 interaction (Li, Wang et al. 2013).

We can speculate that the absence of CLEC2A in the two fibroblast populations that share similarities with XP-C fibroblasts (CAFs and senescent fibroblasts) is not a coincidence. Our results are consistent with the idea that a crosstalk between NK cells and skin fibroblasts via CLEC2A could have major impacts in cancer progression and perhaps also in inflammatory processes.
processes. XP patients could benefit from therapies enhancing NK cell activity. As for the general population, CLEC2A could be a good marker to predict skin cancer outcomes and could guide therapeutic choices. Further clarifications of the mechanisms underlying the regulation of CLEC2A expression may open the door to therapeutic approaches aiming to reestablish CLEC2A surface expression on skin fibroblasts.

**XP-C fibroblasts: Thoughts on responses to infection and lipid metabolism**

The activation of TLR4 receptors by LPS stimuli triggers the internalization of the ligand-receptor complex via the dimerization with surface membrane lipid rafts and directs the complex toward lysosomal degradation or endocytic recycling (Latz, Visintin et al. 2002, Triantafilou, Miyake et al. 2002, Husebye, Halaas et al. 2006). The degradation and recycling processes are important to regulate LPS inflammation and tolerance and are convincingly confirmed in WT fibroblasts in our electron microscopy analysis (page 129) (Murase, Kawasaki et al. 2018). However, XP-C fibroblasts didn’t show an increase in the number of lysosomal organelles after LPS stimuli which imply that XP-C fibroblasts’ response to infection is deregulated. As the expression of TLR4 is not deregulated in XP-C fibroblasts compared to WT fibroblasts at steady state (transcriptomic results) we can suppose that there is an impairment, either in the engagement of the complex TLR4-LPS, or in the trafficking signaling pathway that follows internalization. Because we have no indication of XP-C patients presenting predisposition for chronic inflammatory processes, a default of engagement of TLR4 with LPS may be more likely. Furthermore, our observations of lipid droplets in the cytoplasm of XP-C fibroblasts are consistent with a deregulation in lipid metabolism. Lipids are involved in TLR4-LPS internalization.

In fact, our 2D transcriptomic analysis revealed moderate deregulation in the expression of many genes implicated in the lipid biosynthesis. The expression of genes implicated in the biosynthesis of cholesterol are downregulated in XP-C fibroblasts and genes implicated in the biosynthesis of triacylglycerides (TAG), phosphoglycerides and phosphoinositides are up-regulated (figure 30). Cholesterol and phosphoglycerides are major structural components of biological membranes and TAG are mainly implicated in energy storage in the form of lipid droplets like the ones we saw in the cytoplasm of XP-C fibroblasts (Baenke, Peck et al. 2013). More importantly, cholesterol is one of the main components of lipid rafts directly implicated in the LPS-induced cell activation. Alterations in lipid rafts could impair LPS signaling and explains the lack of lysosomal accumulation that we saw in our electron microscopy analysis. However, a lack of response against infection normally leads to a scenario of recurrent and/or chronic infection that again are not described in XP patients.
Finally, and as already discussed in page 68, fatty acids are essential to fuel tumor growth. We cannot exclude that an accumulation of glycerides within XP-C fibroblasts cytoplasm could support cancer progression and dissemination.

Green and red arrows highlight respectively downregulated and upregulated genes in XP-C fibroblasts comparing to WT fibroblasts. The enzymes involved in catalyzing steps in lipid biosynthetic pathways are indicated in red. Enzyme abbreviations: ACAT, acetyl-CoA acetyltransferase; ACC, acetyl-CoA carboxylase; ACLY, ATP citrate lyase; AGPAT, 1-acylglycerol-3-phosphate O-acyltransferase; COX1/2, prostaglandin-endoperoxide synthase (PTGS); DGAT, diacylglycerol O-acyltransferase; ELOVL, fatty acid elongase; FADS, fatty acid desaturase; FASN, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase; PPAP, phosphatidic acid phosphatase; SCD, stearoyl-CoA desaturase; SPHK, sphingosine-1-kinase. Metabolite abbreviations: α-KG, α-ketoglutarate; CDP-DAG, cytidine diphosphate-diacylglycerol; CER, ceramide; DAG, diacylglycerol; FA, fatty acid; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGE2, prostaglandin E2; PGH2, prostaglandin H2; PI, phosphatidylinositol; PIPx, phosphatidylinositol phosphate; PS, phosphatidylserine; S1P, sphingosine-1-phosphate; SPH, sphingosine; TAG, triacylglyceride.

Figure 30. Schematic overview of the pathways involved in the different lipid classes biosynthesis – cholesterol, Fatty acids (FA), sphingolipids, eicosanoids, acylglycerides, phosphoinositides and phosphoglycerides.
Concluding note from the author

I remember very clearly the first time I heard about a disease called xeroderma pigmentosum. It was thirteen years ago in a genetics class, during my freshman year at the Faculty of Pharmacy of the University of Porto. Now, I’m presenting my thesis work centered on XP disease and it would be nice to say that since that class in 2006 I’ve been focusing on finding a way to put my professional skills at the service of this patients. It was not the case. After that class, life continued for me, I graduated, I worked in a pharmacy, I enrolled in a Master’s program at the Faculty of Pharmacy of the University Paris SUD XI, I graduated again, I worked in the cosmetics industry, all of that without thinking even for a minute of xeroderma pigmentosum. It wasn’t in my plans to work on/with this disease but somehow, the minute I read about this project, it all came to me: the photos of the patients, the schema of the deregulated DNA repair pathway, the videos about little children playing in the backyard at night. I accepted the “XP challenge” without hesitations and it became a part of my life. I hope that the work I present here could bring some benefit to these children in the future.

Besides the important work that some research groups around the world are doing to contribute to the development of new therapies that ultimately improve the quality of life of XP patients, other nonscientific groups provide an enormous help to these patients and their families. They organize gatherings between the children and their families, scientific meetings, and the majority also work as a family support group. They raise funds for research projects and scientific collaborations to help these families acquire the expensive UV protective equipment and to organize activities with the children to avoid social exclusion. Their work is equally as important as ours and deserves to be mentioned in this manuscript. Below a list of the most active XP groups/associations/Charitable Trusts:

XP Family Support Group: https://xpfamilysupport.org/
Teddington Trust: http://www.teddingtontrust.com/
XP Society South Africa: https://www.xpsociety.co.za/
XP Support Group: http://xpsupportgroup.org.uk/
XP Freu(n)de – Mondscheinkinder (Germany): http://www.xerodermapigmentosum.de/
Enfants de la Lune (France): https://www.enfantsdelalune.org/
Aide aux enfants atteindre de xeroderma pigmentosum (Tunisia): http://www.xp-tunisie.org.tn/
References


168


Orphanet. "The portal for rare diseases and orphan drugs." from https://www.orpha.net/consor/cgi-bin/Education_AboutRareDiseases.php?lng=EN.


Weinstein, G. D. and R. J. Boucek (1960). "Collagen and Elastin of Human Dermis**From the Divisions of Dermatology and Cardiology of the Department of Medicine, University of Miami School of Medicine and Howard Hughes Medical Institute, Miami, Florida.This investigation was supported in part by Grants A-2586 and 2G-224, from the N.I.H., U.S. Public Health Service." Journal of Investigative Dermatology 35(4): 227-229.


Appendix
### Differentially expressed genes between WT and XP-C fibroblasts (2D approach) - p≤0.05

