

**UNIVERSITY OF SOUTHAMPTON**

DERMATOPHARMACOLOGY, DIVISION OF INFECTION, INFLAMMATION AND IMMUNITY

School of Medicine

**The role of microRNAs in allergic contact dermatitis and skin responses to ultraviolet radiation**

by

**Eirini Vavatsikou**

Thesis for the degree of Doctor of Philosophy

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# ABSTRACT

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ABSTRACT

DERMATOPHARMACOLOGY, DIVISION OF INFECTION, INFLAMMATION AND IMMUNITY,  
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THE ROLE OF MICRORNAS IN ALLERGIC CONTACT DERMATITIS AND SKIN RESPONSES TO  
ULTRAVIOLET RADIATION

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MicroRNAs are small non-protein coding RNA transcripts that control gene expression in a post-transcriptional manner, affecting several cellular processes including development, differentiation, apoptosis and disease. MicroRNAs are crucially important in the immune system and their deregulation leads to inflammatory diseases or cancer. In particular miR-155 is critical in antigen presentation by dendritic cells; miR-146a is transcribed by NF- $\kappa$ B and miR-125b silences TNF- $\alpha$ . Thus, these microRNAs are implicated in processes that take place in the sensitization process of allergic contact dermatitis (ACD), when the Langerhans cells (LC) migrate from the epidermis to present the hapten to T-cells. In this project, these microRNAs' expression profiles were investigated using an ACD model which involved the application of the allergen 2,4-dinitrochlorobenzene (DNCB) onto skin tissue *ex vivo*, human primary keratinocytes (HPK) and monocyte derived dendritic cells (MoDCs) *in vitro*, in order to elucidate their role in ACD initiation. Migration experiments showed consistent LC depletion after DNCB application (N=8). However, qPCR data from DNCB treated skin explants showed variable or no modulation of miR-155, -125b and -146a. HPK expressed the aforementioned microRNAs but failed to show any significant modulation in their expression. In DNCB stimulated MoDCs, the miR-146a expression was found significantly suppressed ( $p<0.05$ ) in N=8 individuals. This suppression was absent in MoDCs treated with supernatants from DNCB treated HPK but miR-125b was found significantly upregulated ( $p<0.05$ ). These findings reveal that the DNCB has a distinct impact on miR-138 and miR-146a expression in MoDCs that could be used a microRNA signature for antigenicity of potential contact allergens.

Exposure of cells to DNA damaging stimuli, including ionising radiation (IR), adriamycin and ultraviolet radiation (UVR), leads to DNA damage and subsequent upregulation of the p53 protein. p53 activation plays a determinant role in cell cycle arrest/survival or programmed cell death. Post IR or adriamycin insult, p53 triggers transcriptional activation of microRNA-34a (miR-34a) which regulates cell cycle and DNA damage response genes, and represses silent information regulator 1 (SIRT1) thus promoting apoptosis. However, SIRT1 levels remain unaffected by UVR treatment. In this study, it is investigated whether UVR causes transcriptional activation of miR-34a and silencing of SIRT1 translation. HCT116 cells and primary human keratinocytes were exposed separately to UV, ionising radiation and adriamycin. *Ex vivo* human skin was irradiated with ionising radiation and UVR. HCT116 cells and HPK were exposed to IR and adriamycin that led to significant increases in p53 and miR-34a. Even though, UVR caused similar increases in p53, miR-34a expression was significantly suppressed. p53 protein levels were increased by UVR and IR but miR-34a was only induced in the case of IR. In addition, IR and adriamycin reduced SIRT1 protein levels, whereas UVR did not modulate SIRT1 levels. This unique modulation of miR-34a post DNA damage may have important implications for skin carcinogenesis.



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## DECLARATION OF AUTHORSHIP

I, Eirini Vavatsikou

declare that the thesis entitled

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- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

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Date:.....

