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Study of the reciprocal interaction between replicative senescent or UVB-SIPS fibroblasts and squamous cell carcinoma cell lines at different stages of tumourigenesis

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IV. DISCUSSION AND PERSPECTIVES

Cellular senescence is associated with both positive and negative effects according to tissue context. On one hand, cellular senescence is related to tumour suppression. On the other hand, it is well known that senescent cells are involved in several age-related diseases and carcinogenesis. Indeed, it was shown that senescent cells enhance the growth, migration and invasion of cancer cells, notably via their SASP. However, little is known about their impact on skin carcinogenesis, and especially on cSCC development. Interestingly, cSCC is linked to ageing and cumulative sun exposure, which are also characterised by an accumulation of senescent cells. Thus, one can wonder the relationship between these two processes.

In this thesis, we characterised the interactions between human dermal fibroblasts in replicative senescence or in UVB-induced senescence and cSCC cells lines. We also investigated the mechanisms underlying the occurrence of UVB-SIPS in HDFs by studying the signalling pathways activated in this model.

A. Study of the SASP in replicative or UVB-induced senescence HDFs

In senescence studies, attention should be paid to the use of relevant cellular **senescence models**, namely cell type and senescence inducer, which must be ideally selected according to the tissue context. Indeed, some senescence biomarkers, such as the SASP, might be highly modulated in function of these parameters (Coppe et al., 2010; Toutfaire et al., 2017). Since both chronological and UVR-induced ageing affect cSCC development (Krutmann et al., 2017; Leiter et al., 2014), two relevant models of fibroblasts' senescence were compared, namely RS and UVB-SIPS HDFs. Indeed, RS could represent the chronological ageing process, and the UVB-SIPS could represent the photo-ageing of the dermis.

The SASP is generally induced at the **mRNA level** (Coppe et al., 2010). In order to carry out a screening, we thus investigated the expression of SASP-related genes by using a personalised TLDA allowing to simultaneously analyse the expression of 46 genes associated with the SASP. We showed that both models display a common but also a specific significant increase in some SASP-related genes expression. Interestingly, all these genes have been previously mentioned in SASP studies, such as those performed in other strains of senescent dermal HDFs (Freund et al., 2010; Lasry and Ben-Neriah, 2015).

It is well documented that RS and SIPS share common senescence biomarkers (Toussaint et al., 2002a). We highlighted here that RS and UVB-SIPS HDFs display some **common SASP-related gene expression changes**, notably an increase in *IL1B* and *IL6* mRNA expression. In this regard, mass spectrometric analyses and cytokine multiplex assay have shown that IL-1β is overexpressed in supernatants from intrinsically aged dermal HDFs (Waldera Lupa et al., 2015). Moreover, a previous study has also described that *IL1B* mRNA level is increased in UVB-SIPS HDFs (Debacq-Chainiaux et al., 2005). For *IL6*, its mRNA level is increased in RS dermal HDFs compared to young ones (Malaquin et al., 2013). Immunofluorescence staining has also showed that IL-6 expression in senescent stromal cells is significantly increased in skin from old individuals compared to younger individuals

(Ruhland et al., 2016). Finally, IL-6 mRNA and protein levels are significantly increased in dermal HDFs after UVB irradiation compared to non-irradiated HDFs (Brenneisen et al., 1999).

Interestingly, we also demonstrated **specific gene expression changes** between our two models of senescence. Indeed, a significant decrease in *CXCL1* and *CXCL2* gene expression in RS HDFs was highlighted. These decreases are also described in senescent HDFs isolated from chronic wounds using Affymetrix microarray analysis and ELISA quantitation of conditioned medium (Wall et al., 2008). For UVB-SIPS HDFs, we showed an increase in *MMP1* and *MMP3* mRNA level. In this regard, it has been shown that dermis samples from photo-damaged skin overexpress *MMP1* and *MMP3* genes, compared to their matched sun-protected controls (Quan et al., 2013). Another study has described that dermis from photo-aged skin displays higher levels of MMP-1 protein level compared to sun-protected skin from the same individuals (Chung et al., 2001). Supernatant from UVB-irradiated dermal HDFs also display an increase in MMP-1 and MMP-3 protein level compared to non-irradiated HDFs (Brenneisen et al., 1999). MMP-1 and MMP-3 mainly degrade collagen type I, whose precursor is type I pro-collagen (Pittayapruek et al., 2016). Thus, our data regarding the increase in *MMP1* and *MMP3* gene expression in UVB-SIPS HDFs are also consistent with the decreased protein expression levels of type I pro-collagen in photo-damaged dermis compared to subject-matched sun-protected controls *in vivo* (Quan et al., 2013). Finally, an increase in *CTGF*, *IGFBP3* and *TGFB1* mRNA level and both latent and active forms of TGF-β1 was also described in UVB-SIPS HDFs (Debacq-Chainiaux et al., 2005).