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr</th>
<th>baseMean</th>
<th>baseMean</th>
<th>logFC</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEM176A</td>
<td>NM_018487</td>
<td>8,842808</td>
<td>7,706179</td>
<td>12,252694</td>
<td>-5,567242</td>
<td>0.000982</td>
</tr>
<tr>
<td>TMEM176B</td>
<td>NM_014020</td>
<td>8,728515</td>
<td>7,654775</td>
<td>11,949735</td>
<td>-5,545129</td>
<td>0.001905</td>
</tr>
<tr>
<td>APOE</td>
<td>NM_000041</td>
<td>11,659845</td>
<td>10,719523</td>
<td>14,480812</td>
<td>-4,432818</td>
<td>0.016353</td>
</tr>
<tr>
<td>CHI3L1</td>
<td>NM_001276</td>
<td>7,596118</td>
<td>6,631287</td>
<td>10,490613</td>
<td>-4,192727</td>
<td>0.024086</td>
</tr>
<tr>
<td>DBC1</td>
<td>NM_0014618</td>
<td>8,481659</td>
<td>7,610226</td>
<td>11,095958</td>
<td>-3,996130</td>
<td>0.016353</td>
</tr>
<tr>
<td>SFRP2</td>
<td>NM_003013</td>
<td>8,699159</td>
<td>7,823890</td>
<td>11,324963</td>
<td>-3,704781</td>
<td>0.023519</td>
</tr>
<tr>
<td>MAOA</td>
<td>NM_000240</td>
<td>8,333991</td>
<td>7,632653</td>
<td>10,438005</td>
<td>-4,432818</td>
<td>0.016353</td>
</tr>
<tr>
<td>C2</td>
<td>NM_000063</td>
<td>8,784652</td>
<td>8,100108</td>
<td>10,838285</td>
<td>-3,270890</td>
<td>0.018458</td>
</tr>
<tr>
<td>PKD1L2</td>
<td>NM_0052892</td>
<td>7,868430</td>
<td>7,241639</td>
<td>9,748805</td>
<td>-3,242742</td>
<td>0.015994</td>
</tr>
<tr>
<td>KIAA0226L</td>
<td>NM_002511</td>
<td>7,612083</td>
<td>6,896577</td>
<td>9,758602</td>
<td>-3,237318</td>
<td>0.004739</td>
</tr>
<tr>
<td>DMKN</td>
<td>NM_001035516</td>
<td>14,288929</td>
<td>13,622516</td>
<td>16,288168</td>
<td>-3,210723</td>
<td>0.024086</td>
</tr>
<tr>
<td>XLOC_014397</td>
<td>TCONS_00029721</td>
<td>8,356599</td>
<td>7,744401</td>
<td>10,193195</td>
<td>-3,139322</td>
<td>0.001300</td>
</tr>
<tr>
<td>DMKN</td>
<td>NM_003317</td>
<td>11,64999</td>
<td>11,025531</td>
<td>13,521801</td>
<td>-3,077926</td>
<td>0.021521</td>
</tr>
<tr>
<td>DMKN</td>
<td>NM_001190348</td>
<td>11,189766</td>
<td>10,591340</td>
<td>12,985044</td>
<td>-2,985531</td>
<td>0.027858</td>
</tr>
<tr>
<td>PRUNE2</td>
<td>NM_0015225</td>
<td>12,140823</td>
<td>11,570161</td>
<td>13,852812</td>
<td>-2,983723</td>
<td>0.002532</td>
</tr>
<tr>
<td>C3</td>
<td>NM_000064</td>
<td>8,217008</td>
<td>7,624709</td>
<td>9,993903</td>
<td>-2,925882</td>
<td>0.036724</td>
</tr>
<tr>
<td>RSPO4</td>
<td>NM_001029871</td>
<td>7,203580</td>
<td>6,470437</td>
<td>9,403009</td>
<td>-2,886373</td>
<td>0.023369</td>
</tr>
<tr>
<td>C2</td>
<td>NM_001178063</td>
<td>7,927690</td>
<td>7,346606</td>
<td>9,670941</td>
<td>-2,854580</td>
<td>0.024086</td>
</tr>
<tr>
<td>PSG1</td>
<td>NM_006905</td>
<td>10,178272</td>
<td>9,483319</td>
<td>12,263134</td>
<td>-2,852862</td>
<td>0.015994</td>
</tr>
<tr>
<td>SNCA</td>
<td>NM_007308</td>
<td>8,051622</td>
<td>7,569780</td>
<td>9,497147</td>
<td>-2,812024</td>
<td>0.024086</td>
</tr>
<tr>
<td>PKD1L2</td>
<td>NM_0052892</td>
<td>8,018112</td>
<td>7,472486</td>
<td>9,654991</td>
<td>-2,804351</td>
<td>0.017535</td>
</tr>
<tr>
<td>MITF</td>
<td>NM_0198159</td>
<td>10,854708</td>
<td>10,312555</td>
<td>12,481165</td>
<td>-2,777365</td>
<td>0.038489</td>
</tr>
<tr>
<td>PRUNE2</td>
<td>NM_0015225</td>
<td>7,819919</td>
<td>7,221762</td>
<td>9,614393</td>
<td>-2,768323</td>
<td>0.014542</td>
</tr>
<tr>
<td>CLEC2A</td>
<td>NM_001130711</td>
<td>7,181656</td>
<td>6,547449</td>
<td>9,084278</td>
<td>-2,766388</td>
<td>0.022946</td>
</tr>
<tr>
<td>CLDN23</td>
<td>NM_0194284</td>
<td>9,092718</td>
<td>8,592346</td>
<td>10,593831</td>
<td>-2,698022</td>
<td>0.031276</td>
</tr>
<tr>
<td>XPC</td>
<td>NM_004628</td>
<td>9,630079</td>
<td>9,434290</td>
<td>10,217448</td>
<td>-2,680130</td>
<td>0.034283</td>
</tr>
<tr>
<td>CLIC6</td>
<td>NM_053277</td>
<td>7,564427</td>
<td>6,997549</td>
<td>9,265062</td>
<td>-2,640440</td>
<td>0.011741</td>
</tr>
<tr>
<td>XLOC_l2_009281</td>
<td>TCONS_l2_00017559</td>
<td>7,294971</td>
<td>6,696972</td>
<td>9,088968</td>
<td>-2,638447</td>
<td>0.020995</td>
</tr>
<tr>
<td>FAM65C</td>
<td>NM_0080829</td>
<td>9,781153</td>
<td>9,263889</td>
<td>11,332943</td>
<td>-2,619135</td>
<td>0.004850</td>
</tr>
<tr>
<td>PRUNE2</td>
<td>NM_0015225</td>
<td>8,818953</td>
<td>8,273414</td>
<td>10,455571</td>
<td>-2,607414</td>
<td>0.012544</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr_xpc_wt</th>
<th>baseMean_xpc</th>
<th>baseMean_wt</th>
<th>logFC_xpc_wt</th>
<th>adj.P.Val_xpc_wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAFB</td>
<td>NM_005461</td>
<td>11,123765</td>
<td>10,595488</td>
<td>12,708596</td>
<td>-2,556624</td>
<td>0.016353</td>
</tr>
<tr>
<td>MX1</td>
<td>NM_002462</td>
<td>11,200524</td>
<td>10,670367</td>
<td>12,790994</td>
<td>-2,510816</td>
<td>0.030213</td>
</tr>
<tr>
<td>CPXM1</td>
<td>NM_019609</td>
<td>8,807174</td>
<td>8,219211</td>
<td>10,571064</td>
<td>-2,502641</td>
<td>0.033282</td>
</tr>
<tr>
<td>PNLIPRP3</td>
<td>NM_001011709</td>
<td>7,167952</td>
<td>6,677840</td>
<td>8,638289</td>
<td>-2,489950</td>
<td>0.015439</td>
</tr>
<tr>
<td>LAMA3</td>
<td>NM_198129</td>
<td>8,852550</td>
<td>8,378130</td>
<td>10,275808</td>
<td>-2,486232</td>
<td>0.016353</td>
</tr>
<tr>
<td>GLDN</td>
<td>NM_181789</td>
<td>7,491641</td>
<td>7,017463</td>
<td>8,914173</td>
<td>-2,476322</td>
<td>0.001300</td>
</tr>
<tr>
<td>MITF</td>
<td>NM_198159</td>
<td>9,245312</td>
<td>8,756686</td>
<td>10,711190</td>
<td>-2,465933</td>
<td>0.038829</td>
</tr>
<tr>
<td>MAFB</td>
<td>NM_005461</td>
<td>12,978998</td>
<td>12,481799</td>
<td>14,470595</td>
<td>-2,441668</td>
<td>0.027507</td>
</tr>
<tr>
<td>GALNTL2</td>
<td>NM_054110</td>
<td>10,273125</td>
<td>9,790089</td>
<td>11,722322</td>
<td>-2,440417</td>
<td>0.023519</td>
</tr>
<tr>
<td>IFI27</td>
<td>NM_005532</td>
<td>9,512447</td>
<td>9,081769</td>
<td>10,804482</td>
<td>-2,394138</td>
<td>0.048616</td>
</tr>
<tr>
<td>HOXB8</td>
<td>NM_024016</td>
<td>7,378381</td>
<td>6,762853</td>
<td>9,224966</td>
<td>-2,359210</td>
<td>0.034283</td>
</tr>
<tr>
<td>ENPP5</td>
<td>NM_021572</td>
<td>8,756702</td>
<td>8,289175</td>
<td>10,159285</td>
<td>-2,321077</td>
<td>0.031276</td>
</tr>
<tr>
<td>EPB41L4A</td>
<td>NM_022140</td>
<td>7,328327</td>
<td>6,861172</td>
<td>8,729793</td>
<td>-2,255501</td>
<td>0.024086</td>
</tr>
<tr>
<td>AIF1L</td>
<td>NM_001185095</td>
<td>7,277391</td>
<td>6,737147</td>
<td>8,898123</td>
<td>-2,233501</td>
<td>0.018458</td>
</tr>
<tr>
<td>MAB21L1</td>
<td>NM_005584</td>
<td>7,095669</td>
<td>6,537524</td>
<td>8,770105</td>
<td>-2,143817</td>
<td>0.011479</td>
</tr>
<tr>
<td>P2RX7</td>
<td>NM_002562</td>
<td>8,063295</td>
<td>7,626303</td>
<td>9,374271</td>
<td>-2,113096</td>
<td>0.041972</td>
</tr>
<tr>
<td>PPARC1A</td>
<td>NM_013261</td>
<td>7,397582</td>
<td>6,970891</td>
<td>8,677656</td>
<td>-2,010436</td>
<td>0.018387</td>
</tr>
<tr>
<td>OSR2</td>
<td>NM_053001</td>
<td>10,030801</td>
<td>9,532430</td>
<td>11,525915</td>
<td>-2,002182</td>
<td>0.045549</td>
</tr>
<tr>
<td>AKR1CL1</td>
<td>NR_027916</td>
<td>12,447831</td>
<td>12,025318</td>
<td>13,715371</td>
<td>-1,995095</td>
<td>0.036724</td>
</tr>
<tr>
<td>MLPH</td>
<td>NM_024101</td>
<td>7,729478</td>
<td>7,349071</td>
<td>8,870698</td>
<td>-1,911915</td>
<td>0.024086</td>
</tr>
<tr>
<td>ADRA2A</td>
<td>NM_000681</td>
<td>11,382403</td>
<td>10,991532</td>
<td>12,555015</td>
<td>-1,911736</td>
<td>0.018458</td>
</tr>
<tr>
<td>TNFRSF14</td>
<td>NM_003820</td>
<td>12,882001</td>
<td>12,508036</td>
<td>14,003895</td>
<td>-1,882355</td>
<td>0.021922</td>
</tr>
<tr>
<td>SOD2</td>
<td>NM_001024465</td>
<td>12,694861</td>
<td>12,352505</td>
<td>13,721930</td>
<td>-1,867272</td>
<td>0.018458</td>
</tr>
<tr>
<td>MARCO</td>
<td>NM_006770</td>
<td>6,719238</td>
<td>6,272878</td>
<td>8,058319</td>
<td>-1,844429</td>
<td>0.032779</td>
</tr>
<tr>
<td>NPC1</td>
<td>NM_000271</td>
<td>10,323753</td>
<td>9,939615</td>
<td>11,476168</td>
<td>-1,836541</td>
<td>0.021749</td>
</tr>
<tr>
<td>ACS2</td>
<td>NM_018677</td>
<td>11,976638</td>
<td>11,624204</td>
<td>13,033940</td>
<td>-1,832412</td>
<td>0.036556</td>
</tr>
<tr>
<td>SRD5A2</td>
<td>NM_000348</td>
<td>6,633735</td>
<td>6,154948</td>
<td>8,070097</td>
<td>-1,828742</td>
<td>0.006397</td>
</tr>
<tr>
<td>IL1R1</td>
<td>NM_000877</td>
<td>9,268618</td>
<td>8,841669</td>
<td>10,549464</td>
<td>-1,828314</td>
<td>0.015994</td>
</tr>
<tr>
<td>AKR1C1</td>
<td>NM_001353</td>
<td>14,011545</td>
<td>13,622876</td>
<td>15,177553</td>
<td>-1,823560</td>
<td>0.033244</td>
</tr>
<tr>
<td>ANKRD29</td>
<td>NM_173505</td>
<td>9,289354</td>
<td>8,936632</td>
<td>10,347520</td>
<td>-1,806677</td>
<td>0.016353</td>
</tr>
<tr>
<td>DAPK2</td>
<td>NM_014326</td>
<td>9,766765</td>
<td>9,410075</td>
<td>10,836836</td>
<td>-1,787063</td>
<td>0.029951</td>
</tr>
<tr>
<td>GNPTAB</td>
<td>NM_024312</td>
<td>12,326306</td>
<td>12,014012</td>
<td>13,263185</td>
<td>-1,780391</td>
<td>0.025419</td>
</tr>
<tr>
<td>VWCE</td>
<td>NM_152718</td>
<td>12,232482</td>
<td>11,849083</td>
<td>13,382679</td>
<td>-1,756586</td>
<td>0.039798</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Systematic Name</td>
<td>AveExpr</td>
<td>baseMean</td>
<td>logFC</td>
<td>adj.P.Val</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>---------</td>
<td>----------</td>
<td>-------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>HAND2</td>
<td>NM_021973</td>
<td>8,45386</td>
<td>7,948929</td>
<td>-1,739758</td>
<td>0,023519</td>
<td></td>
</tr>
<tr>
<td>TBX5</td>
<td>NM_000192</td>
<td>6,611459</td>
<td>6,192990</td>
<td>-1,737319</td>
<td>0,011741</td>
<td></td>
</tr>
<tr>
<td>C15orf59</td>
<td>NM_001039614</td>
<td>11,634392</td>
<td>11,305984</td>
<td>-1,710900</td>
<td>0,029951</td>
<td></td>
</tr>
<tr>
<td>HIP1R</td>
<td>NM_003959</td>
<td>11,253396</td>
<td>10,900995</td>
<td>-1,709087</td>
<td>0,045474</td>
<td></td>
</tr>
<tr>
<td>NIPAL4</td>
<td>NM_001172292</td>
<td>7,076149</td>
<td>6,686588</td>
<td>-1,683706</td>
<td>0,018387</td>
<td></td>
</tr>
<tr>
<td>RIPK3</td>
<td>NM_004794</td>
<td>11,253396</td>
<td>10,900995</td>
<td>-1,709087</td>
<td>0,045474</td>
<td></td>
</tr>
<tr>
<td>RAB33A</td>
<td>NM_003641</td>
<td>11,903987</td>
<td>11,602730</td>
<td>-1,625221</td>
<td>0,029010</td>
<td></td>
</tr>
<tr>
<td>IFITM1</td>
<td>NM_003641</td>
<td>14,002439</td>
<td>13,628618</td>
<td>-1,631308</td>
<td>0,018458</td>
<td></td>
</tr>
<tr>
<td>STEAP1B</td>
<td>NM_001164460</td>
<td>11,903987</td>
<td>11,602730</td>
<td>-1,625221</td>
<td>0,029010</td>
<td></td>
</tr>
<tr>
<td>MMD</td>
<td>NM_001039614</td>
<td>8,570794</td>
<td>8,253157</td>
<td>-1,671237</td>
<td>0,049084</td>
<td></td>
</tr>
<tr>
<td>SLC29A1</td>
<td>NM_001172292</td>
<td>10,664355</td>
<td>10,288458</td>
<td>-1,651790</td>
<td>0,009313</td>
<td></td>
</tr>
<tr>
<td>DIRAS1</td>
<td>NM_145173</td>
<td>11,950358</td>
<td>11,571936</td>
<td>-1,604594</td>
<td>0,041663</td>
<td></td>
</tr>
<tr>
<td>SLC47A1</td>
<td>NM_018249</td>
<td>7,957923</td>
<td>7,719768</td>
<td>-1,603833</td>
<td>0,029951</td>
<td></td>
</tr>
<tr>
<td>C1orf151-NBL1</td>
<td>NM_001204088</td>
<td>16,126337</td>
<td>15,820512</td>
<td>-1,585648</td>
<td>0,048254</td>
<td></td>
</tr>
<tr>
<td>IFITM1</td>
<td>NM_003641</td>
<td>12,892116</td>
<td>12,529636</td>
<td>-1,582255</td>
<td>0,016668</td>
<td></td>
</tr>
<tr>
<td>AKR1C1</td>
<td>NM_001353</td>
<td>11,950358</td>
<td>11,571936</td>
<td>-1,604594</td>
<td>0,041663</td>
<td></td>
</tr>
<tr>
<td>SLC31A2</td>
<td>NM_001860</td>
<td>11,706060</td>
<td>11,368574</td>
<td>-1,569491</td>
<td>0,017052</td>
<td></td>
</tr>
<tr>
<td>XLOC_i2_013458</td>
<td>TCONS_i2_00025964</td>
<td>6,708555</td>
<td>6,298724</td>
<td>-1,563284</td>
<td>0,045820</td>
<td></td>
</tr>
<tr>
<td>SLCO3A1</td>
<td>NM_013272</td>
<td>8,115865</td>
<td>7,813023</td>
<td>-1,546912</td>
<td>0,048673</td>
<td></td>
</tr>
<tr>
<td>AK123797</td>
<td>AK123797</td>
<td>8,358866</td>
<td>8,005838</td>
<td>-1,544182</td>
<td>0,020995</td>
<td></td>
</tr>
<tr>
<td>MRPS6</td>
<td>NM_032476</td>
<td>14,519547</td>
<td>14,195179</td>
<td>-1,538523</td>
<td>0,032691</td>
<td></td>
</tr>
<tr>
<td>LAYN</td>
<td>NM_178834</td>
<td>13,568881</td>
<td>13,260840</td>
<td>-1,531479</td>
<td>0,021922</td>
<td></td>
</tr>
<tr>
<td>DPR1</td>
<td>NM_000906</td>
<td>7,169741</td>
<td>6,851180</td>
<td>-1,527909</td>
<td>0,029951</td>
<td></td>
</tr>
<tr>
<td>ZCCHC14</td>
<td>NM_015144</td>
<td>8,553508</td>
<td>8,243811</td>
<td>-1,526234</td>
<td>0,018387</td>
<td></td>
</tr>
<tr>
<td>DNM3</td>
<td>NM_001136127</td>
<td>8,266430</td>
<td>7,956137</td>
<td>-1,520638</td>
<td>0,021922</td>
<td></td>
</tr>
<tr>
<td>IL1R1</td>
<td>NM_181078</td>
<td>8,193316</td>
<td>7,937499</td>
<td>-1,498719</td>
<td>0,013265</td>
<td></td>
</tr>
<tr>
<td>ACSL1</td>
<td>NM_001995</td>
<td>10,404482</td>
<td>10,078389</td>
<td>-1,489986</td>
<td>0,024086</td>
<td></td>
</tr>
<tr>
<td>CDH6</td>
<td>NM_004932</td>
<td>7,917944</td>
<td>7,494905</td>
<td>-1,479552</td>
<td>0,039461</td>
<td></td>
</tr>
<tr>
<td>C21orf91</td>
<td>NM_017447</td>
<td>8,225998</td>
<td>7,877825</td>
<td>-1,474789</td>
<td>0,017052</td>
<td></td>
</tr>
<tr>
<td>IL1R1</td>
<td>NM_000877</td>
<td>9,682260</td>
<td>9,383682</td>
<td>-1,469254</td>
<td>0,019640</td>
<td></td>
</tr>
<tr>
<td>ARID5B</td>
<td>NM_032199</td>
<td>13,272581</td>
<td>12,950566</td>
<td>-1,468834</td>
<td>0,025419</td>
<td></td>
</tr>
<tr>
<td>PLXNC1</td>
<td>NM_005761</td>
<td>7,717520</td>
<td>7,417176</td>
<td>-1,459718</td>
<td>0,022176</td>
<td></td>
</tr>
<tr>
<td>GADD45G</td>
<td>NM_006705</td>
<td>8,251830</td>
<td>7,878718</td>
<td>-1,459569</td>
<td>0,025481</td>
<td></td>
</tr>
<tr>
<td>Gene Name</td>
<td>Systematic Name</td>
<td>AveExpr</td>
<td>baseMean</td>
<td>baseMean</td>
<td>logFC</td>
<td>adj.P.Val</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>----------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>ZNF385A</td>
<td>NM_015481</td>
<td>10,563166</td>
<td>10,221513</td>
<td>11,588125</td>
<td>-1,456692</td>