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## Abbreviations

Ab: antibody  
ABC: avidin biotin complex  
AFED: average fold expression difference  
ANOVA: analysis of variance  
AP: activating protein  
APC: antigen presenting cell  
ATM: ataxia telangiectasia mutated  
ATR: ATM- and Rad3-related  
Bcl: B-cell lymphoma  
BCR: B-cell receptor  
BSA: bovine serum albumin  
CCR: CC chemokine receptor  
CD: cluster of differentiation  
CDK: cyclin dependent kinase  
cDNA: complementary DNA  
CHK: checkpoint kinase  
CHS: contact hypersensitivity  
CPD: cyclobutane pyrimidine dimers  
CpG: cytosine guanine  
CXCR: chemokine CXC motif receptor  
DAB: diaminobenzidine  
DAMP: danger associated molecular pattern  
DC: dendritic cells  
DC-SIGN: DC-specific ICAM-3-grabbing nonintegrin  
dDC: dermal dendritic cell  
DEPC: diethylpyrocarbonate  
DMEM: Dulbecco's modified eagle's medium  
DMSO: dimethylsulfoxide  
DNA: deoxyribonucleic acid  
DNA-PK: DNA-dependent protein kinase  
DNCB: dinitrochlorobenzene



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dNTP: deoxynucleotide triphosphate  
DPX: di-N-butyle phthalate in xylene  
EDTA: ethylene-diamine-tetra-acetic acid  
Ep-CAM: epithelial cell adhesion molecule  
FBS: foetal bovine serum  
FED: fold expression difference  
FITC: fluorescein isothiocyanate  
FoxP3: forkhead box p3  
G phase: gap phase  
GTR: glucocorticoid-induced tumor necrosis factor receptor  
GM-CSF: granulocyte macrophage colony stimulating factor  
GRO: growth regulating protein  
GTP: guanine triphosphatase  
HLA: human leucocyte antigen  
HPK: human primary keratinocytes  
HS: human skin  
HSV: herpes simplex virus  
ICAM: inter-cellular adhesion molecule  
IDEC: inflammatory dendritic epidermal cell  
IFN: interferon  
IgG: immunoglobulin G  
IL: interleukin  
iNOS: inducible Isoform of Nitric Oxide Synthase  
IR: ionising radiation  
LC: Langerhans cell  
L-glu: L-glutamine  
LPS: lipo-poly-saccharide  
M phase: mitosis phase  
MAPK: mitogen-activated protein *kinase*  
MDM: mouse double minute  
MHC: major histocompatibility complex  
miR: microRNA  
MMP: matrix metalloproteinase

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MoDC: monocyte derived dendritic cell  
MRN: Mre11/Rad50/Nbs1  
mRNA: messenger RNA  
MSRT: multiscribe reverse transcriptase  
mTOR: mammalian target of rapamycin  
NF-κB: nuclear factor kappa B  
NK: natural killer  
NLR: NOD-like receptor  
NO: nitric oxide  
NOD: nucleotide oligomerization domain  
P/S: penicillin/streptomycin  
PAF: platelet activating factor  
PAFR: platelet activating factor receptor  
PAMP: pathogen associated molecular pattern  
PARP: poly ADP (Adenosine Diphosphate)-ribose polymerase  
PBMC: peripheral blood mononuclear cell  
PBS: phosphate buffer saline  
PE: phycoerythrin  
PTM: post translational modulations  
PVDF: polyvinylidenefluoride  
qPCR: quantitative polymerase chain reaction  
RANKL: receptor Activator of NF-κB Ligand  
Rb: retinoblastoma  
RNA: ribonucleic acid  
ROS: reactive oxygen species  
RPMI: Roswell Park Memorial Institute  
RT: room temperature or reverse transcription  
S phase: synthesis phase  
siRNA: silencing RNA  
SIRT1: silent mating type information regulation 2 homolog  
SOCS: suppressor of cytokine signalling  
SR: scavenging receptor  
STAT: signal transducer and activator of transcription

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TAp63: transcriptionally active p63