The specific SASP-associated gene expression signature observed in our study between RS and UVB-SIPS HDFs is somewhat reminiscent of the "**molecular scars**" observed between RS and subcytotoxic stresses inducing SIPS. This notion has been described in *t*-BHP-, ethanol- and H_2O_2-SIPS HDFs, where mRNA and protein expression changes can be classified into three categories: common with replicative senescence, specific to one kind of stress or specific to several kinds of stresses (Aan et al., 2013; Debacq-Chainiaux et al., 2008; Dierick et al., 2002a; Dierick et al., 2002b; Pascal et al., 2005). Other comparative analyses of the gene signatures in human dermal fibroblasts in RS or in several SIPS models have also identified differentially expressed genes according to the senescence inducer (Hernandez-Segura et al., 2017; Kural et al., 2016). Thus, our results highlight that molecular scars observed after SIPS, in this case UVB-SIPS, might also concern the SASP. This reinforces the need to use complementary models of senescence according to tissue context.

Further studies would be required to **confirm our results at the protein level**. We could perform antibody arrays, ELISA (enzyme-linked immunosorbent assay) or western blotting on senescent cell supernatants isolated from intrinsically or photo-aged skin samples for instance (Rodier, 2013). Regarding the use of cells derived from skin samples, a distinction between site of intrinsic and extrinsic ageing must be considered if possible (Tigges et al., 2014). It should be noted that most of the data regarding SASP composition at the protein level are often obtained through *a priori* methods, and with a limited number of protein candidates. Thus, it would be interesting to perform unbiased proteomic analysis of the secretome of senescent cells. In fact, mass spectrometry-based proteomic techniques, such as SILAC (stable

isotope labeling with amino acid), allow to do so. This approach relies on the use and incorporation of a non-radioactive and stable isotope for labeling in cell culture, and is worth considering for studying SASP composition (Acosta et al., 2013b).

The SASP is mainly described as being composed of secreted proteins (Coppe et al., 2008b). However, senescent cells-derived extracellular vesicles have also been identified. These EVs allow them to interact with surrounding cells (Kadota et al., 2018; Terlecki-Zaniewicz et al., 2018). Thus, it would be interesting to analyse EVs, and particularly exosomes, released by senescent cells. Indeed, EVs influence cell communication, including during the major steps of carcinogenesis, such as EMT, metastasis and angiogenesis (Azmi et al., 2013; Quail and Joyce, 2013). Interestingly, the notion of **SASF**, namely "senescence-associated secreted factors", may represent more effectively the various ways of communication used by senescent cells to communicate with their microenvironment (Bernard, 2018). SASF is also proposed to not only include secreted proteins but also lipids (Loo et al., 2017). Finally, a new mode of communication via intercellular protein transfer is also used by senescent cells. This was shown in senescent human fibroblasts that directly transfer some of their cytoplasmic protein content to neighbouring cells, namely immune cells and epithelial normal and cancer cells, via cytoplasmic bridges *in vitro* (Biran et al., 2015). The mechanisms underlying these interactions need further investigation. It seems that organelles might also be transferred (Biran and Krizhanovsky, 2015).