<td>0.023519</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>NM_003739</td>
<td>14,626305</td>
<td>14,323885</td>
<td>15,533564</td>
<td>-1,455472</td>
<td>0.027246</td>
</tr>
<tr>
<td>SMOG2</td>
<td>NM_022138</td>
<td>6,887499</td>
<td>6,580005</td>
<td>7,809979</td>
<td>-1,452722</td>
<td>0.011741</td>
</tr>
<tr>
<td>ATP8B4</td>
<td>NM_024837</td>
<td>7,339801</td>
<td>7,064066</td>
<td>8,167008</td>
<td>-1,451959</td>
<td>0.031276</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>10,362117</td>
<td>10,107109</td>
<td>11,127143</td>
<td>-1,447602</td>
<td>0.011479</td>
</tr>
<tr>
<td>LOC100506748</td>
<td>XR_109878</td>
<td>10,310825</td>
<td>10,045658</td>
<td>11,106324</td>
<td>-1,444449</td>
<td>0.024086</td>
</tr>
<tr>
<td>KRTDAP</td>
<td>NM_207392</td>
<td>7,196041</td>
<td>6,917244</td>
<td>8,032429</td>
<td>-1,440828</td>
<td>0.024086</td>
</tr>
<tr>
<td>LRG1</td>
<td>NM_015541</td>
<td>10,951348</td>
<td>10,622462</td>
<td>11,938005</td>
<td>-1,422989</td>
<td>0.039665</td>
</tr>
<tr>
<td>ZCCHC14</td>
<td>NM_015144</td>
<td>11,903648</td>
<td>11,591852</td>
<td>12,839038</td>
<td>-1,418797</td>
<td>0.029951</td>
</tr>
<tr>
<td>GNPTAB</td>
<td>NM_024312</td>
<td>9,384816</td>
<td>9,103441</td>
<td>10,228939</td>
<td>-1,417116</td>
<td>0.015994</td>
</tr>
<tr>
<td>EEA1</td>
<td>NM_003566</td>
<td>12,833771</td>
<td>12,557574</td>
<td>13,662362</td>
<td>-1,414254</td>
<td>0.033106</td>
</tr>
<tr>
<td>LOC400043</td>
<td>NR_026656</td>
<td>12,912842</td>
<td>12,573949</td>
<td>13,929521</td>
<td>-1,406491</td>
<td>0.032989</td>
</tr>
<tr>
<td>ABHD5</td>
<td>NM_016006</td>
<td>10,523078</td>
<td>10,219335</td>
<td>11,434306</td>
<td>-1,394236</td>
<td>0.029148</td>
</tr>
<tr>
<td>MBOAT1</td>
<td>NM_001080480</td>
<td>7,808677</td>
<td>7,499054</td>
<td>8,737546</td>
<td>-1,389146</td>
<td>0.025409</td>
</tr>
<tr>
<td>LSS</td>
<td>NM_001001438</td>
<td>9,661136</td>
<td>9,357082</td>
<td>10,573300</td>
<td>-1,383025</td>
<td>0.046477</td>
</tr>
<tr>
<td>STEAP1</td>
<td>NM_012449</td>
<td>11,593063</td>
<td>11,370467</td>
<td>12,260853</td>
<td>-1,372612</td>
<td>0.018387</td>
</tr>
<tr>
<td>EHD3</td>
<td>NM_014600</td>
<td>9,307785</td>
<td>9,005627</td>
<td>10,214260</td>
<td>-1,367688</td>
<td>0.017052</td>
</tr>
<tr>
<td>KIF13B</td>
<td>NM_015254</td>
<td>7,425438</td>
<td>7,137660</td>
<td>8,288772</td>
<td>-1,349626</td>
<td>0.032989</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>9,251408</td>
<td>8,997630</td>
<td>10,012740</td>
<td>-1,346334</td>
<td>0.016353</td>
</tr>
<tr>
<td>FBLN1</td>
<td>NM_001996</td>
<td>16,283068</td>
<td>16,049251</td>
<td>16,984518</td>
<td>-1,332146</td>
<td>0.017052</td>
</tr>
<tr>
<td>LOC100505874</td>
<td>XR_110950</td>
<td>6,774184</td>
<td>6,427229</td>
<td>7,815051</td>
<td>-1,331689</td>
<td>0.027507</td>
</tr>
<tr>
<td>AOX1</td>
<td>NM_001159</td>
<td>11,692814</td>
<td>11,475783</td>
<td>12,343909</td>
<td>-1,324904</td>
<td>0.038090</td>
</tr>
<tr>
<td>MITF</td>
<td>NM_198159</td>
<td>7,391321</td>
<td>7,102825</td>
<td>8,256811</td>
<td>-1,324346</td>
<td>0.038046</td>
</tr>
<tr>
<td>SLC39A8</td>
<td>NM_001135147</td>
<td>8,270826</td>
<td>8,023038</td>
<td>9,014191</td>
<td>-1,316352</td>
<td>0.026943</td>
</tr>
<tr>
<td>PKIG</td>
<td>NM_181805</td>
<td>12,222112</td>
<td>11,908315</td>
<td>13,163501</td>
<td>-1,305076</td>
<td>0.024086</td>
</tr>
<tr>
<td>SIX2</td>
<td>NM_016932</td>
<td>6,675144</td>
<td>6,437547</td>
<td>7,387932</td>
<td>-1,298993</td>
<td>0.013265</td>
</tr>
<tr>
<td>PFKFB3</td>
<td>NM_004566</td>
<td>9,816364</td>
<td>9,568537</td>
<td>10,559845</td>
<td>-1,287585</td>
<td>0.038847</td>
</tr>
<tr>
<td>TDRD6</td>
<td>NM_001010870</td>
<td>7,059928</td>
<td>6,802617</td>
<td>7,831862</td>
<td>-1,285145</td>
<td>0.048405</td>
</tr>
<tr>
<td>ANKRD28</td>
<td>NM_015199</td>
<td>11,065565</td>
<td>10,812643</td>
<td>11,824328</td>
<td>-1,283156</td>
<td>0.029951</td>
</tr>
<tr>
<td>PMP22</td>
<td>NM_000304</td>
<td>15,727830</td>
<td>15,452100</td>
<td>16,555022</td>
<td>-1,271040</td>
<td>0.023519</td>
</tr>
<tr>
<td>CYP7B1</td>
<td>NM_004820</td>
<td>7,346000</td>
<td>7,080424</td>
<td>8,142729</td>
<td>-1,270701</td>
<td>0.024086</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>9,589288</td>
<td>9,351665</td>
<td>10,302159</td>
<td>-1,256390</td>
<td>0.015994</td>
</tr>
<tr>
<td>KCNE3</td>
<td>NM_005472</td>
<td>6,829011</td>
<td>6,574696</td>
<td>7,591957</td>
<td>-1,239226</td>
<td>0.024228</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr_xpc_wt</th>
<th>baseMean_xpc</th>
<th>baseMean_wt</th>
<th>logFC_xpc_wt</th>
<th>adj.P.Val_xpc_wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLCA2</td>
<td>NM_006536</td>
<td>6,994958</td>
<td>6,756860</td>
<td>7,709253</td>
<td>-1,234286</td>
<td>0,018458</td>
</tr>
<tr>
<td>HS3ST2</td>
<td>NM_006043</td>
<td>6,752596</td>
<td>6,519093</td>
<td>7,453105</td>
<td>-1,227123</td>
<td>0,023519</td>
</tr>
<tr>
<td>DSEL</td>
<td>NM_032160</td>
<td>10,126771</td>
<td>9,908924</td>
<td>10,780311</td>
<td>-1,221124</td>
<td>0,032989</td>
</tr>
<tr>
<td>FAM110B</td>
<td>NM_147189</td>
<td>8,916449</td>
<td>8,675265</td>
<td>9,640004</td>
<td>-1,217734</td>
<td>0,017729</td>
</tr>
<tr>
<td>SMARCA2</td>
<td>NM_139045</td>
<td>10,259560</td>
<td>9,969584</td>
<td>11,129489</td>
<td>-1,213285</td>
<td>0,035183</td>
</tr>
<tr>
<td>LOC100144604</td>
<td>NR_021493</td>
<td>8,220937</td>
<td>7,945191</td>
<td>9,048176</td>
<td>-1,204084</td>
<td>0,024086</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>8,552806</td>
<td>8,330738</td>
<td>9,219011</td>
<td>-1,201299</td>
<td>0,011741</td>
</tr>
<tr>
<td>LOC400043</td>
<td>NR_026656</td>
<td>9,986240</td>
<td>9,705866</td>
<td>10,827362</td>
<td>-1,198437</td>
<td>0,047637</td>
</tr>
<tr>
<td>KIAA1522</td>
<td>NM_020888</td>
<td>11,846780</td>
<td>11,581400</td>
<td>12,642917</td>
<td>-1,196520</td>
<td>0,032691</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>10,714812</td>
<td>10,518921</td>
<td>11,302487</td>
<td>-1,189720</td>
<td>0,029951</td>
</tr>
<tr>
<td>PPP4R4</td>
<td>NM_058237</td>
<td>7,066212</td>
<td>6,876589</td>
<td>7,635082</td>
<td>-1,169200</td>
<td>0,045549</td>
</tr>
<tr>
<td>STRADB</td>
<td>NM_018571</td>
<td>10,591050</td>
<td>10,305770</td>
<td>11,446889</td>
<td>-1,168034</td>
<td>0,031276</td>
</tr>
<tr>
<td>UNC93B1</td>
<td>NM_030930</td>
<td>12,125459</td>
<td>11,856231</td>
<td>12,933142</td>
<td>-1,166200</td>
<td>0,037876</td>
</tr>
<tr>
<td>SULT4A1</td>
<td>NM_014351</td>
<td>7,104674</td>
<td>6,794571</td>
<td>8,034981</td>
<td>-1,164982</td>
<td>0,035102</td>
</tr>
<tr>
<td>PLD1</td>
<td>NM_002662</td>
<td>8,082689</td>
<td>7,829937</td>
<td>8,840943</td>
<td>-1,151383</td>
<td>0,034283</td>
</tr>
<tr>
<td>HSD17B7</td>
<td>NM_016371</td>
<td>8,646108</td>
<td>8,417066</td>
<td>9,333232</td>
<td>-1,141851</td>
<td>0,042766</td>
</tr>
<tr>
<td>STRADB</td>
<td>NM_018571</td>
<td>11,885662</td>
<td>11,615801</td>
<td>12,695246</td>
<td>-1,137845</td>
<td>0,037103</td>
</tr>
<tr>
<td>MTMR9LP</td>
<td>NR_026850</td>
<td>11,298343</td>
<td>11,066733</td>
<td>11,993172</td>
<td>-1,137077</td>
<td>0,029151</td>
</tr>
<tr>
<td>LPIN1</td>
<td>NM_145693</td>
<td>13,684510</td>
<td>13,458075</td>
<td>14,363813</td>
<td>-1,120025</td>
<td>0,024086</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>9,897924</td>
<td>9,712262</td>
<td>10,454912</td>
<td>-1,119445</td>
<td>0,034283</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>NM_018896</td>
<td>7,418354</td>
<td>7,213033</td>
<td>8,034317</td>
<td>-1,112736</td>
<td>0,029951</td>
</tr>
<tr>
<td>TBX18</td>
<td>NM_001080508</td>
<td>7,746036</td>
<td>7,507373</td>
<td>8,462022</td>
<td>-1,107939</td>
<td>0,034283</td>
</tr>
<tr>
<td>VAMP4</td>
<td>NM_003762</td>
<td>8,945100</td>
<td>8,682156</td>
<td>9,733994</td>
<td>-1,107272</td>
<td>0,031559</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>10,234256</td>
<td>10,048224</td>
<td>10,792352</td>
<td>-1,102618</td>
<td>0,024086</td>
</tr>
<tr>
<td>TGFA</td>
<td>NM_003236</td>
<td>6,323519</td>
<td>6,037064</td>
<td>7,182885</td>
<td>-1,066069</td>
<td>0,036869</td>
</tr>
<tr>
<td>AADACL2</td>
<td>NM_207365</td>
<td>6,285105</td>
<td>6,011648</td>
<td>7,105475</td>
<td>-1,060664</td>
<td>0,024086</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>7,669168</td>
<td>7,466469</td>
<td>8,277263</td>
<td>-1,054985</td>
<td>0,018458</td>
</tr>
<tr>
<td>pS1TPTE22</td>
<td>AK001299</td>
<td>6,514254</td>
<td>6,272880</td>
<td>7,238734</td>
<td>-1,049598</td>
<td>0,030213</td>
</tr>
<tr>
<td>TBCD1D4</td>
<td>NM_014832</td>
<td>8,517779</td>
<td>8,262259</td>
<td>9,284339</td>
<td>-1,049530</td>
<td>0,032691</td>
</tr>
<tr>
<td>NEDD4L</td>
<td>NM_001144967</td>
<td>7,118408</td>
<td>6,931292</td>
<td>7,679755</td>
<td>-1,033386</td>
<td>0,038489</td>
</tr>
<tr>
<td>A_33_P3329740</td>
<td>A_33_P3329740</td>
<td>12,922944</td>
<td>12,644086</td>
<td>13,759520</td>
<td>-1,032860</td>
<td>0,034283</td>
</tr>
<tr>
<td>ASAH1</td>
<td>NM_177924</td>
<td>14,401283</td>
<td>14,253706</td>
<td>14,844013</td>
<td>-1,031727</td>
<td>0,034283</td>
</tr>
<tr>
<td>NFIL3</td>
<td>NM_005384</td>
<td>11,877366</td>
<td>11,639432</td>
<td>12,591169</td>
<td>-1,027848</td>
<td>0,029951</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr_WT</th>
<th>baseMean_WT</th>
<th>baseMean_XPC</th>
<th>logFC_WT</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEM63A</td>
<td>NM_014698</td>
<td>8,104936</td>
<td>7,903278</td>
<td>8,709911</td>
<td>-1,022290</td>
<td>0,029951</td>
</tr>
<tr>
<td>DNAH7</td>
<td>NM_018897</td>
<td>6,750491</td>
<td>6,547402</td>
<td>7,359758</td>
<td>-1,020667</td>
<td>0,041698</td>
</tr>
<tr>
<td>SLC6A8</td>
<td>NM_005629</td>
<td>9,789853</td>
<td>9,621331</td>
<td>10,295419</td>
<td>-1,018066</td>
<td>0,029951</td>
</tr>
<tr>
<td>LOC255480</td>
<td>NR_038440</td>
<td>6,515405</td>
<td>6,254855</td>
<td>7,297056</td>
<td>-1,014106</td>
<td>0,030213</td>
</tr>
<tr>
<td>ITIH1</td>
<td>NM_002215</td>
<td>6,600710</td>
<td>6,358608</td>
<td>7,327017</td>
<td>-1,008639</td>
<td>0,024086</td>
</tr>
<tr>
<td>SERPING1</td>
<td>NM_000062</td>
<td>10,197568</td>
<td>10,005836</td>
<td>10,772762</td>
<td>-1,008021</td>
<td>0,045223</td>
</tr>
<tr>
<td>KANK2</td>
<td>NM_015493</td>
<td>14,896766</td>
<td>14,682770</td>
<td>15,538752</td>
<td>-1,004161</td>
<td>0,029951</td>
</tr>
<tr>
<td>ATG2A</td>
<td>NM_015104</td>
<td>11,604569</td>
<td>11,386322</td>
<td>12,259311</td>
<td>-1,002344</td>
<td>0,036556</td>
</tr>
<tr>
<td>SWAP70</td>
<td>NM_015055</td>
<td>12,08148</td>
<td>11,769229</td>
<td>12,724906</td>
<td>-0,998037</td>
<td>0,021922</td>
</tr>
<tr>
<td>XLOC_001853</td>
<td>TCONS_00003387</td>
<td>11,438251</td>
<td>11,158489</td>
<td>12,277540</td>
<td>-0,997272</td>
<td>0,032547</td>
</tr>
<tr>
<td>psiTPTE22</td>
<td>AK226145</td>
<td>6,644316</td>
<td>6,399773</td>
<td>7,377943</td>
<td>-0,996076</td>
<td>0,030213</td>
</tr>
<tr>
<td>CTSB</td>
<td>NM_147780</td>
<td>14,348812</td>
<td>14,187638</td>
<td>14,832334</td>
<td>-0,987998</td>
<td>0,045223</td>
</tr>
<tr>
<td>HR</td>
<td>NM_005144</td>
<td>7,292759</td>
<td>7,030197</td>
<td>8,080447</td>
<td>-0,983968</td>
<td>0,038489</td>
</tr>
<tr>
<td>ANKRD26P1</td>
<td>NR_026556</td>
<td>6,758190</td>
<td>6,516692</td>
<td>7,482868</td>
<td>-0,976824</td>
<td>0,029951</td>
</tr>
<tr>
<td>XLOC_004881</td>
<td>TCONS_00010961</td>
<td>7,279615</td>
<td>7,089628</td>
<td>7,849577</td>
<td>-0,975800</td>
<td>0,024086</td>
</tr>
<tr>
<td>PNMA6C</td>
<td>NM_001170944</td>
<td>8,868836</td>
<td>8,684170</td>
<td>9,422834</td>
<td>-0,966702</td>
<td>0,036556</td>
</tr>
<tr>
<td>FLJ11235</td>
<td>NR_027706</td>
<td>6,431994</td>
<td>6,222439</td>
<td>7,060659</td>
<td>-0,949113</td>
<td>0,028846</td>
</tr>
<tr>
<td>TRAK2</td>
<td>NM_015049</td>
<td>10,583076</td>
<td>10,373761</td>
<td>11,211018</td>
<td>-0,943610</td>
<td>0,039798</td>
</tr>
<tr>
<td>TMEM38B</td>
<td>NM_018112</td>
<td>9,905417</td>
<td>9,715580</td>
<td>10,474928</td>
<td>-0,943392</td>
<td>0,034283</td>
</tr>
<tr>
<td>GSN</td>
<td>NM_198252</td>
<td>14,293602</td>
<td>14,066465</td>
<td>14,975013</td>
<td>-0,941580</td>
<td>0,031276</td>
</tr>
<tr>
<td>WDR81</td>
<td>NM_152348</td>
<td>12,806475</td>
<td>12,614864</td>
<td>13,381309</td>
<td>-0,918144</td>
<td>0,029951</td>
</tr>
<tr>
<td>LOC440104</td>
<td>NR_036476</td>
<td>9,730172</td>
<td>9,503000</td>
<td>10,411687</td>
<td>-0,917611</td>
<td>0,036724</td>
</tr>
<tr>
<td>NOXA1</td>
<td>NM_006647</td>
<td>8,250595</td>
<td>8,021977</td>
<td>8,936448</td>
<td>-0,917461</td>
<td>0,018387</td>
</tr>
<tr>
<td>PPFIB2</td>
<td>NM_003621</td>
<td>9,260149</td>
<td>9,060840</td>
<td>9,858076</td>
<td>-0,915841</td>
<td>0,035869</td>
</tr>
<tr>
<td>MTMR9LP</td>
<td>NR_026850</td>
<td>9,533843</td>
<td>9,367220</td>
<td>10,033711</td>
<td>-0,909669</td>
<td>0,036556</td>
</tr>
<tr>
<td>PPFIA4</td>
<td>NM_015053</td>
<td>6,969702</td>
<td>6,758790</td>
<td>7,602439</td>
<td>-0,907753</td>
<td>0,029951</td>
</tr>
<tr>
<td>TPCN2</td>
<td>NM_139075</td>
<td>8,755681</td>
<td>8,550801</td>
<td>9,370321</td>
<td>-0,905939</td>
<td>0,036724</td>
</tr>
<tr>
<td>FLJ43663</td>
<td>NR_015431</td>
<td>7,344772</td>
<td>7,145514</td>
<td>7,942545</td>
<td>-0,903294</td>
<td>0,029700</td>
</tr>
<tr>
<td>DSEL</td>
<td>NM_032160</td>
<td>12,345018</td>
<td>12,189860</td>
<td>12,810491</td>
<td>-0,902891</td>
<td>0,029951</td>
</tr>
<tr>
<td>HSPBAP1</td>
<td>NM_024610</td>
<td>9,008029</td>
<td>8,825165</td>
<td>9,556621</td>
<td>-0,896559</td>
<td>0,047587</td>
</tr>
<tr>
<td>THC2544427</td>
<td>THC2544427</td>
<td>8,134509</td>
<td>7,968431</td>
<td>8,632743</td>
<td>-0,894481</td>
<td>0,034283</td>
</tr>
<tr>
<td>SLC4A7</td>
<td>NM_003615</td>
<td>8,982918</td>
<td>8,784473</td>
<td>9,578252</td>
<td>-0,878985</td>
<td>0,032989</td>
</tr>
<tr>
<td>USP6</td>
<td>NM_004505</td>
<td>9,483944</td>
<td>9,306164</td>
<td>10,017282</td>
<td>-0,871408</td>
<td>0,035764</td>
</tr>
</tbody>
</table>