T<sub>C</sub>cell: cytotoxic T-cell

TCR: T-cell receptor

TF: transcription factor

TGF: transforming growth factor

T<sub>H</sub>cell: helper T-cell

Tip-DC: TNF and inducible nitric oxide synthase–producing dendritic cell

TLR: toll like receptor

TNF: tumour necrosis factor

UTR: translated region

UVR: ultraviolet radiation

*XPA: xeroderma pigmentosum protein A*

ΔNp63: transcriptionally inactive



# 1. Introduction

## 1.1. SKIN STRUCTURE AND FUNCTIONS

The skin is the largest organ of the human body. It is a barrier against the external environment, thus its homeostasis is pivotal to maintaining good health. The skin comprises the epidermis and the dermis, beneath which lies the subcutis. The epidermis is a cell-rich layer and the predominant cell type within the epidermis is the keratinocyte. Approximately 10% of the epidermal cells are Langerhans cells and melanocytes (Haake *et al.* 2001; Rees 2004). Based on morphology, the epidermis consists of, from the deepest layer to the uppermost, the stratum basale, the stratum spinosum, the stratum granulosum and the stratum corneum (Micali *et al.*, 2001). The stratum basale consists of proliferating cells which subsequently begin to differentiate as they are pushed upwards to form the stratum spinosum. As the cells continue to move upwards, the keratinocytes gradually flatten and become anucleate and the keratin filaments within the cells aggregate to form keratohyalin granules, giving a coarse-like appearance to the stratum granulosum (Haake *et al.*, 2001). When the keratinocytes move to the most superficial layer of the epidermis, they become corneocytes (which is their terminal state of differentiation), acquire a flat polyhedral shape and are surrounded by lipids which have been released from lamellar bodies in the granular cell layer (Lee *et al.*, 2006). Eventually, at the skin surface they lose their intercellular junctions and are slowly desquamated (Madison, 2003).

Langerhans cells are epidermal dendritic antigen presenting cells, which contain characteristic tennis racket-shaped granules called Birbeck granules (Cumberbatch, 2000; Udey *et al.*, 2001). These cells reside in the suprabasal and basal layers of the epidermis, distributed in a way to scan efficiently for antigenic agents (Cumberbatch, 2000; Numahara *et al.*, 2001). When an area of skin is exposed to a variety of exogenous agents (infection, allergens), the Langerhans cells at that site will take up and antigens before travelling to the local draining lymph node and maturing to become fully-functional antigen-presenting cells (Udey *et al.*, 2001; Janeway *et al.*, 2005). Melanocytes are dendritic, neural crest derived cells in the basal layer of the epidermis and in the hair follicles (Lin and Fisher, 2007; Rees,

2003; Rees, 2004). These pigment cells are responsible for hair and skin colour and produce melanin which they transfer in melanosomes along their dendrites to neighbouring keratinocytes. (Lin and Fisher, 2007; Boissy, 2003).

The dermis is responsible for the nourishment and mechanical support of the epidermis. It is composed of a connective tissue matrix, which is synthesized by fibroblasts. The dermis is divided into two zones, the upper papillary and the lower reticular dermis (Sorrell, 2004). The papillary dermis is more poorly organized, containing small collagen bundles and proteoglycans, while the reticular dermis is thick with a rigid network of collagen bundles and a smaller concentration of proteoglycans (Sorrell, 2004). Blood vessels form a vascular plexus within the dermis, providing the skin with nutrients and oxygen (Haake *et al*, 2001). Skin appendages reside in the dermis and project up into the epidermis; these include sweat glands that function in thermoregulation and hair follicles (with their sebaceous glands that secrete sebum) from which hairs protrude out of the skin.