B. Impact of senescent HDFs on cSCC cell lines

After addressing the expression of SASP-related genes in senescent HDFs, we investigated whether these fibroblasts could impact cSCC cell lines. Indeed, carcinogenesis is not only explained by cell-autonomous properties of cancer cells, but involves the tumour microenvironment as well. Indeed, the surrounding cells influence **tumour development**, with an impact on initiation, dissemination, intravasation, extravasation, and metastatic colonisation processes for instance (Hanahan and Weinberg, 2011; Lambert et al., 2017). Moreover, they are associated with the reorganisation of the stromal network that may further impact cancer cell migration and metastasis formation for example (Clark and Vignjevic, 2015). A senescent microenvironment is also associated with detrimental roles in carcinogenesis. These deleterious effects are either made directly (through communication via secreted factors for instance) or indirectly (via the recruitment of other cell types or the induction of ECM reorganisation) that further impact tumour development (Di Mitri and Alimonti, 2016; Sieben et al., 2018). These various kinds of interactions can be studied *in vitro* through various methods. The role of secreted factors can be evaluated with the use of conditioned media or indirect co-cultures. Juxtacrine interactions, namely interaction between adjacent cells, can be assessed with direct co-culture experiments. The use of matrices-deposited by senescent cells is also described.

The role of senescent HDFs has been previously described in **skin carcinogenesis**. HDFs from aged donors secrete sFRP2 that induces melanoma cell invasion (Kaur et al., 2016). Conditioned media from senescent HDFs also increase the growth of pre-

cancerous melanoma cells and the invasion of invasive melanoma cells (Kim et al., 2013). Moreover, MMPs, secreted by human replicative senescent dermal HDFs, promote migration of post-senescence emergent keratinocytes, an early event in skin cancer development (Malaquin et al., 2013). IL-6 secreted by senescent stromal mouse cells promotes a tumour-permissive microenvironment that contributes to tumour promotion (Ruhland et al., 2016). Finally, it has been shown that senescent HDFs increase the migration of cSCC cells (including the A431 cell line). However, conditioned media collection was performed after seven days of incubation, which is quite long, since 48 or 72h of conditioning remains the usual protocol. This effect was attributed to chemerin, an unreported SASP factor (Farsam et al., 2016). Thus it would be interesting to check its expression in the senescent fibroblasts used in our study.

In our research project, we analysed the impact of RS and UVB-SIPS HDFs **conditioned media** on the growth, migration and invasion of cSCC cell lines at different stages of tumourigenesis. A limited impact was observed, with only a significant growth increase for PM1 incubated with UVB-SIPS conditioned medium. These negative results might reflect the interactions within the cSCC environment, since we used skin-derived senescent HDFs and cSCC cell lines. In a previous study, however, stimulation of keratinocytes with senescent HDFs conditioned media was achieved from the beginning of the culture until post-senescence emergence, which corresponds to about 45 days of stimulation (Malaquin et al., 2013). This long-term exposure, compared to short-term exposure, supports a pro-EMT effect of senescent fibroblast secretome on keratinocytes. This might partly explain why we observed limited effects since cSCC cell lines were only exposed for 24 hours with conditioned media, even if short-term exposure remains the usual protocol (Kaur et al., 2016; Kim et al., 2013). We also investigated the impact of conditioned media from senescent HDFs on the gene expression of EMT markers, namely *CDH1*, *VIM* and *SNAI2*, in cSCC cell lines. No significant changes were observed which is consistent with the absence of effect on migration and invasion after 24h of incubation with conditioned media.

The impact of senescent HDFs after **indirect co-cultures** was also analysed. Indirect co-culture allows bidirectional exchanges with cSCC cell lines. Our co-culture protocol was validated with a positive control, which is the positive impact of RS WI-38 HDFs on MDA-MB-231 cell line growth (Krtolica et al., 2001). Again, a very limited impact of senescent HDFs on cSCC growth was detected. First, we highlighted a significant increase in PM1 growth after one day of co-culture with UVB-SIPS HDFs. This result is consistent with the effect observed for UVB-SIPS HDFs-conditioned media. The growth of a pre-neoplastic spontaneously immortalized keratinocyte cell line with non-tumorigenic properties, HaCaT, was also increased after direct co-culture with bleomycin-SIPS dermal HDFs (Pazolli et al., 2009). Second, we also noticed a significant increase in MET4 growth at day 1 and 6 of co-culture with RS HDFs. However, no effect was shown in the other conditions tested.