_(Continued on next page)_
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr_xpc_wt</th>
<th>baseMean_xpc</th>
<th>baseMean_wt</th>
<th>logFC_xpc_wt</th>
<th>adj.P.Val_xpc_wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>XLOC_l2_009140</td>
<td>ENST00000449602</td>
<td>6,711726</td>
<td>6,500453</td>
<td>7,345547</td>
<td>-0,869460</td>
<td>0,029951</td>
</tr>
<tr>
<td>IFIT3</td>
<td>NM_001549</td>
<td>10,905647</td>
<td>10,691554</td>
<td>11,547926</td>
<td>-0,862504</td>
<td>0,032691</td>
</tr>
<tr>
<td>RASSF2</td>
<td>ENST00000478553</td>
<td>7,402318</td>
<td>7,230145</td>
<td>7,918837</td>
<td>-0,855942</td>
<td>0,034283</td>
</tr>
<tr>
<td>NMUR1</td>
<td>NM_006056</td>
<td>6,695553</td>
<td>6,485803</td>
<td>7,324801</td>
<td>-0,854985</td>
<td>0,045223</td>
</tr>
<tr>
<td>SLFN12</td>
<td>NM_018042</td>
<td>7,808354</td>
<td>7,635134</td>
<td>8,328016</td>
<td>-0,851712</td>
<td>0,024228</td>
</tr>
<tr>
<td>A_33_P3320283</td>
<td>A_33_P3320283</td>
<td>6,167111</td>
<td>5,983770</td>
<td>6,720333</td>
<td>-0,847826</td>
<td>0,038090</td>
</tr>
<tr>
<td>IFIT3</td>
<td>NM_001549</td>
<td>10,905647</td>
<td>10,691554</td>
<td>11,547926</td>
<td>-0,862504</td>
<td>0,032691</td>
</tr>
<tr>
<td>RASSF2</td>
<td>ENST00000478553</td>
<td>7,402318</td>
<td>7,230145</td>
<td>7,918837</td>
<td>-0,855942</td>
<td>0,034283</td>
</tr>
<tr>
<td>NMUR1</td>
<td>NM_006056</td>
<td>6,695553</td>
<td>6,485803</td>
<td>7,324801</td>
<td>-0,854985</td>
<td>0,045223</td>
</tr>
<tr>
<td>SLFN12</td>
<td>NM_018042</td>
<td>7,808354</td>
<td>7,635134</td>
<td>8,328016</td>
<td>-0,851712</td>
<td>0,024228</td>
</tr>
<tr>
<td>A_33_P3320283</td>
<td>A_33_P3320283</td>
<td>6,167111</td>
<td>5,983770</td>
<td>6,720333</td>
<td>-0,847826</td>
<td>0,038090</td>
</tr>
<tr>
<td>TAP1</td>
<td>NM_000593</td>
<td>12,513287</td>
<td>12,338011</td>
<td>13,09114</td>
<td>-0,837762</td>
<td>0,046661</td>
</tr>
<tr>
<td>AFF1</td>
<td>NM_005935</td>
<td>8,536550</td>
<td>8,366713</td>
<td>9,046063</td>
<td>-0,825478</td>
<td>0,045456</td>
</tr>
<tr>
<td>SPTBN4</td>
<td>NM_020971</td>
<td>7,321016</td>
<td>7,125126</td>
<td>7,908637</td>
<td>-0,825229</td>
<td>0,030357</td>
</tr>
<tr>
<td>IL6ST</td>
<td>NM_002184</td>
<td>11,738022</td>
<td>11,568056</td>
<td>12,247921</td>
<td>-0,820602</td>
<td>0,045223</td>
</tr>
<tr>
<td>B3GNT7</td>
<td>NM_145236</td>
<td>8,346694</td>
<td>8,152430</td>
<td>8,929487</td>
<td>-0,819915</td>
<td>0,041792</td>
</tr>
<tr>
<td>WIPI1</td>
<td>NM_017983</td>
<td>7,321016</td>
<td>7,125126</td>
<td>7,908637</td>
<td>-0,825229</td>
<td>0,030357</td>
</tr>
<tr>
<td>RSC1A1</td>
<td>NM_002184</td>
<td>11,738022</td>
<td>11,568056</td>
<td>12,247921</td>
<td>-0,820602</td>
<td>0,038489</td>
</tr>
<tr>
<td>TLE4</td>
<td>NM_002087</td>
<td>17,646867</td>
<td>17,493995</td>
<td>18,105480</td>
<td>-0,742778</td>
<td>0,035201</td>
</tr>
<tr>
<td>LOC100131089</td>
<td>NR_040059</td>
<td>7,365161</td>
<td>7,217807</td>
<td>7,807221</td>
<td>-0,735099</td>
<td>0,037978</td>
</tr>
<tr>
<td>CTSA</td>
<td>NM_00308</td>
<td>16,029775</td>
<td>15,900448</td>
<td>16,417758</td>
<td>-0,712584</td>
<td>0,041596</td>
</tr>
<tr>
<td>HERC2</td>
<td>NM_004667</td>
<td>9,56451</td>
<td>9,420094</td>
<td>10,005520</td>
<td>-0,711452</td>
<td>0,046661</td>
</tr>
<tr>
<td>PTPN11</td>
<td>NM_002834</td>
<td>12,286044</td>
<td>12,130894</td>
<td>12,751492</td>
<td>-0,710959</td>
<td>0,049203</td>
</tr>
<tr>
<td>STARD9</td>
<td>NM_020759</td>
<td>6,848940</td>
<td>6,693324</td>
<td>7,313990</td>
<td>-0,690058</td>
<td>0,037876</td>
</tr>
<tr>
<td>CR627426</td>
<td>CR627426</td>
<td>6,885925</td>
<td>6,740786</td>
<td>7,321340</td>
<td>-0,684458</td>
<td>0,046477</td>
</tr>
<tr>
<td>DTX3L</td>
<td>NM_138287</td>
<td>8,639698</td>
<td>8,493522</td>
<td>9,078227</td>
<td>-0,616665</td>
<td>0,048328</td>
</tr>
<tr>
<td>TFI2</td>
<td>NM_006528</td>
<td>12,033816</td>
<td>12,853899</td>
<td>9,573569</td>
<td>3,513599</td>
<td>0,036556</td>
</tr>
<tr>
<td>CDH13</td>
<td>NM_001257</td>
<td>11,195536</td>
<td>11,928949</td>
<td>8,995297</td>
<td>2,853326</td>
<td>0,049387</td>
</tr>
<tr>
<td>C9orf167</td>
<td>NM_017723</td>
<td>9,105734</td>
<td>9,787507</td>
<td>7,060415</td>
<td>2,803456</td>
<td>0,049203</td>
</tr>
<tr>
<td>LHX9</td>
<td>NM_020204</td>
<td>8,201783</td>
<td>8,770490</td>
<td>6,495663</td>
<td>2,595684</td>
<td>0,018458</td>
</tr>
<tr>
<td>SGK223</td>
<td>NM_001808026</td>
<td>9,687380</td>
<td>10,207730</td>
<td>8,126332</td>
<td>2,225773</td>
<td>0,035102</td>
</tr>
<tr>
<td>HOXC10</td>
<td>NM_017409</td>
<td>9,168054</td>
<td>9,688024</td>
<td>7,608144</td>
<td>2,221504</td>
<td>0,011741</td>
</tr>
<tr>
<td>LRRC17</td>
<td>NM_005824</td>
<td>8,668098</td>
<td>9,136689</td>
<td>7,262325</td>
<td>2,198570</td>
<td>0,018458</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr</th>
<th>baseMean</th>
<th>baseMean</th>
<th>logFC</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP3</td>
<td>NM_001013398</td>
<td>15,831254</td>
<td>16,308410</td>
<td>14,399787</td>
<td>2,155716</td>
<td>0,036724</td>
</tr>
<tr>
<td>PRDM16</td>
<td>NM_022114</td>
<td>7,658894</td>
<td>8,066146</td>
<td>6,437137</td>
<td>2,132355</td>
<td>0,017744</td>
</tr>
<tr>
<td>CRIP1</td>
<td>NM_001311</td>
<td>13,761095</td>
<td>14,079410</td>
<td>12,806152</td>
<td>2,118895</td>
<td>0,035102</td>
</tr>
<tr>
<td>FBN2</td>
<td>NM_001999</td>
<td>11,395501</td>
<td>11,586963</td>
<td>10,821116</td>
<td>1,996619</td>
<td>0,028023</td>
</tr>
<tr>
<td>PCDH10</td>
<td>NM_020815</td>
<td>7,612941</td>
<td>8,017974</td>
<td>6,397841</td>
<td>1,914916</td>
<td>0,031559</td>
</tr>
<tr>
<td>CCDC85C</td>
<td>NM_001144995</td>
<td>8,265251</td>
<td>8,653081</td>
<td>7,101760</td>
<td>1,888633</td>
<td>0,024086</td>
</tr>
<tr>
<td>FAM176A</td>
<td>NM_003149</td>
<td>9,555894</td>
<td>9,951428</td>
<td>8,397841</td>
<td>1,876005</td>
<td>0,024086</td>
</tr>
<tr>
<td>STAC</td>
<td>NM_003149</td>
<td>9,555894</td>
<td>9,951428</td>
<td>8,397841</td>
<td>1,876005</td>
<td>0,024086</td>
</tr>
<tr>
<td>COL12A1</td>
<td>NM_004370</td>
<td>11,301945</td>
<td>11,725786</td>
<td>10,030423</td>
<td>1,696694</td>
<td>0,024086</td>
</tr>
<tr>
<td>MKX</td>
<td>NM_173576</td>
<td>10,354661</td>
<td>10,764472</td>
<td>9,124430</td>
<td>1,568389</td>
<td>0,032691</td>
</tr>
<tr>
<td>TNFSF4</td>
<td>NM_003326</td>
<td>8,105701</td>
<td>8,436452</td>
<td>6,146562</td>
<td>1,568389</td>
<td>0,024086</td>
</tr>
<tr>
<td>A_24_P49597</td>
<td>A_24_P49597</td>
<td>13,185993</td>
<td>13,567436</td>
<td>12,041665</td>
<td>1,573126</td>
<td>0,046661</td>
</tr>
<tr>
<td>SSTR1</td>
<td>NM_001049</td>
<td>9,941097</td>
<td>10,280798</td>
<td>8,921994</td>
<td>1,568389</td>
<td>0,023691</td>
</tr>
<tr>
<td>ATP6V1G3</td>
<td>NM_133326</td>
<td>7,249093</td>
<td>7,616603</td>
<td>6,146562</td>
<td>1,550011</td>
<td>0,024086</td>
</tr>
<tr>
<td>LRRC17</td>
<td>NM_001031692</td>
<td>7,469203</td>
<td>7,788708</td>
<td>6,510688</td>
<td>1,549754</td>
<td>0,029148</td>
</tr>
<tr>
<td>NEK7</td>
<td>NM_133494</td>
<td>8,037290</td>
<td>8,338803</td>
<td>7,132751</td>
<td>1,484517</td>
<td>0,046735</td>
</tr>
<tr>
<td>WNK4</td>
<td>NM_032387</td>
<td>7,070086</td>
<td>7,379785</td>
<td>6,140989</td>
<td>1,459853</td>
<td>0,049111</td>
</tr>
<tr>
<td>LOC100506995</td>
<td>XR_108482</td>
<td>7,472281</td>
<td>7,802612</td>
<td>6,481290</td>
<td>1,451416</td>
<td>0,048076</td>
</tr>
<tr>
<td>RPS26</td>
<td>NM_001029</td>
<td>14,985825</td>
<td>15,332524</td>
<td>13,945729</td>
<td>1,423332</td>
<td>0,03569</td>
</tr>
<tr>
<td>PABPC4L</td>
<td>NM_001114734</td>
<td>9,139647</td>
<td>9,479693</td>
<td>8,119509</td>
<td>1,398016</td>
<td>0,038489</td>
</tr>
<tr>
<td>LOC100505633</td>
<td>NR_038849</td>
<td>10,801113</td>
<td>11,049807</td>
<td>10,055032</td>
<td>1,394106</td>
<td>0,029661</td>
</tr>
<tr>
<td>PLAC8</td>
<td>NM_016619</td>
<td>7,889364</td>
<td>8,230568</td>
<td>6,865754</td>
<td>1,390903</td>
<td>0,018458</td>
</tr>
<tr>
<td>LOC100505633</td>
<td>NR_038849</td>
<td>10,588031</td>
<td>10,839648</td>
<td>9,833182</td>
<td>1,390146</td>
<td>0,027246</td>
</tr>
<tr>
<td>ALCAM</td>
<td>NM_001627</td>
<td>11,018966</td>
<td>11,344224</td>
<td>10,043194</td>
<td>1,381376</td>
<td>0,045820</td>
</tr>
<tr>
<td>LOC100505633</td>
<td>NR_038849</td>
<td>11,917962</td>
<td>12,162280</td>
<td>11,185006</td>
<td>1,367263</td>
<td>0,037646</td>
</tr>
<tr>
<td>RCN3</td>
<td>NM_020650</td>
<td>12,091638</td>
<td>12,356885</td>
<td>11,295899</td>
<td>1,354628</td>
<td>0,028280</td>
</tr>
<tr>
<td>RPS26</td>
<td>NM_001029</td>
<td>16,931566</td>
<td>17,275911</td>
<td>15,89531</td>
<td>1,354207</td>
<td>0,030207</td>
</tr>
<tr>
<td>SIX1</td>
<td>NM_005982</td>
<td>9,985266</td>
<td>10,240607</td>
<td>9,196865</td>
<td>1,336013</td>
<td>0,038489</td>
</tr>
<tr>
<td>C1orf53</td>
<td>NM_001024594</td>
<td>10,504821</td>
<td>10,768280</td>
<td>9,714446</td>
<td>1,322209</td>
<td>0,029010</td>
</tr>
<tr>
<td>SSPN</td>
<td>NM_005086</td>
<td>11,989330</td>
<td>12,252697</td>
<td>11,199228</td>
<td>1,310725</td>
<td>0,029064</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr</th>
<th>baseMean</th>
<th>baseMean</th>
<th>logFC</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOC100131262</td>
<td>XR_132952</td>
<td>11,866404</td>
<td>12,148606</td>
<td>11,019798</td>
<td>1,298083</td>
<td>0.036556</td>
</tr>
<tr>
<td>XLOC_010933</td>
<td>THC2654170</td>
<td>9,189199</td>
<td>9,436043</td>
<td>8,448666</td>
<td>1,279250</td>
<td>0.037585</td>
</tr>
<tr>
<td>CDC42EP3</td>
<td>NM_006449</td>
<td>8,272014</td>
<td>8,558628</td>
<td>7,412171</td>
<td>1,261356</td>
<td>0.032993</td>
</tr>
<tr>
<td>COL8A2</td>
<td>NM_005202</td>
<td>11,100668</td>
<td>11,400015</td>
<td>10,202626</td>
<td>1,257557</td>
<td>0.035102</td>
</tr>
<tr>
<td>PCDH7</td>
<td>NM_002589</td>
<td>9,170461</td>
<td>9,547493</td>
<td>8,039365</td>
<td>1,245412</td>
<td>0.049203</td>
</tr>
<tr>
<td>ABI3BP</td>
<td>NM_001542</td>
<td>9,940817</td>
<td>10,276910</td>
<td>8,932539</td>
<td>1,235387</td>
<td>0.049600</td>
</tr>
<tr>
<td>XLOC_009583</td>
<td>TH2695809</td>
<td>7,522396</td>
<td>7,727524</td>
<td>6,907013</td>
<td>1,225280</td>
<td>0.046661</td>
</tr>
<tr>
<td>ENST00000457049</td>
<td>ENST00000457049</td>
<td>10,135919</td>
<td>10,341014</td>
<td>9,520633</td>
<td>1,198257</td>
<td>0.026001</td>
</tr>
<tr>
<td>GLI2</td>
<td>NM_005270</td>
<td>7,945477</td>
<td>8,132515</td>
<td>7,384362</td>
<td>1,151299</td>
<td>0.032779</td>
</tr>
<tr>
<td>HIC1</td>
<td>NM_006497</td>
<td>10,137476</td>
<td>10,355054</td>
<td>9,484740</td>
<td>1,144536</td>
<td>0.046593</td>
</tr>
<tr>
<td>HMGN2</td>
<td>NM_005517</td>
<td>13,049900</td>
<td>13,267520</td>
<td>12,397038</td>
<td>1,141629</td>
<td>0.031265</td>
</tr>
<tr>
<td>EZH2</td>
<td>NM_004456</td>
<td>9,309292</td>
<td>9,565436</td>
<td>8,540859</td>
<td>1,133202</td>
<td>0.021922</td>
</tr>
<tr>
<td>TMEM200A</td>
<td>NM_0052913</td>
<td>11,955128</td>
<td>12,47276</td>
<td>11,078684</td>
<td>1,119390</td>
<td>0.040772</td>
</tr>
<tr>
<td>ENST00000450495</td>
<td>ENST00000450495</td>
<td>9,882456</td>
<td>10,077377</td>
<td>9,297693</td>
<td>1,110639</td>
<td>0.030213</td>
</tr>
<tr>
<td>NEXN</td>
<td>NM_144573</td>
<td>9,512094</td>
<td>9,808532</td>
<td>8,622780</td>
<td>1,104567</td>
<td>0.023519</td>
</tr>
<tr>
<td>HOXB2</td>
<td>NM_002145</td>
<td>11,751995</td>
<td>11,987152</td>
<td>11,046524</td>
<td>1,078748</td>
<td>0.036556</td>
</tr>
<tr>
<td>HMGN2</td>
<td>NM_005517</td>
<td>15,836119</td>
<td>16,070226</td>
<td>15,137999</td>
<td>1,067063</td>
<td>0.036724</td>
</tr>
<tr>
<td>CLIC4</td>
<td>NM_0013943</td>
<td>10,805262</td>
<td>11,027510</td>
<td>10,138520</td>
<td>1,052216</td>
<td>0,020643</td>
</tr>
<tr>
<td>KDEL3</td>
<td>NM_016657</td>
<td>11,195222</td>
<td>11,397625</td>
<td>10,588012</td>
<td>1,050462</td>
<td>0,018309</td>
</tr>
<tr>
<td>TRMT5</td>
<td>NM_0020810</td>
<td>10,675884</td>
<td>10,858038</td>
<td>10,129423</td>
<td>1,047575</td>
<td>0.032691</td>
</tr>
<tr>
<td>SH3GL3</td>
<td>NM_003027</td>
<td>6,861173</td>
<td>7,082383</td>
<td>6,197542</td>
<td>1,044876</td>
<td>0.032691</td>
</tr>
<tr>
<td>A_33_P3349840</td>
<td>A_33_P3349840</td>
<td>9,157626</td>
<td>9,318325</td>
<td>8,675531</td>
<td>1,033104</td>
<td>0.037666</td>
</tr>
<tr>
<td>NDT1</td>
<td>NM_001543</td>
<td>14,783885</td>
<td>15,014166</td>
<td>14,093041</td>
<td>1,017426</td>
<td>0.024086</td>
</tr>
<tr>
<td>HMGN2</td>
<td>NM_005517</td>
<td>13,354392</td>
<td>13,537040</td>
<td>12,806448</td>
<td>1,010829</td>
<td>0,048254</td>
</tr>
<tr>
<td>LRRC8D</td>
<td>NM_018103</td>
<td>10,101676</td>
<td>10,308048</td>
<td>9,482559</td>
<td>0,994593</td>
<td>0,038489</td>
</tr>
<tr>
<td>KCTD1</td>
<td>NM_198991</td>
<td>11,189016</td>
<td>11,447116</td>
<td>10,421918</td>
<td>0,979205</td>
<td>0,016353</td>
</tr>
<tr>
<td>CLIC4</td>
<td>NM_013943</td>
<td>11,152413</td>
<td>11,343449</td>
<td>10,579307</td>
<td>0,967379</td>
<td>0,031276</td>
</tr>
<tr>
<td>CPE</td>
<td>NM_001873</td>
<td>12,461945</td>
<td>12,669744</td>
<td>11,838547</td>
<td>0,951188</td>
<td>0,032691</td>
</tr>
<tr>
<td>POPDC3</td>
<td>NM_0022361</td>
<td>10,686453</td>
<td>10,865732</td>
<td>10,148615</td>
<td>0,949366</td>
<td>0,028011</td>
</tr>
<tr>
<td>CEBPA</td>
<td>NM_004364</td>
<td>7,171892</td>
<td>7,402467</td>
<td>6,480164</td>
<td>0,948691</td>
<td>0,049600</td>
</tr>
<tr>
<td>LOC643650</td>
<td>NR_033957</td>
<td>7,064100</td>
<td>7,270745</td>
<td>6,444168</td>
<td>0,901195</td>
<td>0,035102</td>
</tr>
<tr>
<td>NFE2L3</td>
<td>NM_004289</td>
<td>7,207324</td>
<td>7,382161</td>
<td>6,682813</td>
<td>0,892188</td>
<td>0,027858</td>
</tr>
<tr>
<td>CELF1</td>
<td>NM_001172640</td>
<td>11,081758</td>
<td>11,269220</td>
<td>10,519371</td>
<td>0,889811</td>
<td>0,026943</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Systematic Name</th>
<th>AveExpr_xpc_wt</th>
<th>baseMean_xpc</th>
<th>baseMean_wt</th>
<th>logFC_xpc_wt</th>
<th>adj.P.Val_xpc_wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI3BP</td>
<td>NM_015429</td>
<td>7,683497</td>
<td>7,915669</td>
<td>6,986979</td>
<td>0,879860</td>
<td>0,024086</td>
</tr>
<tr>
<td>SNORD114-6</td>
<td>NR_003198</td>
<td>6,962188</td>
<td>7,126676</td>
<td>6,468726</td>
<td>0,860216</td>
<td>0,034283</td>
</tr>
<tr>
<td>GATSL3</td>
<td>NM_001037666</td>
<td>10,247236</td>
<td>10,386076</td>
<td>9,830716</td>
<td>0,833349</td>
<td>0,049203</td>
</tr>
<tr>
<td>MEX3A</td>
<td>NM_001093725</td>
<td>6,988128</td>
<td>7,159774</td>
<td>6,473191</td>
<td>0,826047</td>
<td>0,029951</td>
</tr>
<tr>
<td>LOX</td>
<td>NM_002317</td>
<td>14,291690</td>
<td>14,432939</td>
<td>13,867942</td>
<td>0,823170</td>
<td>0,036724</td>
</tr>
<tr>
<td>LEPRE1</td>
<td>NM_022356</td>
<td>12,853167</td>
<td>12,975679</td>
<td>12,485629</td>
<td>0,803174</td>
<td>0,038771</td>
</tr>
<tr>
<td>SIKE1</td>
<td>NM_001102396</td>
<td>9,931223</td>
<td>10,086985</td>
<td>9,463936</td>
<td>0,780671</td>
<td>0,028846</td>
</tr>
<tr>
<td>ZCCHC17</td>
<td>NM_016505</td>
<td>10,711379</td>
<td>10,876770</td>
<td>10,215207</td>
<td>0,770869</td>
<td>0,030357</td>
</tr>
<tr>
<td>COPZ2</td>
<td>NM_016429</td>
<td>12,520875</td>
<td>12,658954</td>
<td>12,106637</td>
<td>0,754871</td>
<td>0,036556</td>
</tr>
<tr>
<td>HIST1H4D</td>
<td>NM_003539</td>
<td>8,037786</td>
<td>8,183747</td>
<td>7,599902</td>
<td>0,680841</td>
<td>0,032779</td>
</tr>
<tr>
<td>LPCAT2</td>
<td>NM_017839</td>
<td>7,836494</td>
<td>7,961408</td>
<td>7,461752</td>
<td>0,665767</td>
<td>0,049111</td>
</tr>
<tr>
<td>KCTD1</td>
<td>NM_198991</td>
<td>8,357303</td>
<td>8,533384</td>
<td>7,829059</td>
<td>0,663043</td>
<td>0,039798</td>
</tr>
</tbody>
</table>
Genetic therapy of Xeroderma Pigmentosum: analysis of strategies and translation