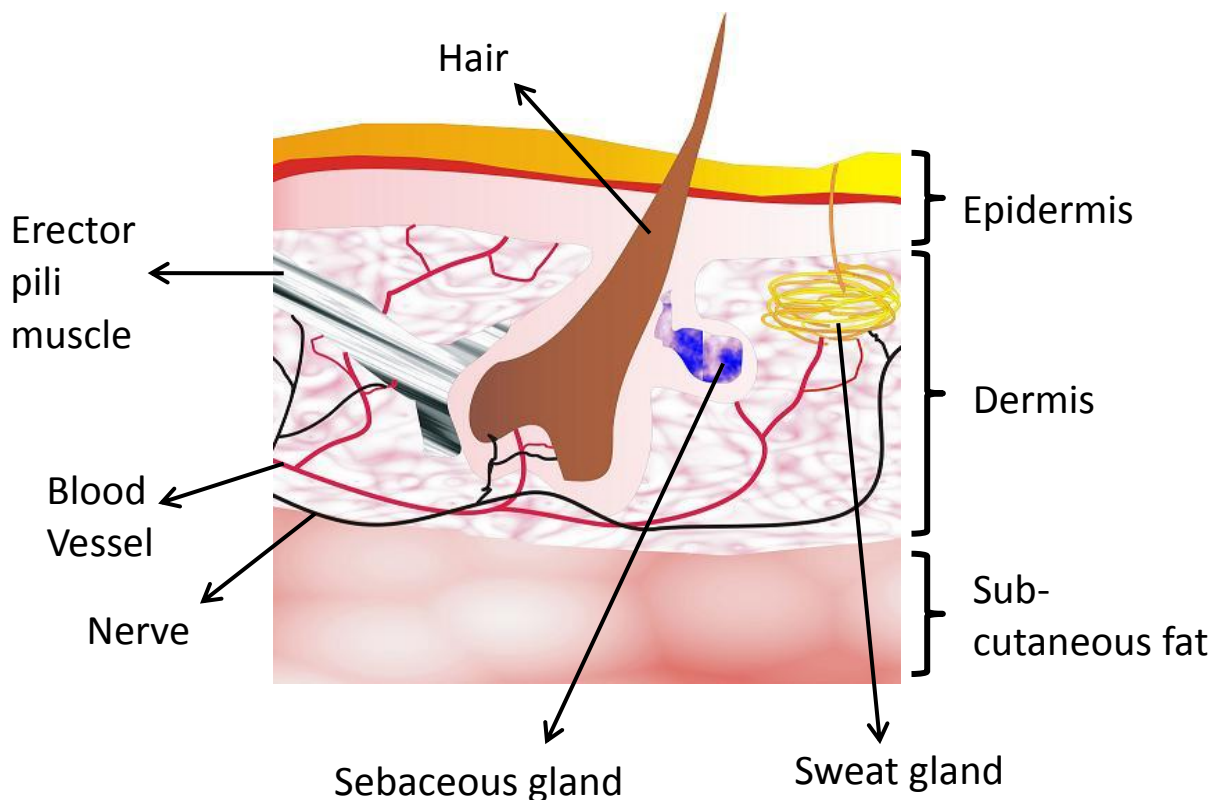


Figure 1-1 **Skin Structure.**

## 1.2. THE SKIN IMMUNE SYSTEM

### 1.2.1. Innate immunity and inflammation

In order to comprehend the skin immune system, it is essential to define the immune system as a whole. The immune system is divided into the innate and the acquired (or adapted) immune systems. The innate immune system protects the organism from external harmful stimuli such as mechanical damage, pathogens, extreme temperatures and hazardous chemicals. To achieve this protection the innate immune system has four distinct ways to defend the organism; i) the mechanical barrier of viscous mucus in mucosal surfaces and tight junctions between epithelial cells, like the corneocytes in the stratum corneum of the skin, ii) the chemical barrier of proteolytic enzymes, antimicrobial peptides in saliva and tears as well as low pH in the stomach, iii) a microbiologic barrier formed by local flora in the gut and skin and iv) inflammatory responses (Gallo and Nizet, 2008; Janeway *et al*, 2005).

When a pathogen crosses the physical barriers of the innate immune system it has to be rapidly recognised and eliminated in the site of infection. To achieve this, the innate immune system is armed with pattern recognition receptors (PRRs) that are expressed by macrophages, neutrophils, dendritic cells (DCs), B-cells, monocytes, mast cells, natural killer (NK) and epithelial cells (Janeway *et al*, 2005; Cooper *et al*, 2009). They recognise a broad spectrum of pathogen associated molecular patterns (PAMPs) (repeating molecules in antigenic components) and danger associated molecular patterns (DAMPs) (Turvey and Broide, 2010). PRRs are: toll like receptors (TLR) on surface or endosome cell membranes, nucleotide oligomerisation domain (NOD) like receptors (NLR) in the cytoplasm, membrane bound receptors of the C-lectin family (e.g. Dectin-1, the mannose receptor, DC-SIGN (CD209), langerin (CD207)), soluble C-lectin receptors called collectins, RNA helicases in the cytoplasm and membrane bound scavenger receptors (e.g. CD36, SR-A1, SR-A2, SR-B2) (Sioud, 2006; Geijtenberg and Gringhuis, 2009; Dam and Brewer, 2010). When these receptors encounter a pathogenic molecule they take two different courses of action, one is phagocytosis and the other is signalling (Yokoyama and Colonna, 2008). Phagocytosis is a process during which the pathogenic organism or molecule is engulfed by the cell's

membrane forming a vesicle called a phagosome that will mature to a lysosome containing proteolytic enzymes and NO particles that will effectively kill or disintegrate the pathogenic material (Ramanchandra *et al*, 2009). On the other hand, the signalling pathways entail activation of several transcription factors like AP-1, NF- $\kappa$ B, SOCS-1 whose downstream genes include a vast variety of soluble mediators that are pro-inflammatory cytokines (for example IL-1, IL-18, IL-6) and chemokines (for example MIP-1a, MIP-1b, RANTES) (Underhill and Gantner, 2004; Turvey and Broide, 2010). The role of cytokines is to alert the surrounding environment about the ongoing infection. The secreted chemokines' role is to attract neutrophils and monocytes from circulating blood to the area of infection so that further phagocytosis takes place and the hinderance of further infection. During inflammation the sufferer experiences redness, swelling and sometimes pain in the affected area. This is caused by the increased blood flow and permeability across the blood vessels which facilitates infiltration of cytokines, complement, antibodies and circulating lymphocytes in the site of infection (Barrington *et al*, 2001; Janeway *et al*, 2005). If the infection is successfully controlled then wound healing occurs. Otherwise the infection becomes systemic and more elements of the immune system partake in managing it (see next section).