These results highlight that RS and UVB-SIPS HDFs might differentially affect cSCC cell lines growth at different **tumourigenesis stages**. It is worth noting that the impact of the cancer cell tumourigenesis stage is unfortunately rarely investigated within the same study, yet this is an important question to address. Indeed, cancer cells progressively change during the multistep of tumour progression, and this

modulates their reciprocal heterotypic signalling with the surrounding cells over time (Hanahan and Weinberg, 2011).

Finally, it would be interesting to completely address the impact of senescent HDFs on cSCC cell lines after co-cultures, and particularly regarding their impact on the expression of EMT markers. This could be done by the analysis of the protein level of these markers by western blotting. It is important to note that cancer cells rarely undergo a complete EMT program. Thus, if an effect is observed, it is likely that EMT will be only partial, with the expression of mesenchymal traits while some epithelial traits will still be expressed (Brabletz et al., 2018; Nieto et al., 2016). We can also consider the fact that senescent cells may also impact cSCC cells indirectly, by modifying the ECM for instance (Pittayapruek et al., 2016). Indeed, senescent cells are known to secrete MMPs that can disrupt the ECM organisation. Besides this, the physical interactions and the mechanical forces between cancer cells and their microenvironment are important in carcinogenesis, including during cSCC development (Ratushny et al., 2012), and especially during the metastatic process (Wirtz et al., 2011). However, this readout cannot be addressed with co-culture experiments but rather with 3D matrices.

Altogether, the effects observed in our study appear to be limited and might reflect the **particular microenvironment** of cSCC. In fact, we have evaluated various strategies to possibly increase these effects. The concentration of conditioned media and their collection after increased time points (48h and 72h) still showed a limited impact on cSCC cells. Moreover, we also used HDFs with a very low or very high proportion of SA-βgal positive cells in order to further increase the differences between the two states but this gave the same results. Thus, the limited impact of senescent HDFs on cSCC cells in our study has been confirmed with several complementary approaches, namely the use of conditioned media and also co-cultures. Our data are questioning the well-described pro-carcinogenic effects of senescent cells described on other cancer cells. Since these effects are mainly attributed to the SASP whose composition is modulated by senescence inducers and cell types, this reinforces the importance of taking into account tissue context in senescence studies.

C. Impact of cSCC cell lines on HDFs

C.1. Impact of cSCC cell lines on the senescence status of HDFs

We then analysed the impact of cancer cell lines on **young and replicative senescent HDFs**. This is poorly studied in senescence studies that rather look at the impact of senescent cells on cancer cells. We investigated the senescence status of HDFs, by measuring the proportion of SA-βgal positive cells and the expression of SASP-related genes. Our results suggest that cSCC cell lines reinforce HDF senescent traits after indirect co-cultures. We showed that co-cultures with PM1, MET1 and MET4 cell lines increase the proportion of SA-βgal positive cells detected in young (but not in RS) HDFs when compared with the control condition (alone).