Maria Goncalves-Maia and Thierry Magnaldo
Lille Sciences, Institute for Research on Cancer and Aging, Lille, France

1. Introduction: rare genetic diseases

Diseases are considered rare by the European Commission when affecting less than 1/2,000 citizens and it is estimated that 80% of them have a genetic origin [1,2]. According to Orphanet, a comprehensive portal for rare diseases (www.orpha.net), between 6,000 and 7,000 rare genetic diseases have been identified. One of the most known ‘legacies’ from the Orphanet organization is that ‘rare diseases are rare, but rare disease patients are numerous’ reinforcing the idea that millions people worldwide are affected by a rare disease.

Research on rare genetic diseases is sometimes seen as a lesser priority compared to more common ones; but researchers have shown over the years that rare diseases may also be considered as human models to better understand the physiology and mechanism of the human body. For instance, a major breakthrough in the understanding of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway emerged from the study of the rare genetic disorder tuberous sclerosis complex (TSC) [3].

With respect to skin, 80% rare genodermatoses are caused by a single gene and, in some cases, mutations in different genes can cause the same phenotype [4]. Unlike TSC, rare genodermatoses may also be relevant models to study the signaling pathways involved in cutaneous homeostasis. Gorlin syndrome, for instance, a rare genetic disease, has proved to be a valuable model in the study of cancer development and progression [5]. Due to its location, skin is a very accessible organ and therefore a good candidate to attempts of corrective gene therapy both in vivo and in vitro in the absence of any satisfactory pharmacological treatment [6]. In this review, we will focus on one of these diseases, xeroderma pigmentosum, its current rare strategies, and the progresses that have been done in the field of factors contributing to its development as well as gene therapy approaches aiming to improving patient’s health conditions.

2. The xeroderma pigmentosum genetic

Xeroderma Pigmentosum (XP) is a rare autosomal recessive genetic disease. The frequency of XP newborns varies according to the geographical location; frequency of XP newborns is estimated at approximately 1/250,000 in the USA, 1/20,000 in Japan, and 23/1,000,000 in Western Europe [7,8]. Clinical manifestations include hypersensitivity to sunlight, pigmentation abnormalities, progressive neurological degeneration in most complementation groups (except in XP-C, XP-E, and XP-V, see below) and early development of cancers in sun exposed areas of the skin. The onset of skin cancer in XP patients is at average before 10 years [9]. XP patients present incidences of skin carcinoma basal cell carcinoma, BCC squamous cell carcinomas (BCC), as well as melanocyte cancers (melanoma), increased up to 10,000-fold for BCC and SCC and 2000-fold for melanoma compared to the general population[10]. These clinical traits are directly related to a repair
2.1. NER mechanism

There are five major steps involved in the NER mechanism [13,14].

(1) Recognition of the lesion-induced DNA distortion. Proteins involved in this step depend on the location of the lesion in either nonactive or active DNA lesions located on nonactive DNA regions (global genome), are recognized by the XPC-HR23B/CEN2 complex with the help of XPF protein in a process known as global genome repair (GGR); when the...
lesion is located in the transcribed strand of active DNA, the RNA polymerase II is stalled by the DNA distortion, leading to a NER subpathway called transcription-coupled NER repair (TC-NER or TCR).

(d) DNA ‘opening’. The ATP-dependent helicases XPB and XPD belong to the general transcription factor II H (TFIIH). Following the recognition step (f), recruitment of TFIIH leads to unwinding of the double-strand DNA around the lesion to form a bubble. The XPA protein is thought to make a platform allowing the intervention of the DNA endonucleases (XPF and XPC, step (3)).

(3) DNA incision. After stabilization of the repair complex and DNA unwinding, the damaged strand is incised in S’ and 3’ by the ERCC1/XPF complex and the XPG single-strand endonucleases, respectively. The DNA fragment bearing the lesion (about 10 nucleotides) is eliminated.

(4) Gap filling. The remaining single DNA strand is then used as a replication template for the DNA polymerases ε/δ/κ.

(5) Ligation. The newly synthesized strand is then covalently ligated by either DNA ligase 1 or 3.

The respective role of XP proteins in the different steps of NER mechanism are summarized below.

2.1.1. Recognition of the DNA distortion

2.1.1.1. The ‘master’ XPC. XPC (625 kDa) is a protein that combines with NER proteins to bind DNA distortions. In previous studies, Sugawara showed that the purified XPC protein exhibits a general affinity for DNA sequences that alter the classical Watson–Crick chemical conformation of the double-strand DNA helix, rather than lesions themselves. XPC acts by recognizing distortions of the DNA helix caused by specific nucleotide modifications [13]; early work by Kim and colleagues, showed that CPD and 6-4 PP are responsible for NER inactivation, extending from 7-30° to 44°, respectively [14]. The ratio of CPD to 6-4 PP is changing in the same UV dose has been estimated to about 9:1. In vitro experiments, however, have shown that XPC preferentially binds to double-stranded DNA bearing highly distorting bulky lesions, notably 6-4PP and cyclobutane intra-strand crosslink. Nevertheless, XPC is not alone to ensure capacity of recognizing a wide range of lesion-Induced DNA distortion. In addition of NER proteins, by which it is stabilized, XPC is actually helped by the DNA-binding DDB2 protein (XPD, damaged DNA-binding protein 2), which favors DNA lesion recognition through modification of chromatin conformation [15,16].

2.1.1.2. The ‘helper’ XPE. The DNA damage binding complex, or UV-DBI, is composed of damage-specific binding proteins DDB1 (127 kDa) and DDB2/XPE (48 kDa) as well as the ubiquitin ligases CULIN4, (88 kDa) and ROC1 also known as RBC1 (12 kDa), respectively. DDB1 facilitates DDB2 damage recognition, helping the XPC binding to the distortion. After UV exposure, both DDB2 and XPC are ubiquitinated by the complex but only DDB2 is degraded. XPC then presents enhanced DNA-binding affinity [21,22].

In vitro experiments, that is, in naked DNA bearing different types of lesions, have shown that DDB2 preserves a better affinity than XPC for DNA lesions such as CPDs, that is, leading to lower DNA distortions than 6-4 PP and CsPP [19,23]. The XPC complex itself can poorly recognize CPD lesions presumably because of the small helix distortion [24]. Under this circumstance, the XPF complex facilitates recruitment of the NER factors, including XPC [25]. X-F patients usually do not suffer from neurological abnormalities are photodermatosis, and develop skin cancers at a much higher rate than individuals from the general population. However, onset of skin cancers may occur later than in XP-C patients, that is, in adulthood [26].

2.1.2. The DNA unwinds XPB and XPD

XBP and XPD are the two ATP-dependent helicases associated to the TFIIH complex which is essential for the initiation of transcription. XBP and XPD are also involved in NER where they operate to unwind the double-strand DNA around the distorting lesion [27]. XP-B (extremely rare) and XP-D patients develop severe neurodegenerative problems early in life, leading to psychomotority problems and metabolic limitations. Mutations in XBP and XPD can cause not only XP but also other genetic diseases: Cockayne syndrome (CS) and trichothiodystrophy (TTD). CS is a developmental and neurological disorder, and XPC/CS patients have a combination of cutaneous, developmental, and neurological abnormalities. Although TTD may be associated to severe photo sensitivity, susceptibility toward skin cancer has never been associated with this syndrome [28,29].

Regarding the NER mechanism, XPB is believed to be the first helicase in place, opening the DNA strand and allowing positioning of XPD. Following double-strand DNA coiling, XPD stays in place enabling the denaturation of DNA strands to allow XPA, RPA, and XPG to take place [30].

2.1.3. The XPA stabilization companion

XPA is a DNA-binding protein that, together with replication protein A (RPA) makes a platform for the interventions of the two endonucleases XPF and XPG (please see below) [31]. The most severe form of XP-A is prone to the development of neurological problems with aging [14,22,23]. XPA is sometimes called the central element of the NER mechanism because of its interactions with multiple proteins/complexes involved in this pathway: TFIIH, RPA, XPC, RAD51B, XPE, XPG (proliferating cell nuclear antigen (PCNA) [14].

2.1.4. DNA microsurgery: the single-strand DNA scissors XPF and XPG

XPF/ERCC1 and XPG are the two endonucleases responsible for the excision of the DNA lesion. XPF/ERCC1 cuts the 5’ and XPG the 3’ to release a fragment of single-strand DNA of about 30 nucleotides. Recent studies have shown that XPG and XPF do not incise the DNA simultaneously. XPF/ERCC1 incision initiates the process, allowing immediate replicative synthesis until the 3’ end before XPG cuts. The process is complete with the ligation step by DNA ligase 3 or 1 depending on the cell cycle phase [32,33]. X-G and X-P patients are photosensitive,
but usually less prone to skin cancers than other XP groups of genetic complementation. Those patients also present neurological and developmental abnormalities such as mental retardation and microcephaly [11,35,36].

2.2. Defects in translesion DNA replicative synthesis in XP-V patients

XP-V is not, per se, a group of genetic complementation of XP XP-A to XP-GI. Unlike in XP-A to XP-G patients, the NER mechanism is functional in XP-V patients. Instead, photoreactivation and high incidence of skin cancers in XP-V results from mutations in the XPF gene which encodes the TLS DNA polymerase β [37]. TLS polymerases belong to the Y family of DNA polymerases and substitute the replicative DNA polymerases when the latter are blocked by specific DNA lesions/distortions. DNA pol β is able to replicate UV-induced CPD DNA lesions in an error-free manner [38,39]. However, specific abrogation of DNA pol β activity in XP-V cells leads to error-prone translesional synthesis by other unfaulty Y DNA polymerases, hence resulting in levels of mutagenesis and skin cancer susceptibility [40].

3. XP and the immune system

In addition to NER defect, it has been hypothesized that immune deficiency could contribute to the high prevalence of skin cancers in XP patients. A few studies on natural killer (NK) cell activity, which ensure the innate immune defense, were done in XP patients. Despite normal numbers of circulating NK cells in XP patients, the NK cytotoxic capacity was found decreased in XP patients compared with young individuals from the general population [41-43]. In another study, XP-deficient mice were daily irradiated with supraphysiological doses of UV (500 mJ/m² or 5000 mJ/m²) for one, three, or five days. 24 h after three or five daily irradiations, 60 and 30% decreased NK activities were described, respectively [44]. In spite of these early findings, no additional clear evidence of an alteration of the innate immunity have ever been associated with the XP syndrome yet.

4. Diagnostic of the XP disease

XP is not only a very rare disease but also, it is characterized by the variability of clinical traits and their extent between the different groups of genetic complementation. These are the two main factors that may hamper robust clinical diagnostic of the disease. In addition to clinical expertise, cell and molecular biology techniques are often required to confirm clinical diagnosis and further on, strengthen our knowledge on the relationships between genotypes and phenotypes connected to the XP syndrome.

4.1. Assessing UV cell survival and DNA repair capacities by NER

4.1.1. Cell survival

XP cells may be isolated from a small, non-photo-exposed skin biopsy. The simplest way to formalize UV sensitivity is based on measurement of UV cell survival, most often by clonal analysis [45]. The lethal UV doses necessary to kill XP cells are variable among the groups of genetic complementation. Determination of UV sensitivity is quite tedious but remains a robust and essential parameter in the stepwise assessment of a putative XP clinical syndrome. UV-cell survival, however, is too variable to conclude on the group of genetic complementation. For instance, XP-E cell survival may be close to normal.

4.1.2. Measuring the levels of GGR and TC-NER

XP cells fall into seven groups of genetic complementation that are all deficient with variable extents in the NER process. XP-C and XP-D solely suffer from GGR defect with about 10 and 50% residual repair. Other groups of genetic complementation present significant incapacies in both GGR and TC-NER. Unscheduled DNA synthesis (UDS) is a simple laboratorial procedure that allows the measure of nucleotide incorporation levels associated with a DNA repair process after DNA damage [46]. In a quite similar way, the levels of RNA recovery synthesis (RRS) can be measured after UV irradiation (TC-NER). The principle is to incorporate labeled nucleotides in DNA patches synthesized upon either GGR or TC-NER. It is of importance to distinguish failures to repair by GGR, by TC-NER, or both. Indeed, as mentioned above, the different groups of genetic complementation of XP can be distinguished by their alteration to perform either GGR or TC-NER, or both. Of note, among NER pathologies, only patients suffering from the CS present repair defect limited to TC-NER (but not GGR).

Up to a few years ago, skin cells (fibroblasts) from patients presenting with a suspicion of XP were cultured in standard conditions, before being irradiated or vivo in the presence of labeled radioactive nucleotides (HETDR (GGR) or HEDtR (TC-NER). Upon repair, labeled nucleotides were incorporated either in the DNA or the RNA of irradiated cells that were then processed for, and subjected to autoradiography or the less tedious liquid scintillation counting [47,48]. Incorporation of a allyl-cysteine-conjugated nucleoside, analog of thymidine or uridine (called either 5-ethynyl-β-D-uracil (EdU) or 5-ethynyluridine (EUE), respectively) are now nonradioactive techniques alternative to radioactive measures of UDS or RRS [48,49].

4.2. Determination of XP groups of genetic complementation

The determination of the complementation group of a given patient helps to connect the relation between genotype and phenotype data and may also contribute to the development of new targeted pharmacological therapeutics through the identification of pathways affected in addition of solely DNA repair by NER.

4.2.1. History

4.2.1.1. Genetic complementation by cell fusion. Well before discovering the identity of genes whose mutation are responsible for the different forms of XP, researchers used to fuse cells (using the Sendai virus and polyethylene glycol, PEG) from (two) patients with distinctive or identical phenotypic
traits. The fusion was generally made between one XP cell for which the complementation groups was already known and XP cell from unknown complementation group. Following UV irradiation, isolated or fused cells were fed with medium containing labeled DNA or RNA nucleosides and the levels of UDS were measured. Restoration of UDS indicated that the tested cell from unknown group of complementation is complemented by an XP cell from an identified group of complementation. In contrast, failure to restore UDS after cell fusion implied that both cells were from the same group of genetic complementation.

The appraisal and recovery of both GGR and TCNER levels can be assessed following metabolic incorporation of labeled nucleosides after UV irradiation [50]. In other words, cells from a patient showing low levels (10%) of GGR but normal levels of TCNER were assumed as XP-C cells. Cells suffering from both GGR and TCNER could be assumed to belong to the XP-A, XP-B, XP-D, and XP-E groups of genetic complementation. In any case, these analyses of DNA repair by NER should be confirmed by genetics.

4.2.2. Genetic complementation by gene transfer of NER genes

Cloning of genes whose mutations are responsible for either GGR or TCNER, or both, contributed to a great facilitation of molecular diagnostic of NER defects. One of the first complementation assays to this end was based on the introduction by transfection in patient's cells of a reporter plasmid following UV irradiation of the naked DNA (host cell reactivation assay). Cells containing a reporter plasmid that had been transfected with a candidate complementation gene could further indicate the group of genetic complementation of the patient cells [51].

Development of gene transfer using retroviral recombinant vectors greatly facilitated the determination of the complementation group of genetic diseases, in general, and of the XP syndrome in particular. As described below, the input of using transfective retroviruses also significantly contributed to ex vivo gene therapy approaches based on gene transfer. As mentioned above, the various NER genes can be transferred independently to patients' cells in order to determine their group of genetic complementation after assessment of UDS and IRS levels [52].

4.3. Determination of NER genes mutation by DNA sequencing

As for any other identified monogenic syndrome, DNA sequencing and next-generation sequencing are now the most efficient techniques to determine the identity of the gene implicated in the suspected disease and the nature and location of the mutation [32]. Although quite variable, clinical traits of XP patients can usually target the identification of the gene(s), candidate for identification of a mutation. For instance, patients presenting with high photosensitivity and early onset of skin cancer but lacking neurological disorders may be thought to belong to either XP-C or XP-E groups of genetic complementation. However, due to the much higher frequency of XP-C over XP-E patients in Europe and North Africa, the XP-C gene is the one to be first sequenced. Interestingly, a XP-C founder mutation c1643_1644delCTG (p. Val548AlaX32) has been found in 87% of North African patients involved in a clinical/genetic study, and the same mutation was found in a Sudanese family [35,34]. This finding strongly contributes to the molecular diagnosis of XP patients and family relatives. Other founding mutations in the XP-C gene have been described in Japan, Guatemala, and also in black Mahori populations [35-38].

5. Current therapies

Although no curative treatment is available for the XP syndrome, some preventive actions and palliative therapies may help limit skin cancer development and progression. All the therapeutic strategies described in this chapter are known for their role in cancer prevention or progression but do not solve the incapacity to remove DNA lesions by NER in XP patients.

5.1. Sun avoidance

Sun avoidance and protection is the best way to delay cancer manifestations in XP patients. Protection of UV radiation implies no outdoor activities during the day, use of sunglasses, hat with neck protection, woolen clothes, and use of sunscreen with high sun protection factor (SPF). When families can afford it, windows may be covered with screens absorbing UV radiation from the sun [59]. Availability of high SPF sunscreens may improve the cosmetic results. In France, once the diagnosis of XP is confirmed, the cost of such sunscreens can now be reimbursed by Social Security. Otherwise, sunscreens from industrial sponsors may be provided to the patients through charity associations. In order to assess the in vivo efficacy of sun protective formulations including not only sunscreen but also other therapeutic substances such as antioxidants or inhibitors of pathways with aberrant activation in XP cells, Garcia et al. developed a model of humanized mouse allowing morphogenesis of human XP skin. This model overcomes ethical problems and constitutes a valuable tool to measure, in the long term (up to 20 weeks), the consequences of either acute or chronic UVB irradiations.