### 1.2.2. Adaptive immunity

When it comes to adaptive immunity, memory is acquired. This memory is carried by T-cells and B-cells. Both cell types come from the same hematopoietic progenitor cell; during differentiation, some express T cell receptor (TCR) and become T-cells in the thymus, while some express B cell receptor (BCR) and become B-cells in the bone marrow (Alam and Gorska, 2003). The genes coding for TCR and BCR undergo gene rearrangement which is different in each T and B-cell (Janeway *et al*, 2005). This means that each cell recognises a different antigen. T-cells that recognise self-antigens are forced to undergo apoptosis in the thymus; a similar process occurs for self-recognising B-cells in the bone marrow. The unique gene rearrangements of TCR and BCR though are transient and will only become a part of permanent immunological memory upon activation of T-cells and B-cells respectively (Alam and Gorska, 2003).



In the case of a pathogen that has managed to bypass the defences of the innate immune system or its components don't fall within the template recognised by the PRRs, DCs bind the antigen with phagocytic receptors, process it and present it through MHC I or MHC II molecules (Liu, 2001; Ueno *et al*, 2010). MHC I (major histocompatibility complex) is expressed on every nucleated cell of the human body and it serves as an intracellular non-self-presence alarm that is detected by NK cells, which engage in cytotoxic killing if they recognise a cell as non-self (Bonilla and Oetgen, 2010). TCR of CD8<sup>+</sup> cells recognise the complex of MHC I and intracellular antigen. MHC I molecules bind to CD8 and T-cell activation signalling commences by CD3 (through Zap70) (Bonilla and Oetgen, 2010). The MHC I:antigen complex invites cytotoxic cells such as NK or Tc-cells (cytotoxic T-cells) to kill the cell bearing it. MHC II is expressed by antigen presenting cells (APC: DCs, macrophages, B-cells) and gut, lung and skin epithelial cells (Albanesi *et al*, 1998; Janeway *et al*, 2005; Mallegol *et al*, 2005; Kreisel *et al*, 2010). Cells use MHC II to present extracellular antigens (soluble or attached to a pathogen) to the TCR compartment of CD4<sup>+</sup> T-cells. Similarly to MHC I driven presentation, CD4 molecules bind to MHC II molecules and CD3 initiates the T-cell activation process (Bonilla and Oetgen, 2010). This interaction is the landmark of the first signal of T-cell activation (priming). A T-cell can only be activated by an APC that is presenting its cognate antigen. The APC undergoes maturation during antigen uptake and processing, which means it can no longer process new antigens, secretes a range of cytokines and expresses co-stimulatory molecules; these co-stimulatory molecules (e.g. CD40, CD80, CD86) react with different receptors on the surface of T-cells (e.g. CD28) that have received the first activation signal (Janeway *et al*, 2005; Tan and O'Neill, 2005). This communication between APCs and T-cells is determinative as far as the further action of the T-cell is concerned; they can become effector, cytotoxic, regulatory or memory T-cells. There are many effector T-cell types characterised, the main types are T<sub>H</sub>1 CD4<sup>+</sup>, T<sub>H</sub>2 CD4<sup>+</sup>, T<sub>H</sub>17 CD4<sup>+</sup>, T<sub>C</sub>1 CD8<sup>+</sup>, T<sub>C</sub>2 CD8<sup>+</sup> and CD4<sup>+</sup> follicular T-cells; there is a specific cytokine milieu that promotes the differentiation of a naïve T-cell to one of these effector T-cell types (see Table 1.1) (Romagnani *et al*, 2002; Woodland and Dutton, 2003; Shrikant *et al*, 2009; Wan and Flavel, 2009; Cyster, 2010). As mentioned above, each antigen is presented through MHC I to CD8<sup>+</sup> T-cells or MHC II CD4<sup>+</sup> T-cells according to its location (intracellular or extracellular space respectively). It has been reported that some antigens can be cross-

presented by MHC II to CD8<sup>+</sup> T-cells, therefore under the same cytokine secretions (Table 1.1) T<sub>H</sub>1 CD4<sup>+</sup> and T<sub>C</sub>1 CD8<sup>+</sup> are generated, with one type being essential to the other, for example influenza virus produces intracellular antigens thus causing a CD8<sup>+</sup> response that was shown to be impaired in CD4<sup>+</sup> null mice (Riberdy *et al*, 2000; Novy *et al*, 2007; Baumier and Rothman, 2009; Shrikant *et al*, 2009). Of course the predominant T-cell type was CD8<sup>+</sup>.