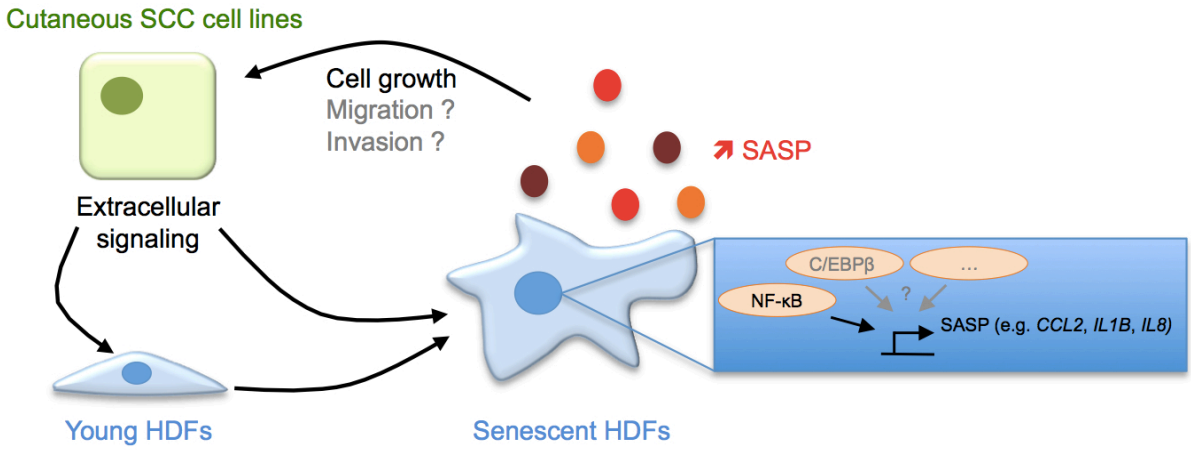

Fig. IV.1: Possible signalling between cutaneous squamous cell carcinoma (cSCC) cell lines and dermal HDFs.
Co-cultures between cSCC cell lines and dermal human diploid fibroblasts (HDFs) allow bi-directional exchanges. Extracellular signalling from cSCC cell lines induces senescence and senescence-associated secretory phenotype (SASP) expression in young HDFs and reinforces SASP in replicative senescent HDFs. This might involve an activation of nuclear factor-kappa B (NF-κB) and/or other transcription factors such as C/EBPβ (CCAAT/enhancer binding protein beta) in HDFs. It results in cSCC cell growth, and may impact migration and invasion. Overall these exchanges are modulated by the SCC cell line tumourigenesis stage. Source: Toutfaire et al., 2018.

Next, SASP expression at the mRNA level was studied by TLDA. Interestingly, our screening showed that co-cultures with PM1 and MET1 mostly induced a significant gene expression increase in young and RS HDFs, when compared with their respective controls. These results were confirmed for *CCL2*, *IL1B* and *IL8* mRNA expressions on a larger sample size and with A431, another cSCC cell line. Our data regarding the promotion of SASP-related gene expressions after co-culture with cancer cells are particularly interesting in the senescence context. Indeed, since senescent cells already overexpress SASP genes, this effect would reinforce their senescent state and SASP would be even more pronounced in senescent cells surrounding tumours.

The over-expression of SASP-related genes was **more pronounced in co-culture with MET1**, the highly invasive primary cSCC cell line. Similarly, CCL2, IL-1β and IL-8 are also up-regulated at the mRNA and protein levels in dermal HDFs co-cultured or exposed to conditioned media from several invasive melanoma cell lines when compared to a non-invasive one (Li et al., 2009). These results suggest that invasive cells may have higher impact on surrounding cells compared to pre-neoplastic ones.

The **mechanisms inducing SASP**-related over-expression during co-culture were then investigated. Our results strongly suggest that NF-κB, a master regulator of the mRNA expression of most SASP factors (Malaquin et al., 2016), is activated in HDFs during co-cultures. Indeed, we detected a significant increase in nuclear p65 staining in young and RS HDFs co-cultured with PM1 and MET4. As a reminder, NF-κB activation relies on the activation of IKK complex, which triggers the phosphorylation and subsequent degradation of IκB (Fig. I.21). A significant decrease in IκBα is thus reminiscent of NF-κB activation (Capece et al., 2017), and was observed in total protein extracts from young and RS HDFs co-cultured with PM1, MET1 and MET4. In order to confirm the functional involvement of NF-κB in the overexpression of SASP-related genes, we could test whether chemical inhibitors of NF-κB signalling would block their expression. Thus, treating HDFs with BAY 11-7082, an inhibitor of IKK complex that selectively and irreversibly inhibits the phosphorylation of IκBα, would result in the blocking of NF-κB signalling. Finally, other mechanisms might also be involved in the expression of the senescence secretome, such as C/EBPβ, the other major transcription factor regulating the expression of SASP factors (Malaquin et al., 2016).

Altogether, our results have highlighted an interaction between HDFs and cSCC cell lines, which is summarised in Fig. IV.1.