Briefly, a fibroblast matrix populated with dermal fibroblasts isolated from the patient skin is used as the dermal component of the bioengineered skin. Patient keratinocytes are then seeded on the fibroblast matrix to form the epidermal layer. After keratinocyte confluence, the bioengineered skin is grafted onto the back of the immune-deficient mouse to replace its own skin [60]. As described below, this alternative model of study is also highly relevant to assess innovative therapeutic approaches, notably gene therapy.

While total avoidance of sun exposure is undoubtedly beneficial to delay skin cancer, it is also accompanied by vitamin D deficiency. The amount of vitamin D needed by the human body is estimated at 1000 IU/day. Lower vitamin D intake contributes to bone diseases and demineralization, fractures, hypertension, multiple sclerosis, and also depression. In contrast, higher serum concentrations of 25(OH)D, (active metabolite of vitamin D) are associated with lower rates of some
types of cancer like breast, ovarian, and prostate cancers. In the general population, the daily intake of vitamin D is about 200 IU. In the presence of sunlight, the human body is capable of synthesizing the remaining 800 IU/day through a photochemical reaction [61,62]. In XP patients subjected to strict photo-protection levels of circulating 25(OH)D3 are decreased compared to people normally exposed to the sun. To prevent deficiency, patients may then receive an external source of vitamin D (50,000 IU once a month, per os) [63].

5.2. Tumor excision, demabrasion, and chemical peels

In order to limit cancer cell dissemination, XP patients must be regularly followed by the dermatologist and have frequent surgical ablation of cancerous lesions. Because of the necessity to take large margins around lesions, especially in the case of melanoma, XP patients occasionally need plastic reconstructive surgery including grafts. Skin pieces destined to be grafted are taken from non-sun-exposed areas such as abdomen and buttock [64,65]. Demabrasion and chemical ablation were used some decades ago as a prophylactic approach. The potential advantage of these procedures was the promotion of constant re-epithelialization of the skin with cells that arise from those in the deeper layers of epidermis and therefore less touched by UV light. However, the efficacy of such approach was never really demonstrated and therefore both procedures were discontinued [66,67].

5.3. Laser and photodynamic therapy (PDT)

Full-face laser resurfacing and PDT can also be considered as prophylactic measures for the prevention of nonmelanoma skin cancers in general population and therefore are suitable to be used in XP patients. CO2 laser resurfacing was the first to be proposed for the treatment of actinic keratosis (SCC precancerous lesions) but now, the pulsed and fractionated lasers are preferred for their better clinical reliability [68]. Several studies actually showed that laser treatments result in lower incidence of nonmelanoma skin cancers and long-term recurrence-free intervals [66,69].

PDT involves the use of a photosensitizing agent that upon light activation is able to destroy cancer cells. Photofrin, a first-generation systemic photosensitizer, was used at a dose of 1 mg/kg with 20D J/cm² and 630 nm red light to effectively treat BCCs [70]. However, because of the high incidence of scarring, photofrin should be avoided in children, that constitutes a huge part of XP patients, and topical AAL should be used instead. AAL is a precursor of the endogenous photosensitizer protoporphyrin IX and has been successfully used to treat SCC in XP patients both with red and blue light (417–432 nm) activation [71,72]. However, in one study of an XP-A patient, treatment of SCCs on eyelid, conjunctiva and cornea with sodium salt hematoporphyrin derivative photosensitizer resulted in higher incidence of SCC development [73]. Using a therapy involving light in XP patients is delicate and still controversial mainly because some XP patients present defects in the repair of oxidative DNA damage occurring upon photodynamic radiation.

5.4. Retinoids

Retinoids have been used as chemoprophylactic agents to avoid the formation of cutaneous carcinomas in XP and other patients with high risks of skin cancer. Studies showed that high doses (2 mg/kg) of oral daily intake during 2 years of a retinoid derivative, isotretinoin, significantly decreased (60%) the occurrence of skin cancers in XP patients. However, following discontinuation of the treatment, a burst of de novo cancerous lesions was observed [74,75]. In addition, systemic treatment with 2 mg/kg/day of isotretinoin was associated to severe side effects such as development of skeletal abnormalities and mucocutaneous toxic effects. The benefits/risk balance of retinoid treatment has been the subject of debates and is now practically abandoned [76]. How exactly retinoid may act as chemoprotector in XP patients remains unclear. However, it is conceivable that they exert their anti-cancer effect through: (i) their pro-extracellular matrix activity; (ii) the maintenance of stem cells (bearing mutagenic DNA lesions) in quiescence, then avoiding replicative mutagenesis [77,78]; (iii) the inhibition of matrix degrading enzymes such as matrix metalloproteinases [79].

5.5. 5-Fluorouracil

5-Fluorouracil (5-FU) is a modified form of Uracil. 5-FU acts as an anticancer drug by (i) integrating the RNA molecules and blocking transcription; (ii) by interacting with the enzyme thymidylate synthase (TS) and hence inhibiting all production of thymidine triphosphate (TTP) from uracil. These effects result in cell death by apoptosis. In skin, topical 5-FU presents good efficacy and cosmetic properties for actinic keratoses and superficial carcinoma. 5-FU administration may also be considered as an alternative treatment to delicate surgical resection [80,81].

5.6. T4 endonuclease V

In 1975, Tanaka et al. showed that treatment of XP cells with a bacteriophage T4 endonuclease V (T4Ns), restored normal levels of UDS [82]. Later on, in 2001, Daniel Yarmush set up a clinical trial based on a liposomal formulation containing 1 mg/L of T4Ns. Thirty XP patients from different groups of genitic complementation were treated for 1 year with topical application of the formulation without constraint on life conditions. Results showed a 30% decrease of BCC and a 70% decrease of actinic keratoses. No immune reaction was observed in the group of treated patients. These encouraging results however were significant only in patient <18, suggesting that the enzyme amount and/or activity was limited when too many DNA lesions accumulated prior to treatment [83]. Approval of the T4Ns formulation (Dimericine®) as a medic-line for at-risk patients is currently under appraisal by the FDA.

5.7. Imiquimod

Imiquimod is an immune response modifier that proved to be effective in the treatment of skin malignancies and
premaligancies [84]. In XP patients, the use of 5% topical imiquimod proved to be successful for BCC treatment [85,86]. Besides the effect of imiquimod in the resolution of BCCs, in XP patients, topical use of imiquimod also improved pigmentation alterations as well as defects in the skin texture [82]. Topical administration of imiquimod formulations could represent a valuable approach to limit the development of malignant lesions and also to improve cosmetic aspects.

6. Potential of gene therapy

One of the first organisms known to perform ‘survival gene therapy’ is probably the K cells of Escherichia coli which was shown to exchange genetic material through the pill ‘accompanyment mechanism’ [87]. Indeed as early as in the sixties, observations emerged that mixing bacteria from different origins could generate subpopulations resistant to hostile media of culture, for example, lacking a specific nutrient or having to face an antibiotic killing substance such as neomycin (or geneticin in prokaryotes). Transfer of the TNS episome, which bears the gene encoding neomycin phosphotransferase, confers neomycin resistance and allows therefore the growth of transformed bacteria in presence of neomycin [88,89].

The concept of ‘gene therapy’ in eukaryotic cells then grew up, based on accumulating knowledge on the genome, especially the human genome, as well as the identification of mutations responsible for rare human diseases [90]. For more than 30 years, gene therapy research has brought new hopes to the persons suffering from genetic disorders and still lacking curative therapy. Two main approaches have been explored until today. The first approach is schematically relying on the transfer of genetic material usually based on the capacity of defective retroviruses (either derived from the mouse Moloney leukemia virus (MLV) or the human immunodeficiency virus (HIV)) to insert foreign DNA into a host genome [90]. The second ‘general’ approach relies on genome editing. Using techniques based on either nonhomologous end joining (NHEJ) of DNA or homologous recombination (HR) of DNA, both private companies and academic laboratories deployed much work to define optimal conditions of therapeutic HR in primary cells [91,92].

6.1. Addition of the therapeutic gene

Twenty-five years after the first clinical trial, there are successful stories that encourage and raise hope on using gene therapy to treat and cure diseases [93]. Severe combined immunodeficiency (SCID) is due to the lack of adenosine deaminase (ADA) and is a fatal disorder. The transduction of the ADA gene via a retroviral vector into the blood cells isolated from 10 independent patients allowed basically to cure the disease [94]. Interestingly also, some success of a gene therapy approach was reported in patients suffering from with Leber’s congenital amaurosis (LCA), a rare form of inherited blindness. Progressive loss of rod and cone function is due to the lack of the RPE65 isomerase required to form light-sensitive pigment. The administration of a vector expressing RPE65 allowed a gain of light sensitivity and, in some cases, recovery of visual acuity [95].

As many other rare genetic diseases, XP has been thought for a long while as an excellent candidate disease toward gene therapy approaches. As for many other genetic diseases, several technical approaches have been proposed to correct the impact of defective genes and therefore restore their function. One of the latest strategies rely on genome edition in order to correct the genetic defect in cellulo without adding in, or removing from, any component of the host genome. Promising aspects of the CRISPR/CAS 9 technology are discussed below. Among all groups of genetic complementation, the XP-C group is the most frequent group of genetic complementation (about 50% XP patients, in Europe) and due to the lack of neurological problems early appeared as the best candidate for ex vivo gene therapy [79,96,97].

6.2. Corrective gene transfer using recombinant retroviruses in XP cells

First attempts to correct XP cells were based on the use of replication-defective recombinant MLV retroviruses [91,98,99] (Figure 2). These studies demonstrated that RV transduction of XP cells (XPG, XPO) of patient’s fibroblasts restored the survival and NER capacity in UV-irradiated cultures [92]. Following these proofs of principle, setting up conditions of isolation and culture of XP keratinocytes, Amaddeo-Bergard and colleagues reported efficient genetic correction of XP-C keratinocytes [100]. One step beyond the previous studies, the authors made transduced XP-C keratinocytes retained their capacity to stratify and differentiate, a condition sine qua non in the perspective of regeneration in vivo of genetically corrected skin. As in previous reports, however, it’s study, was seriously flawed by (i) the limited sustainability of expression of the therapeutic gene (XPC); (ii) the method of selection of transduced cells, based on the expression of the neomycin phosphotransferase which confers G418 (geneticin) resistance. As in many other laboratories, the authors had then to face the problem of high survival of the success of the approach including graft of corrected cells in vivo [101].

In contrast, the selection procedure is mandatory in XP cells destined to be grafted in a ease and non-connected cells remain prone to cancer.

Taking these specifications into account, in 2007, Bergoglio et al. [102] reported a selection strategy to be compatible with grafts in humans. The procedure is based on the expression of a small surface antigen CD24 together with the gene of interest (here the GFP reporter gene). In the human epidermis, CD24 is naturally restricted to non-differentiated keratinocytes that occupies the suprabasal epidermal layers [103]. Since RV infection is limited to dividing cells (naturally negative for CD24), ectopic expression of CD24 in transduced cells allows to sort them out using an antibody recognizing specifically the antigen. Cell sorting of CD24-positive cells thus results in the purification of both transduced cells (expressing ectopic CD24) and cells that
naturally express CD24 (post mitotic cells). However, cells that naturally express CD24 are not able to divide anymore and therefore are lost after one to two cell passages [103]. Importantly enough, Bengio and colleagues demonstrated that selected populations were enriched in stem cells and could be serially propagated for more than 150 population doublings [104]. Transduced population retained normal capacities of growth and differentiation in the long term (22 weeks) in vivo, after skin regeneration on the back of the athymic mouse [102]. Following these encouraging results, and using the same procedure, Warrick et al. reported efficient genetic correction of epidermal XPC stem cells in the long term (~100 population doublings). Corrected cells recovered normal NER levels in vitro, as well as in a preclinical skin humanized murine model in vivo [103]. This was the first demonstration that human epidermal stem cells can be genetically corrected and selected based on the ectopic expression of a cell surface epitope naturally expressed in postmitotic keratinocytes. In addition, to avoid any problem of insertional mutagenesis, or partial activation of oncogenes (LM0) as described in the case of ex vivo gene therapy of X-linked SCID [106], sequences of insertion of the genomic DNA were determined. Genome-wide analysis 20PD and 120PD after transduction did not reveal any particular integration spots nor donor selection after 120PD [105]. Attempts to correct XPC phenotype were also made using recombinant adenovirus (AdXPA) as a vehicle for the delivery of human XPA cDNA to a XPA−/− mice. Skin injection of the AdXPA virus in XPA−/− mice led to complete recovery of defective DNA repair. After UV irradiation, parental XPA+/+ mice developed numerous skin tumors while mice injected with AdXPA did not develop skin tumors [107]. These results show the protective effect of JP genes transfer against cancer development in animal models of some forms of the XP pathology. Although this approach could avoid ex vivo corrective gene transfer and subsequent grafting of corrected cells, the sustainability of the therapeutic gene as well as immune activation could appear limiting in the cure perspective of the XP.

6.3. Genome editing

Thanks to previous researches and innovative techniques, notably those connected to next-generation sequencing, numerous germline mutations responsible for orphan genetic diseases have been identified, opening a new avenue toward attempts to bowdlerize genetic correction rather than corrective gene addition.

The principle of genome editing is to target a DNA sequence bearing a specific germline DNA mutation in situ in order to replace this sequence by its correct version, that is, the sequence considered as the "wild-type" one [93,102]. Different methodologies have been developed to target specific genome sequences. Here, we will focus on the different procedures based on the use of, in principle, "smart and faithful" DNA endonucleases. Two major technical difficulties emerged in the course of numerous researches on DNA micro
surgery. First, the efficiency may be extremely poor, especially in human primary cells; second, the specificity of DNA recombination, that is, the absolute necessity to verify absence of "off-site" recombination, which could be as mutagenic as IV insertion mutagenesis.

Specific engineered nucleases are constructed to generate double-strand breaks (DSBs) on specific genome regions and in doing so, activate DNA repair pathways notably the HR. A "donor" matrix with the adequate sequence allows the correction of mutations, during the DNA repair [91,92].

6.3.1. Mega endonucleases
Meganucleases (MENs) are highly specific DNA cleaving enzymes, recognizing large (>14bp) sequences. The main family of MENs are the homing endonucleases (HEs) that are naturally present in bacteria and eukaryotes [108]. The function of MENs is to induce HR following the introduction of DNA DSB, in a process called "homing" devoted to cell protection. Engineering artificial MEN with sequence specificity allows targeting of a huge number of genome locations [109]. Derivative l-Cel endonucleases were designed to cleave different regions of the XPC gene with success in CHO-H1 and MRC5 immortalized cells [110,111]. These encouraging results, however, were attenuated by the sensitivity of meganucleases to DNA methylation. As a first instance, targeting a XPC founder mutation located downstream of DNA sequences with fully methylated Cpg dinucleotides remained unsuccessful in transformed cell lines unless a demethylating procedure (5AzA-dc) and transfection of a DNA unwinding enzyme (helicase) were applied [112,113]. Obviously, under these conditions, meganuclease approach is not valid yet for genetic therapy in XP cells but it remains interesting for other genetic targets located away from CpG islands.

6.3.2. TALE-nucleases
The transcription activator-like effector (TALE) protein of lachnemonas is able to recognize specific DNA sequences via the TALE DNA-binding domain composed of a variable number of 34 amino acids called TALE repeats. These repeats are identical except for two hypervariable amino acids at positions 12 and 13, called repeat-variable diresidues (RVD) and required for target-site specificity [92,114,115]. Genetic modifications of the RVD domain lead to changes in DNA sequences targets of TALE. In theory, such modifications may allow editing virtually any sequence in mammalian cells, including in the XPC gene [111,116-118]. However, some TALEs are also sensitive to methylation which impedes specific sequence recognition. A methyl-insensitive TALE DNA binding domain was selected in order to edit mutation in the XPC gene in an XP-C transformed cell line in presence of a DNA helicase. The approach resulted in an accurate correction of the XPC locus, leading to restoration of the NER repair and UVC survival [112].

6.3.3. CRISPR/Cas9
In 2012, Doudna and Charpentier published a paper reporting a reproducible dual-RNA-guided DNA endonuclease. The process is based in the CRISPR/Cas9 systems that make part of the adaptive immune system in bacteria and protects against viral infections and bacteriophages. CRISPR is a ribonucleoprotein complex composed of the specificity determiner CRISPR RNA (crRNA) and the assisting transactivating crRNA (tracrRNA). CRISPR complex assembles with the Cas9 endonuclease to cleave DNA sequences complementary to the crRNA [119]. Engineering single-guide RNA (sgRNA) from the fusion of crRNA and tracrRNA is easier than programing mega or TALE nucleases. The CRISPR-Cas9 technique has been applied to correct or introduce mutations in a large panel of organisms including mammals [120-124]. Until now, the CRISPR-Cas9 technology has been applied successfully to correct mutations in the DYSTOPHIN gene responsible for Duchenne muscular dystrophy and the cystic fibrosis transmembrane conductance regulator [125,126]. In near future, correction of XPC mutations using the CRISPR-Cas9 will certainly be possible but even using this technology, selection of corrected cells will remain mandatory. In the mouse model of catenatin (bearing a mutation in the Cysc gene) zygotic injection, a CRISPR-Cas9-specific construct led to stable and invariable correction of the original phenotype [127].

The risk of off-target mutagenesis and the ethical problems besides the transmission of a corrected sequence of the genome to the progeny are the two main obstacles regarding the genetic correction of diseases using engine nucleases. Off-target mutations could lead to unwanted chromosomal translocations, DNA insertions or deletions. To avoid this problem, is essential to choose unique target sequences without any homology elsewhere in the genome together with the genome-wide screening for DSB caused by off-target CRISPR-Cas9. Many research groups are working to solve all these technical problems. This will allow to make a step forward in the correction of genetic diseases in patient somatic cells [128,129]. Ethical problems, however, must be addressed, notably through the development of a framework able to regulate attempts to edit the human genome.

Eric Lander (Massachusetts Institute of Technology, Boston, USA) proposed four main specifications that need to be taken in consideration regarding genetic therapy:

- Technical issues. Before start thinking of the development of genetically human models, specificity of targeting must be ensured. We are not there yet.
- Balance between medical benefits and risks. Obviously, proposing to correct the variant of the ADPKD gene, a risk factor for Alzheimer's disease, is rather different than modifying non-pathologic traits such as eye or hair color.
- Who should make the call? Parents hold the right to make decisions about their own children but should these decisions interfere with future generations, an expert would necessarily be recruited to assist the decision.
- Morality. How far can we go? If we open a precedent even for medical reasons, will we be able to go back or to resign the approach in the future? What would be the implications of editing genome in future generations?