We have also investigated the impact of cSCC cell lines on **UVB-SIPS HDFs and CTL-SIPS HDFs**. The proportion of SA-βgal positive cells in UVB-SIPS HDFs and CTL-SIPS HDFs was significantly different after 1 day of culture (with or without cSCC cells). Surprisingly, this difference between UVB-SIPS and CTL-SIPS HDFs was reduced after 6 days of culture. Indeed, the proportion of SA-βgal positive cells increased in CTL-SIPS HDFs between day 1 and day 6, which may be related to the fact that the level of confluence was higher in CTL-SIPS HDFs. This was previously reported to be associated with an increase in the proportion of SA-βgal positive cells (Debacq-Chainiaux et al., 2009). Moreover, we did not detect an impact of co-culture with cSCC cells in CTL-SIPS HDFs, such as the one observed in young HDFs after 6

days of co-culture. This may be explained by the fact that the basal level of SA-βgal positive cells was higher in CTL-SIPS HDFs compared to young HDFs, which did not allow us to highlight a possible impact.

The analysis of SASP-related gene expression by TLDA and qPCR showed the same result, namely an absence of difference between CTL-SIPS and UVB-SIPS HDFs. This may suggest that CTL-SIPS HDFs become senescent in the co-culture condition. Thus, it would be informative to evaluate other senescence biomarkers, such as $p16^{Ink4a}$ or $p21^{Waf-1}$ expression for instance, to check the senescence status of these cells in comparison with UVB-SIPS HDFs. Finally, we highlighted that co-culture with PM1, MET1, MET4 and A431 cells highly increase SASP-related gene expression in both CTL-SIPS and UVB-SIPS HDFs. This is consistent with the results obtained in young and RS HDFs.

It would be also interesting to analyse the expression of other SASP-related genes after co-cultures, such as genes encoding for MMPs for instance. The **confirmation** of these results at the protein level would be also very interesting but it is tricky to investigate. Indeed, co-culture medium comes from both HDFs and cSCC cells so the origin of the secreted molecules is unknown. We could imagine to perform intracellular cytokine staining for instance. This is a flow cytometry-based assay that detects the production and accumulation of cytokines within the endoplasmic reticulum after treatment with an inhibitor of protein transport. Brefeldine A is a molecule that retains the cytokines inside the cell for instance (Lamoreaux et al., 2006). However, we would not be sure that these cytokines are indeed secreted under normal conditions.

The overexpression of several SASP-related genes after co-culture with cSCC cells is particularly interesting given that these genes encode cytokines that are widely known to be involved in **carcinogenesis**. Indeed, CCL2, also known as MCP-1 (monocyte chemoattractant protein-1), has a key role in the infiltration of inflammatory cells and in tumour immunity (Kalluri, 2016). Regarding IL-1β, which is a secreted form of IL-1, an involvement in inflammatory response (Kalluri and Zeisberg, 2006) and in tumour invasiveness is described (Dinarello, 1996). Finally, IL-8 has pro-angiogenic activities and is also involved in tumour progression and in the activation of an innate immune response (Coppe et al., 2010; Di Mitri and Alimonti, 2016; Kalluri, 2016). Thus, if the genes overexpressed after co-culture are also overexpressed at the protein level, they may further represent pro-tumourigenic signals to cSCC cells directly or indirectly (by activating other cell types).

Finally, it would be interesting to address the impact of senescent HDFs on **normal human keratinocytes** and the other way around effect by using indirect co-cultures. We have tried to answer to this question during this thesis. The choice of the co-culture medium was tricky. Indeed, our preliminary results suggest that keratinocyte growth medium modulates SASP expression in HDFs, and that the addition of serum induces the differentiation of keratinocytes. Thus, further optimizations would be required to address this point.

C.2. cSCC cell lines and cancer-associated fibroblasts

Fibroblasts constitute a crucial component of the tumour microenvironment and have been termed CAFs (Kalluri, 2016). They are involved in tumour progression, with the induction of proliferation, migration, invasion, and angiogenesis for instance (Ishii et al., 2016). The involvement of CAFs in **cSCC development** has been described. $FSP1^+$ (fibroblast specific protein-1, another marker of CAF) fibroblasts secrete CCL2 that may maintain local inflammation, and promote skin carcinogenesis in DMBA/TPA- (7,12 Dimethylbenzanthracene/12-O-Tetradecanoyl Phorbol 13-Acetate) induced skin cancer in mouse (Zhang et al., 2011). It has also been shown that CAFs increase the migration and invasion of cSCC cell line *in vitro* (Labernadie et al., 2017). They can also remodel the stromal ECM by secreting MMPs, which could further facilitate cSCC invasion (Nissinen et al., 2016).

The activation and recruitment of CAFs to the tumour are dependent on growth factors released by cancer cells (Kalluri, 2016). Therefore, we investigated whether co-cultures with cSCC cell lines could activate HDFs into CAFs. We analysed the expression of two main CAF markers, αSMA and FAP. Our **preliminary results** suggest that cSCC cell lines co-cultures with young, RS, CTL-SIPS and UVB-SIPS HDFs are not associated with CAF activation. Since this was only performed once, it would be interesting to confirm these results by performing at least two other independent experiments.

Understanding the mechanisms linking CAF activation and fibroblast senescence is little explored. The underlying link between these two events seems to be the CSL protein (also known as RBP-Jκ, recombination signal binding protein for immunoglobulin kappa J region, a key effector of the canonical NOTCH signalling). In fact, CSL down-modulation (after environmental insults like UVA radiation exposures) can induce senescence in human dermal fibroblasts. Then, subsequent loss of p53 can block senescence and enhance CAF marker expression. These findings support a combined CSL-p53 control of CAF activation (Procopio et al., 2015; Procopio et al., 2016).

D. Crosstalk between cSCC cell lines and HDFs

A bidirectional crosstalk is described between **cancer cells and CAFs**. It further increases some important capabilities of tumour cells, such as growth and invasion (Hanahan and Weinberg, 2011). Indeed, signals (chemokines for example) released by cancer cells enhance the ability of CAFs to secrete pro-tumourigenic factors. These ones exerting back a strengthening effect on cancer cells as a positive loopback (Mishra et al., 2011).

Interestingly, these kinds of interactions have been described between **skin** cancer cells and CAFs. For instance, pro-inflammatory factors are overexpressed by dermal HDFs co-cultured with invasive melanoma cell lines. Then, these melanoma cell lines display an increase in their invasive potential if they are co-cultured with HDFs, but not upon stimulation with fibroblast-conditioned media (Li et al., 2009). An education of dermal fibroblasts by carcinoma cells is also observed in K14/HPV16 mouse. This

model represents the multistep of squamous skin carcinogenesis since animals develop hyperplastic and then dysplastic lesions that progress to invasive SCC. This study has shown that fibroblasts display an induction of their inflammatory gene signature after incubation with conditioned media from a skin carcinoma cell line *in vitro* and *ex vivo*. Interestingly, an increase in nuclear p65 staining in CAFs is observed in skin section from K14/HPV16 mouse but not in control mice skin. Then, these inflammatory fibroblasts further enhance tumour growth and vascularisation (Erez et al., 2010).

Thus, our data regarding the increase in pro-inflammatory gene expressions in HDFs after co-culture with cSCC cells may suggest a bidirectional crosstalk reminiscent of the one occurring between CAFs and cancer cells. First, we need to validate that senescent HDFs have indeed a functional impact on cancer cells. If any, we could investigate the related signalling pathways and the known mediators involved in this process. Then, the blocking of these mediators, with neutralising antibodies for instance, would confirm their involvement.

Finally, it is known that tumours are associated with an **accumulation of senescent cells** in adjacent tissues (He and Sharpless, 2017; Naylor et al., 2013; Sieben et al., 2018). This is described in hepatocellular carcinoma and ovarian cancer for example (Sieben et al., 2018). Thus, one can wonder why these senescent cells accumulate at these sites. One explanation might be that cancer cells induce senescence in the surrounding stroma. In fact, our data suggest that cSCC cell lines induce and reinforce senescence in HDFs. A previous study has indeed shown that tumour cells produce local signals that induce non-cell-autonomous $p16^{Ink4a}$ expression in the tumour-associated stroma in mice *in vivo* (Burd et al., 2013). Thus, it would be very informative to perform $p16^{Ink4a}$ labelling in human skin biopsies, from actinic keratosis, up to invasive SCC lesions, and to evaluate whether if an accumulation of senescent cells is observed around these pre-cancerous and cancerous sites.