7. Conclusions
XP is a rare genetic disease affecting 1/500,000 newborns worldwide. Although there are some preventive treatment, there is no cure for these patients and they may present
compromised life expectancy due to multiple skin cancers early in life. The idea of genetically correct XP cells, proved to be possible for different approaches from retroviral vectors to the use of TALEN-nucleases and possibly CRISPR-Cas9 technique. We were able to create safe strategies allowing correction of XP cells in a manner compatible with perspective of skin grafts in the patients. More recent techniques such as the CRISPR-Cas9 also hold much promise toward local cutaneous gene therapy of the XP. However, since skin is the largest organ of the human body, its full transplantation following genetic correction remains difficult to envisage. According to technical, as well as legal and ethical policies, genome editing in parental gametes would probably appear as the best choice toward limitation of cancer susceptibility associated to the disease.

6. Expert opinion

XP is very rare disease still lacking any efficient care. During the last thirty years, laboratory studies on cells isolated from XP patients or from genetically engineered mouse models provided valuable insights into the mechanistic understanding of DNA repair following genotoxic attacks, notably the short, mutagenic UV radiation.

In contrast, very few progresses toward patients care emerged to date from laboratory studies. Here, we summarize the essential traits of the XP syndrome and try to shed some light on potential innovative therapies. We have developed approaches of ex vivo gene therapy of epidermal stem cells isolated from patients carrying the XP-C form of the disease. Encouraging results have been obtained at the preclinical level, suggesting that palliative skin replacement using genetically corrected ex vivo cells could limit occurrence of skin cancer in sun exposed areas and contribute to improve patients' health conditions. In this context, our purpose is not to fully replace the skin of XP-C patients, but rather, as a first attempt, to contribute to reconstructive skin surgery using genetically corrected epidermal stem cells.

Research is going fast and the emergence of genome edition techniques, such as CRISPR-Cas9-mediated recombination may now appear more elegant and safe than simple gene complementation using retroviral or lentivirus vectors. Theoretically, the use of genetic recombination holds real promise toward genetic correction of monogenic diseases. Some improvements, however, remain to be taken into account. First, the specificity of the recombination process raises some problems. Indeed, as for retroviral insertion in the host genome, cells subjected to the CRISPR-Cas9 recombiantion technology should be checked for genome integrity by next-generation sequencing techniques. If proved safe, in situ recombination of genetic regions bearing a specific gene mutation would avoid insertional mutagenesis and, potentially, distal activation or extinction of genes at the vicinity of the inserted viral genome. In spite of the attractiveness of the method, whether genome recombination will be efficient in human primary cells in general and, in XP-C cells in particular, still remains uncertain. More work is needed before answering this question. Besides approaches of genetic complementation by insertional gene transfer or genome editing, we propose pharmacological strategies aiming to reduce the permissivity of skin microenvironment toward cancer development in XP patients. For instance, accumulation of reactive oxygen species observed in XP-C fibroblasts could be counteracted using antioxidants such as epigallocatechin gallate (EGCG); as well as stimulation of immune responses could prevent cancer cell colonization and invasion. We believe that combinations of those different approaches could contribute to limit cancer development in XP-C patients.

Funding

This work was supported by the French Government (National Research Agency, ANR, CNRS, INSERM) through the Investments for the Future LABEX SIGNALIFE program reference 1 ANR-11-LABX-0029-01; INS (Université de Nice Sophia Antipolis) and Association l'Enfant Tournou. MFRM was supported by the Fondation de l'Encadrement de l'Assurance Maladie de la Région Provence-Alpes-Côte d'Azur. The European Community's 7th Framework Programme (FP7) under Grant Agreement n° 241436. The Eurogene-XP project was supported by the French Ministry of Health (INSERM, AP-HP) and the European Commission in the framework of the Sixth Framework Program (FP6) under grant agreement n° 037955.

Declaration of Interest

The authors have no relevant affiliations or financial involvement with any organization or entity in a financial conflict of interest in the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received, or pending, or royalties.

References

Papers of special note have been highlighted as either of interest (+) or of considerable interest (+++) to readers.


106. The first demonstration of human epidermal stem cells correction and selective via epidermal expression of the cell surface selection epitope CDO.4. After genetic correction, selected epidermal stem cells proved to retain their normal proliferation, differentiation, and strafication potential to develop a normal, UV-exposed normal human epidermis after remodeling on the athymic mouse.


The first attempt to use gene editing for the correction of XP-C phenotype using engineered nucleases in transformed human XP-C cells overexpressing a DNA helicase.


The first description of a programmable dual-RNA-guided DNA endonuclease based on the CRISPR-Cas9 system, a new era in genome editing. The transposition of a bacterial immunity-associated mechanism to yeast cells forms the normal human epidermis after remodeling on the athymic mouse.


A Real-Time Cytotoxicity Assay as an Alternative to the Standard Chromium-51 Release Assay for Measurement of Human NK and T Cell Cytotoxic Activity

Julien Fasly,1 Kyriaki Tsalkitzi,1 Maria Goncalves-Maia,2 and Véronique M. Braud1

1Institut de Pharmacologie Moléculaire et Cellulaire, Université Côte d’Azur, CNRS, UMR 7375, Valbonne, France
2Institute for Research on Cancer and Aging, Université Côte d’Azur, Nice, France

This unit describes the monitoring and quantification of cellular cytotoxicity using a non-radioactive and real-time cytotoxicity assay. The extent of target-cell lysis is monitored over time by imaging and quantifying live fluorescent target cells using a cell-imaging multimode reader. This assay is performed in a 96 well plate in optimized culture conditions at 37°C in the presence of 5% CO₂. The basic protocol describes natural killer cell-mediated cytotoxic assay that can be adapted to include antibodies blocking inhibitory NK receptors or triggering antibody-dependent cell-mediated cytotoxicity (ADCC). The assay is also suitable for antigen specific T-cell cytotoxic assays. Until now, the standard chromium 51 (⁵¹Cr) release assay has remained the sole sensitive assay but its major drawbacks include cost and hazard of handling radioactivity. The real-time cytotoxic assay is therefore an effective alternative providing a robust and sensitive assay that accurately monitors lysis of target cells over time. © 2017 by John Wiley & Sons, Inc.

Keywords: cell cytotoxic assay • antibody-dependent cell-mediated cytotoxicity • natural killer cells • cytotoxic T cells

INTRODUCTION

This unit describes a non-radioactive and real-time cytotoxicity assay that provides reliable measurement of NK and T-cell-mediated cytotoxicity (Fasly, Tsalkitzi, Salavagione, Hamouda-Tekaya, & Braud, 2017). Assessment of cellular cytotoxicity is essential in research and in the clinic when characterizing immune responses and monitoring efficacy of immunotherapies. So far, the standard ⁵¹Cr-release assay has remained the most sensitive cytotoxic assay (Thorn, Palmer, & Mascon, 1974). Target cells are loaded with ⁵¹Cr, and release of ⁵¹Cr in the media measured after 4-hour incubation with cytotoxic effector cells is proportional to the lysis of target cells (see UNITS 7.1B & 7.2B; Nelson, Kurnan & Serbousek, 2001, Whiteside, 2001b). However, due to the cost and hazard of handling radioactivity, it has become a major issue to develop alternative non-radioactive assays with comparable sensitivity. Previous attempts to provide such assays have failed. Assays that measure enzymes or proteases released by dead cells cannot differentiate
target from effector cell death (Korzenniewski & Callewaert, 1983). Measurements of the release of fluorescent molecules upon target cell death lack sensitivity mainly because of the autofluorescence of the media (Hennina, Dakubu, Mukkala, Sitari, & Logren, 1984; Kummerow et al., 2014; Lichterfeld, Biddison, Schulz, Vogt, & Martin, 1994, Neri, Mariani, Meneghetti, Catanii, & Facchini, 2001). And the flow cytometry-based assays can monitor degradation of effector NK or T cells but not target cell lysis (Alen, Malenfant, & Atfield, 2004), or they can record apoptosis of target cells at a single time-point but not throughout the assay (Fieger et al., 1995). The recent development of cell-imaging multimode readers now provides a novel opportunity to set up such alternative cytotoxic assay to ⁵¹Cr-release assays.

In the Basic Protocol, target cells are loaded with the cell-permeable calcine-AM dye hydrolyzed by intracellular esterases into fluorescent calcine retained in the cytoplasm. Labeled cells are washed and incubated with increasing numbers of cytotoxic NK cells. In control wells, NK cells are replaced by non-labeled target cells to provide similar density of cells in the wells. NK cell-mediated cytotoxicity is measured by quantifying the number of fluorescent target cells in each well every 10 to 15 min over 4 hr. Alternate Protocol 1 includes the addition of mAbs that block key ligand/receptor pairs that modulate effector functions of NK cells. An example is shown using an anti-MHC class I mAb blocking MHC class I interaction with inhibitory killer-cell Immunoglobulin-like receptors and HLA-E interaction with CD94/NKG2A. Alternate Protocol 2 describes an antibody-dependent cell-mediated cytotoxicity (ADCC) assay using Rituximab, a human/mouse chimeric anti-CD20 mAb administered to patients. ADCC is a key mechanism of action of therapeutic mAbs resulting in depletion of tumor cells. Alternate Protocol 3 describes an antigen-specific T-cell cytotoxic assay. Target cells are pulsed with antigens peptides that bind to specific MHC class I molecules before calcine labeling. Washed target cells are then incubated with cytotoxic T cells (CTLs) restricted to a particular MHC class I molecule loaded with a specific antigenic peptide.

**STRATEGIC PLANNING**

The protocol for assessing cellular cytotoxicity requires the use of effector cells that are prepared either on the day of the assay, the day before the assay, or even cultured for several weeks before the assay. Effector cells include peripheral blood mononuclear cells (PBMCs) that can be readily obtained from whole blood by density gradient centrifugation (see UNIT 72; Fuss, Kanof, Smirh, & Zola, 2000), highly purified natural killer cell populations isolated from PBMCs by magnetic separation (see UNIT 77; Whiteside, 2001a), and NK and cytotoxic T cells expanded in culture over several weeks (see UNITS 77 & 79; Whiteside, 2001a; Yssel & Spins, 2002).

The assay is performed using a Cytation 5 (BioTek) cell imaging multimode plate reader, which has a chamber that needs to be set at 37°C and an atmosphere supplemented with 5% CO₂ at least 15 min before the assay.

**QUANTIFICATION OF NATURAL KILLER CELL-MEDIATED CYTOTOXIC ACTIVITY USING A REAL-TIME DIGITAL BIO-IMAGING ASSAY**

In this protocol, the specific lysis of target cells by natural killer cells is monitored over time using a non-radioactive cytotoxic assay. Target cells are labeled with calcine-AM and become fluorescent. They are incubated with effector NK cells at different effector to target (E:T) ratios. A cell-imaging multimode reader is used to count the number of fluorescent cells over time in each well of a 96-well plate. The percentage of specific lysis of target cells is calculated using a ratio of cell numbers in wells with effector cells normalized to the starting number of cells at t₀ over the cell numbers in wells without effector cells normalized to the starting number of cells at t₀.
Materials

K562 erythroleukemic cells (ATCC, cat. no. CCL-243; DSMZ, cat. no. ACC-10)
SUDHL4 B cell lymphoma cells (ATCC, cat. no. CRL-2957; DSMZ, cat. no. ACC-495)
OciLy19 B cell lymphoma cells (DSMZ, cat. no. ACC-528)
Human PBMC: freshly isolated (see UNIT 7.2; Fuss et al., 2009) or from liquid
nitrogen storage (see UNIT 4.3; Sterich & Eiermann, 2013)
Highly purified human NK cell populations isolated by magnetic separation (see
UNIT 7.7; Whiteside, 2001a)
Human NK cell lines expanded following allogenic stimulation (see UNIT 7.7
Whiteside, 2001a)
IMDM/10% FBS: complete Iscove’s Modified Dulbecco’s Medium (APPENDIX 2A)
supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml
penicillin and 100 μg/ml streptomycin
500 ng/ml (500 μM) calcine-AM cell-permeant dye (Molecular Probes), stored at
−20°C
15-ml and 50-ml conical tubes
96-well imaging microplate with lid, black with clear flat bottom and tissue
culture-treated (Falcon)
Cytation 5 multi-mode plate reader (BioTek) for cell imaging, connected to a
source of CO₂, holding a GFP filter cube (Excitation 469/35/Emmision 525/39,
mirror-497), a 463 nm LED, and a 4× phase-contrast objective.
Gen5 software (BioTek)
Multichannel (12-channel) pipette with 200-μl disposable tips

Additional reagents and equipment for obtaining fresh PBMC from whole blood
(see UNITS 7.1 & 14.32; Fuss et al., 2009, Sterich & Eiermann, 2013), frozen PBMC
(see UNIT 14.32; Sterich & Eiermann, 2013), highly purified human NK cells (see
UNIT 7.7; Whiteside, 2001a), and expanded human NK cell lines (see UNIT 7.7,
Whiteside, 2001a)

NOTE: All reagents and materials used in the preparation of the cells and during the
assay must be sterile.

Label target cells with calcine-AM

1. Culture K562, SUDHL4, and OciLy19 target cells to a density of 2 × 10^5 cells/ml
in IMDM/10% FBS.

2. On the day of the assay, count target cells (APPENDIX 3A & 4B).

3. Take a bit more than the required number of cells (2 × 10^4 calcine-labeled cells per
well) for labeling with calcine: 1–2 × 10^6 target cells.

4. Wash cells twice in IMDM/10% FBS 1–2 × 10^6 target cells, centrifuging 5 min at
500 × g, 20°C. Discard supernatant and suspend the cell pellet to a density of 10^6
cells/ml in IMDM/10% FBS.

5. Add 1 μl calcine-AM stock solution per 10^6 cells (final concentration 0.5 μM), mix
gently, and incubate the cells at room temperature in the dark for 15 min.

6. Wash cells twice with 20 ml IMDM/10% FBS, centrifuging 5 min at 500 × g, 20°C.

7. Suspend the cells in IMDM/10% FBS to a density of 2 × 10^6 cells/ml.

When manipulating calcine-labeled target cells, direct bright light must be avoided.
Prepare unlabeled target cells for control wells

8. Take the required number of target cells that will be added to control wells (0.5 to 2 \times 10^6 cells per well). Wash them twice in IMDM/10% FBS, centrifuging 5 min at 500 \times g, 20°C.

9. Discard supernatant and suspend the cell pellet in IMDM/10% FBS to a density of 2 \times 10^5 cells/ml for the 10:1 effector to target (E:T) ratio. Prepare additional dilutions at 1 \times 10^6 cells/ml and 5 \times 10^5 cells/ml in IMDM/10%FBS for 5:1 and 2.5:1 E:T ratios, respectively.

Prepare effector cells

10. Transfer the required number of effector PBMC or NK cells (0.5–2 \times 10^5 cells per well) to a conical tube. Wash the cells twice in IMDM/10% FBS, centrifuging 5 min at 500 \times g, 20°C.

11. Discard supernatant and suspend the cell pellet in IMDM/10% FBS at a density of 2 \times 10^6 cells/ml, for the 10:1 effector to target (E:T) ratio. Prepare additional dilutions at 10^6 cells/ml and 2 \times 10^5 cells/ml in IMDM/10% FBS for 5:1 and 2.5:1 E:T ratios, respectively.

Perform real-time cytotoxic assay

12. Switch on the Cytation 5 (BioTek) reader and Gen5 software (BioTek) on the associated computer. Calibrate according to manufacturer’s instructions. Set temperature at 37°C and the chamber atmosphere to 5% CO₂.

13. Transfer 100 \mu l (2 \times 10^5) calcine-labeled target cells to each well of a 96-well imaging microplate (black with clear flat bottom and tissue culture-treated) with lid. Include replicates of two or three wells for each condition.

An example of layout is shown in Figure 7.42.1A.

14. Centrifuge the 96-well plate for 2 min at 50 \times g, 20°C, with low brake.

Cells should be distributed homogeneously in the wells.

15. Cover the plate with aluminum foil and transfer to the Cytation 5.

16. Set the protocol of the Cytation 5 to focus on the calcine-labeled target cells, with the following parameters: the plate type used in the assay, including the use of the lid, image as detection mode, the GFP channel, the wells to be analyzed, and 4 pictures per well.

17. Place the plate in the Cytation 5, and focus on the calcine-labeled target cells.

18. Adjust settings (e.g., LED density, integration time, and camera gain) to obtain a focused picture with sufficient contrast over background. Save settings.

19. Set a time course of 4 hr with pictures taken every 10 or 15 min.

20. Set the processing of the images to image stitching with an average fusion method, and register the cell count of the 4 pictures in each well.

21. Remove the plate from the Cytation 5.

22. Add 100 \mu l unlabeled target and effector cells at each selected E:T ratio (10:1; 5:1; 2.5:1), and mix using a multichannel pipette.

An example of layout is shown in Figure 7.42.1A.

Experimental wells contain calcine-labeled targets and effector cells, while control wells contain calcine-labeled targets and unlabeled targets at the same E:T ratios as effector cells.
Figure 7.42.1 NK cell-mediated cytotoxicity assay (A) Plate setup, (B) Time-course of NK cell-mediated lysis of K562 erythroblastic target cells.

23. Centrifuge the 96-well plate for 2 min at 50 × g, 20°C, with low brake. Cover the plate with aluminum foil during transfer to the Cytation 5.

24. Put the plate in the Cytation 5, and start the protocol.

25. At the end of the 4 hr assay, calculate the cell count in each well at each time point during the 4 hr incubation, and register the data.

26. Analyze further the data using Gen5™ software (BioTek). Calculate the percentage of lysis as follows: % lysis = \[1 - (\text{experimental well at } t \div \text{control well at } t0)\] × 100 (Fig. 7.42.1B).

MONITORING OF THE REGULATION OF NK CELL-MEDIATED CYTOTOXIC ACTIVITY USING A REAL-TIME DIGITAL BIO-IMAGING ASSAY

In this alternate protocol, the modulation of NK cell lysis by inhibitory NK receptors is monitored using mAbs blocking the interaction with their ligands, the MHC class I molecules.

ALTERNATE PROTOCOL

Immunologic Studies in Humans

7.42.5

Current Protocols in Immunology

Supplement 118

209
210

Figure 7.42.2  Modulation of NK cell-mediated cytotoxicity by inhibitory receptors. (A) Plate set up. (B) Time-course of NK cell-mediated lysis of SUDHL4 B-cell lymphoma cells in the presence of blocking anti-MHC class I mAb.

Additional Materials (also see Basic Protocol)
1 mg/ml anti-MHC class I mAb, DX17 clone, mlgG1.K (BD Biosciences)
1.1 ng/ml isotype mlgG1, MOPC 21 clone (Sigma-Aldrich)

Perform real-time cytotoxic assay
1. Prepare calcein-labeled target cells, unlabeled target cells, and effector cells as described in Basic Protocol.
2. Prepare a solution of 200 μg/ml blocking mAbs (isotype mlgG1 or anti-MHC class I mAb) in IMDM/10% FBS.
3. Transfer 10 μl of medium alone, diluted isotype mlgG1, or anti-MHC class I mAb to a 96-well imaging microplate. For each condition, include duplicates or triplicates with and without effector NK cells. The final concentration of the mAb during the assay should be 10 μg/ml.

An example layout is shown in Figure 7.42.2A.
4. Transfer 100 μl (2 x 10^5) calcein-labeled target cells to each well of the 96-well imaging microplate, and mix using a multichannel pipette. Incubate at 37°C for 15 min.
5. Switch on CytoFluor 5 (BioTek) and Gen 5 software (BioTek) on the associated computer. Perform the calibration according to manufacturer instruction. Set temperature at 37°C and the chamber atmosphere to 5% CO₂.

An example of the plate layout and analysis is shown in Figure 7.42.2.
MEASUREMENT OF ADCC USING A REAL-TIME DIGITAL BIO-IMAGING ASSAY

This alternate protocol describes the measurement of antibody-dependent cell-mediated cytotoxicity (ADCC) which is triggered upon cross-linking by an antibody specific for a cell-surface antigen on target cells and binding to activating Fc receptors on NK cells. The unit describes ADCC using increasing concentrations of the chimeric human/mouse anti-CD20 mAb Rituximab that binds to CD20-expressing B lymphoma targets and to CD16 (FcγRIIIa) expressed by NK cells (see UNIT 7.42.7 & 14.39; Nelson et al., 2001; Warren, 2013).

Additional Materials (also see Basic Protocol)

1 mg/ml anti-CD20 mAb, Rituximab (Genentech) in PBS containing 0.1% BSA, prepared from a 10 mg/ml stock and stored at 4°C
1.19 mg/ml human myeloma IgG1 (Sigma Aldrich)

Perform real-time cytotoxic assay

1. Prepare calcine-labeled target cells, unlabeled target cells, and effector cells as described in Basic Protocol.

SUDHL4 and Ocyly19 cells can be used as target cells in this protocol because they express CD20. K562 erythroleukemic cells are not used in ADCC because they do not express CD20.

The number of effector cells is calculated for one E:T ratio.

2. Prepare serial 10-fold dilutions of isotopic IgG1 and Rituximab from 200 μg/ml to 0.0002 μg/ml in IMDM/10% FBS.

![Image of ADCC assay](image)

Figure 7.42.3 ADCC assay (A) Plate set up (B) Time-course of ADCC of SUDHL4 B-cell lymphoma cells in the presence of 1 μg/ml anti-CD20 mAb (Rituximab) (C) ADCC in the presence of increasing concentrations of Rituximab.

Current Protocols in Immunology

Supplement 118

211
3. Transfer 10 μl of medium alone, or each dilution of antibody (isotype IgG1 or Rituximab) to a 96-well imaging microplate. For each condition, include duplicates or triplicates with and without effector cells. The final concentrations of mAb during the assay should be 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 μg/ml.

An example layout is shown in Figure 7.42A.

4. Transfer 100 μl (2 × 10⁶) calcia-labeled target cells in each well of the 96 well black with clear flat bottom tissue culture-treated imaging microplate. Mix using a multichannel pipette, and incubate for 15 min at 37°C.

5. Switch on CytoVation 5 (BioTek) and Gen5 software (BioTek) on the associated computer. Perform the calibration according to manufacturer instruction. Set temperature to 37°C and the chamber atmosphere to 5% CO₂.

6. Follow Basic Protocol, steps 14 to 26, except assay unlabeled target and effector cells at one E:T ratio, between 10:1 to 5:1.

An example of the plate layout and analysis is shown in Figure 7.42.

**QUANTIFICATION OF T CELL-MEDIATED CYTOTOXIC ACTIVITY USING A REAL-TIME DIGITAL BIO-IMAGING ASSAY**

This alternate protocol describes the lysis by specific cytotoxic T-cell clones of B lymphoblastoid cells that are loaded with a 9-mer antigenic peptide binding a specific MHC class I molecule expressed by the target cells.

**Additional Materials (also see Basic Protocol)**

- B-lymphoblastoid cells immortalized by Epstein Barr virus (MHC class I type must be known)
- 10 mM 9-mer synthetic peptides that binds to identified MHC class I molecules, prepared in DMSO, stored at −20°C
- Cloned human T-cell lines (UNIT 7.19; Yssel & Spits, 2002)

**Pulse B-lymphoblastoid target cells with antigenic 9-mer peptide**

1. Maintain B-lymphoblastoid target cells in IMDM/10% FBS at 2 × 10⁶ cells/ml.

2. On the day of the assay, count target cells (APPENDIX 3A a b), and transfer 2 × 10⁶ target cells to a conical tube.

3. Wash the target cells twice in IMDM/10% FBS, centrifuging 5 min at 500 × g, 20°C.

4. After the second wash, discard supernatant, suspend the cell pellet in 200 μl IMDM/10% FBS, and split the sample into two tubes.

5. Prepare 1 mM 9-mer peptide solution in IMDM/10% FBS and 10% DMSO in IMDM/10% FBS (i.e., 10-fold dilution of 10 mM 9-mer stock solution or DMSO).

6. Add 4 μl of 1 mM 9-mer peptide solution to the target cells (final concentration 20 μM) or 4 μl 110% DMSO to unspun control target cells, mix gently, and incubate the target cells for 1 hr at 37°C.

7. Wash target cells twice in IMDM/10% FBS, centrifuging 5 min at 500 × g, 20°C. Discard the supernatant.

8. After the second wash, suspend the cell pellet in IMDM/10% FBS to a density of 10⁶ cells/ml in IMDM/10% FBS.
Figure 7.42. A) Plate setup. (B) CTL-mediated lysis of an HLA-A*0301-expressing 10^5 cell line loaded with 20 μM influenza nucleoprotein NP265-273 at the indicated E:T ratio after 4 hr incubation.

**Label target cells with calcine**

9. Add 1 μl calcine stock solution per 10^6 cells (final concentration 0.5 μM), mix gently, and incubate the cells for 15 min at room temperature in the dark.

When manipulating calcine-labeled target cells, direct bright light must be prohibited.

10. Wash cells twice with 20 ml IMDM/10% FBS, centrifuging 5 min at 500 × g, 20°C.

11. Suspend the labeled target cells in IMDM/10% FBS to a density of 2 × 10^5 cells/ml.

**Prepare unlabeled target cells for control wells**

12. Transfer the required number of B-lymphoblastoid target cells that will be added to control wells (12, 6, 3, 2, and 1 × 10^6 cells per well) to a conical tube. Wash them twice in IMDM/10% FBS, centrifuging 5 min at 500 × g, 20°C.

13. Discard supernatant and suspend the cell pellet in IMDM/10% FBS at a density of 12 × 10^6 cells/ml, for the 60:1 effector to target (E:T) ratio. Prepare additional dilutions at 6 × 10^6 cells/ml, 3 × 10^6 cells/ml, 2 × 10^6 cells/ml, and 10^5 cells/ml in IMDM/10% FBS for 30:1, 15:1, 10:1 and 5:1 E:T ratios, respectively.
Prepare effector cells

14. Transfer the required number of effector cytotoxic T cells (12, 6, 3, 2, and 1 × 10^6 cells per well) to a conical tube, and wash them twice in IMDM/10% FBS, centrifuging 5 min at 500 × g, 20°C.

15. Discard supernatant and suspend the cell pellet in IMDM/10% FBS at a density of 12 × 10^6 cells/ml for the 66:1 effector to target (E:T) ratio. Prepare additional dilutions at 6 × 10^6 cells/ml, 3 × 10^6 cells/ml, 2 × 10^6 cells/ml, and 10^6 cells/ml in IMDM/10% FBS for 30:1, 15:1, 10:1 and 5:1 E:T ratios, respectively.

Perform real-time cytotoxic assay

16. Follow Basic Protocol, steps 12 to 26, except assay unlabeled target and effector cells at E:T ratios 30:1, 15:1, 10:1 and 5:1.

An example of the plate layout and analyses is shown in Figure 7.42.4.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5

Calcin AM stock solution

Calcine AM cell-permeant dye (Molecular Probes): pack of 20 × 50 μg, MW: 994.87

Suspend in DMSO at 500 ng/μl

Store aliquots at −20°C, and avoid freeze-thaw cycles.

IMDM/10% FBS

Complete IMDM medium (APPENDIX 2A) supplemented with:

10% (v/v) heat-inactivated fetal bovine serum (FBS)

100 U/ml penicillin

100 μg/ml streptomycin

Store up to 4 weeks at 4°C

COMMENTARY

Background Information

Cell-mediated cytotoxicity is a major effector function of natural killer (NK) cells and cytotoxic T cells (CTL). While CTLs are restricted to a specific MHC class I molecule presenting an antigenic peptide, generally of 9-mer length, NK cells detect the absence of MHC class I molecules (natural cytotoxicity) or target cells coated with antibodies (ADCC). The standard 51Cr-release assay has remained the most sensitive assay to measure cytotoxic activity (Thorn et al., 1974). Target cells are loaded with 51Cr, washed, and incubated for 4 hr with NK cells or CTLs. Supernatants are harvested and 51Cr radioactivity is measured using a γ-radiation scintillation counter. The percentage of lysis is calculated as experimental lysis minus spontaneous lysis without effector cells divided by maximum lysis in detergent minus spontaneous lysis (see UNIT 7.18; 7.72 & 111; Neder et al., 2001; Whiteside, 2001b: Wendlerich, Shearer, Livingston & Brooks, 2006). Due to the cost and hazards associated with 51Cr, however, alternative non-radioactive assays with similar sensitivity are needed. In the past two decades, methods that measure the release of enzymes or proteins by dead target cells have been used, but they lack sensitivity and specificity as the death of effector NK and T cells is measured concurrently with target cell death (Corey et al., 1997; Korzeniewski & Callewaert, 1983). Methods that detect fluorescent molecules released into supernatants upon target cell lysis also lack sufficient sensitivity and reproducibility (Kummerow et al., 2014; Lichti et al., 1994; Neri et al., 2001). Many cells are refractory to loading and the autofluorescence of the media decreases sensitivity of the assays (Hammar et al., 1984). More recently, flow cytometry assays have been used in place of 51Cr release assay. The most common is the detection of CD107a (LAMP-1) at the cell surface of NK cells or CTLs, which correlates with
degradation (Alter et al., 2004). This assay fails to detect target cell death. Flow cytometry assays that detect fluorescent target cell killing have been developed, but these assays only detect target cells undergoing apoptosis and not the entire target cell killing over the course of the assay (Fligner et al., 1995).

With cell-imaging multimode readers, we can now visualize target cell killing by NK cells and CTLs over the entire length of the assay. Target cells are labeled with calcine-AM, which is hydrolyzed by intracellular esterases into highly fluorescent calcine retained in the cytoplasm. Fast and accurate quantification of fluorescent target cells is performed using images of each well registered every 10 to 15 min. This assay provides a time course of NK or CTL killing. Results show that in most assays, the majority of cell killing occurs within the first 2 hr.

Critical Parameters and Troubleshooting

The real-time, digital bio-imaging assay can be used to measure the cytotoxic activity of several types of effectors cells. Robust measurement of NK cell killing is obtained using PBMCs readily isolated from blood by density gradient centrifugation or NK cells readily purified from blood by magnetic separation. Frozen cells can also be used and provide efficient NK cell killing. When availability of NK cells is limited, they can be expanded in culture over several weeks by allogenic stimulation and in the presence of IL-2. NK cells purified by magnetic separation are stimulated with irradiated allogenic PBMCs and B-cell lines in complete medium supplemented with 500 U/ml human IL-2. Exponential expansion is obtained over several weeks. NK cell lines are then cultured in medium without IL-2 for about a week to recover from activation before being used in the real-time cytotoxic assay. Importantly, this assay does not depend on effectorc cell viability at the time of the assay, as only target cell death is measured. Similarly, CTL clones and lines can be used from expanded cultures. The E:T ratios used will depend on the frequency of specific CTL clones within a CTL line.

Target cells can be adherent or in suspension. They should be maintained in exponential growth before labeling to reduce spontaneous cell death. Generally, calcine-AM easily permeates all types of target cells. The concentration of calcine has been optimized to obtain sufficient intensity of the fluorescent signal over the background and throughout the course of the assay. Importantly, a significant number of cells express multidrug resistance systems that actively extrude calcine. To retain calcine, we have tested inhibitors—e.g., indomethacin and MC571—that efficiently block multidrug resistance transporters. In preliminary experiments, these inhibitors show an insignificant inhibition of NK cell killing, suggesting that they may be used to maintain calcine in target cells during the killing assay (unpublished data). Alternatively, target cells expressing multidrug resistance transporters can be stably transfected with vectors coding for fluorescent proteins, including GFP, RFP, and mCherry.

The assay is performed under optimized cell culture conditions at 37°C in an atmosphere supplemented with 5% CO2 using a cell-imaging multi-mode plate reader CytoFlex 5 developed by BioTek. To ensure accurate cell counts, 2 x 10⁶ fluorescent target cells per well was identified as an optimum number of cells. Four images per well are taken and stitched together to allow cell counting over most of the well surface. Importantly, the cell density in each well must be near 90% confluence to avoid cell aggregation (Fassler et al., 2017). The addition of a minimum of 10⁵ effector cells or unlabeled target cells is therefore required (E:T ratio). This may be a limiting factor when effector cells are readily purified from blood or when CTL clones that efficiently kill their targets are used. In these situations, the number of fluorescent target cells per well can be increased to a 4 x 10⁶ cells.

Anticipated Results

The main advantage of this assay over current assays is the monitoring of cytotoxicity throughout the assay. The kinetics allow the visualization of different killing behaviors between conditions.

Time Considerations

The real-time cytotoxic assay has revealed that half maximum killing is reached within 30 min and maximum killing is obtained after 2 hr. The assay can therefore be shortened to 2 hr. The assay has a simplified procedure since supernatants need not be harvested. The isolation or culture of effector cells are therefore the longest steps.

Acknowledgements

This work was supported by Centre National de la Recherche Scientifique, Cancéropole PACA, Fondation ARC pour la recherche sur le cancer, and Agence Nationale de la Recherche (ANR).
Conflict of interest

VMI and JF benefited from a reimbursement from BioTek from attending a symposium. KT and MCIM have no conflict of interest to disclose.

Literature Cited


Basal Cell Carcinoma in Gorlin’s Patients: a Matter of Fibroblasts-Led Protumoral Microenvironment?

Yannick Gasche1, 2, Florence Brelle1, Sophie Rouxset1, 2, Sahar Al-Gharaghiu1, 2, Maria Goncalves-Mais1, 2, Elodie Burti-Vallin1, Stéphane Barney1, Sabine Scarfato1, 2, Martial Rust1, Nicolas Savet1, Marie-Françoise Avril1, Thierry Magnan1, 2

1 INSERM U1081—CNRS UMR7294—UNS, Nice, France, 2 Université de Nice-Sophia Antipolis, Faculté de Médecine, Nice, France, 3 CNRS FRE2939, Université de Paris Sud—Institut Gustave Roussy, Villejuif, France, 4 CNRS UMR 1097, Neurosciences Paris-Saclay Institute, Gif-sur-Yvette, France, 5 INSERM U916 & Institut Bergonie, Laboratoire de génétique moléculaire, Bordeaux, France, 6 Service de Dermatologie, Université Paris 5 AMRH, Paris, France

Received: August 5, 2015
Accepted: December 1, 2015
Published: December 22, 2015

Copyright: © 2015 Gasche et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Basal cell carcinoma (BCC) is the commonest tumor in human. About 70% sporadic BCCs bear somatic mutations in the PATCHED1 tumor suppressor gene which encodes the receptor for the Sonic Hedgehog morphogen (SHH). PATCHED1 germline mutations are associated with the dominant Nevoid Basal Cell Carcinoma Syndrome (NBCCS), a major hallmark of which is a high susceptibility to RCCs. Although the vast majority of sporadic RCCs arises exclusively in an exposed skin areas, 40 to 60% BCCs from NBCCS patients develop in non-photo-exposed skin areas. Since overwhelming evidences indicate that microenvironment may both be modified by- and influence the epidermal tumor, we hypothesised that NBCCS fibroblasts could contribute to BCCs in NBCCS patients, notably those developing in non-photo-exposed skin areas. The functional impact of NBCCS fibroblasts was then assessed in organotypic skin cultures with control keratinocytes. Onset of epidermal differentiation was delayed in the presence of primary NBCCS fibroblasts. Unexpectedly, keratinocyte proliferation was severely reduced and showed high levels of nuclear P53 in both organotypic skin cultures and in fibroblast-conditioning experiments. However, in spite of downregulated levels of senescence-associated β-galactosidase activity in keratinocytes cultured in the presence of medium conditioned by NBCCS fibroblasts, we failed to observe activation of P16 and P21 and then of bona fide features of senescence. Constitutive extraction of P53 in WT keratinocytes resulted in an invasive phenotype in the presence of NBCCS fibroblasts. Finally, we found that expression of SHH was limited to fibroblasts but was dependent on the presence of keratinocytes. Inhibition of SHH binding resulted in improved epidermal morphogenesis. Altogether, these data suggest that the repertoire of diffuse factors (including SHH) expressed by primary NBCCS fibroblasts generate stress affecting keratinocytes behavior and epidermal homeostasis. Our findings suggest that defects in dermoepidermal interactions could contribute to BCC susceptibility in NBCCS patients.