E. Signalling pathways activated in UVB-SIPS HDFs

Our results suggest a modulation of the senescent state of HDFs after co-cultures with cSCC cells. This has been shown in young HDFs that display an increase in the proportion of SA-βgal positive cells and an increase in SASP-related gene expression. Regarding RS HDFs, they also showed an increase in SASP-related gene expression. Thus, the question that remains open is which signalling pathways are activated in these co-cultured fibroblasts, in comparison with “normal” conditions, namely in the absence of cSCC cells.

SIPS models are useful for studying the molecular pathways involved in the onset of the senescent phenotype. However, the signalling pathways involved in the occurrence of UVB-SIPS in HDFs are poorly understood, even under “normal” conditions.

In the last part of this thesis, we investigated two major pathways that could be involved in the appearance of senescence in UVB-SIPS: the TGF-β1 signalling pathway and the DNA damage response pathway.

E.1. **TGF-β1 signalling**

It is known that TGF-β signalling plays a central role in the establishment of senescence in several SIPS models through the canonical **Smad** pathway (Acosta et al., 2013a; Hubackova et al., 2016). In this subject, the impact of neutralising antibodies against TGF-β1 itself or TGF-βRII in UVB-SIPS HDFs suggests an involvement of TGF-β1 signalling in the appearance of the senescent phenotype (Debacq-Chainiaux et al., 2005). Therefore, we investigated whether UVB-SIPS in dermal HDFs is related to the activation of the canonical Smad pathway.

We performed western blotting to determine the protein abundance of Smad2, Smad3 and their phosphorylated forms, and of Smad4 at different time points after UVB exposures inducing SIPS. In order to define protein localisation, we analysed total protein extracts, but also nuclear and cytoplasmic extracts. Immunofluorescence staining was also performed to highlight a possible nuclear translocation of these proteins. We did not show any sign of activation of this Smad pathway.

Several studies have investigated TGF-β signalling in dermal HDFs exposed to UVB. They have shown that the transcription factor AP-1 is induced after UVB irradiation, which increases inhibitory Smad7 expression and decreases TGF-βRII synthesis. Then, the phosphorylation of Smad2/3 is reduced, which impaired the whole Smad signalling (Cavinato and Jansen-Durr, 2017). These data have also been confirmed in human skin *in vivo* (Han et al., 2005; Quan et al., 2004).

This suggests that other TGF-β1-associated signalling pathways might be activated in the UVB-SIPS model, such as $p38^{MAPK}$ (Zhang, 2017). Indeed, it would be interesting to investigate $p38^{MAPK}$ possible activation. In fact, the inhibition of $p38^{MAPK}$ decreases the proportion of SA-βgal positive cells and SA-gene overexpression in H_2O_2-SIPS model for instance (Debacq-Chainiaux et al., 2016). Finally, it is known that an upstream event of TGF-β activation during senescence is NOTCH1 activation (Hoare et al., 2016; Ito et al., 2017). Thus, it might be interesting to investigate its activation.

E.2. **DNA damage response**

Senescence can also be triggered by unrepaired DNA damage and persistent DDR signalling activation in various SIPS models (Bielak-Zmijewska et al., 2018). This is made through the DDR-dependent control of both the growth arrest and the SASP (Malaquin et al., 2016; Sulli et al., 2012).

We investigated whether UVB-SIPS model is related to the persistence of DNA damage and DDR pathway activation. We analysed by immunofluorescence staining the expression of CPDs, (6-4)PPs, and P-H2A.X. Then, we studied by western blotting the protein abundance of some components of the DDR pathway (namely ATM, ATR, BRCA1, Chk1, Chk2 and p53) and their phosphorylated forms in total protein extracts from HDFs in UVB-SIPS at different time points.

We showed that HDFs in UVB-SIPS display long-term maintenance of DNA damage. This suggests that repair mechanisms are probably overwhelmed and not able to