Table 1-1 T-cell functions in immune responses

T-cell Type	Cytokines milieu from APC	Phenotype	Secreted cytokines by T-cell	Functions
Effector T-cells	IL-12, IFN- $\gamma$	T <sub>H</sub> 1 CD4 <sup>+</sup>	IFN- $\gamma$ , TNF- $\beta$ , (IL-21)	Maximise cell killing by macrophages and cytotoxic CD8 <sup>+</sup> T-cells. Activate B-cells (when secreting IL-21).
	IL-4	T <sub>H</sub> 2 CD4 <sup>+</sup>	IL-4, -5, -6, -13, -21	Activate B-cells. Recruit eosinophils and activate mast cells.
	IL-1, IL-6, IL-23, TGF- $\beta$	T <sub>H</sub> 17 CD4 <sup>+</sup>	IL-17, -22, -16	Recruit neutrophils. Antagonise T <sub>H</sub> 2 responses. Promote autoimmune disorders.
	Upregulation of Bcl-6 ( $\pm$ IL-21)	Follicular T-cells CXCR5 <sup>+</sup>	IL-21	Activate B-cells for antigen secretion in germinal centers.
Cytotoxic T-cells	IL-12, IFN- $\gamma$	T <sub>C</sub> 1 CD8 <sup>+</sup>	IFN- $\gamma$ , TNF- $\alpha$	Kill cells through FasL, TRAIL, TWEAK receptors and by releasing proteolytic enzymes in the cytosol of the infected/damaged cell.
	IL-4	T <sub>C</sub> 2 CD8 <sup>+</sup>	TNF- $\alpha$ , IL-4, -5	Same as T <sub>C</sub> 1 but also activate B-cells.
T-regs	IL-10, TGF- $\beta$	CD4 <sup>+</sup> , CD25 <sup>+</sup> , Foxp3 <sup>+</sup> and CD8 <sup>+</sup> , CXCR5 <sup>+</sup> , GITR <sup>+</sup> , Foxp3 <sup>-</sup>	IL-10, TGF- $\beta$	Suppress activation of the immune response to self-antigens. Negative feedback mediators of inflammation.
Memory	(see text)	CD8 <sup>+</sup> CD62L <sup>+</sup> CCR7 <sup>+</sup> and CD4 <sup>+</sup> CD45 <sup>+</sup>	(see text)	Reside at locations of pathogen entry (lungs, skin) and remain quiescent until re-exposure to their cognate antigen when they are activated and turn into effector or cytotoxic T-cells.

The adapted immune response can be predominantly cell-mediated or humoral. A cell-mediated immune response is governed by cytotoxic T-cells and phagocytosis by

macrophages. A humoral immune response involves the activation of B-cells for the production of antibodies (soluble immunoglobulins). A B-cell is activated when it is exposed to its cognate antigen and then activated by a T-cell (see Table 1.1) that has encountered the same antigen. Following activation, B-cells will switch the class of the immunoglobulins they produce (from IgM or IgD to either IgG, IgE or IgA) in the germinal centre if it is necessary and clonal expansion takes place (Bonilla and Oetgen, 2010; Cyster, 2010). B-cells will then secrete antibodies that bind to the antigen; the latter process is called opsonisation and it promotes the phagocytosis and clearance of the antigen from the host (Janeway *et al*, 2005). Once the antigen is cleared from the site of infection and wound healing occurs, activated T- or B-cell populations will contract and form memory cells that will remain close to sites of antigen entry (Alam and Gorska, 2003). Upon re-exposure to the antigen, they are activated and re-gain the characteristics of the cells they originated from, for instance a CD8<sup>+</sup> memory T-cell, will multiply and engage in killing the infected cells as it did in the first infection (Alam and Gorska, 2003).

### 1.2.3. The keratinocyte as an instigator of inflammation

The skin is an immunologically dynamic organ. In addition to the involvement of the cells of the systemic immune system (neutrophils, macrophages, lymphocytes) in immune responses in skin it is thought that the keratinocytes and the skin DCs play important roles in cutaneous immunity, especially in response to external antigens. For example, it is the keratinocytes which first encounter exogenous agents that come into contact with the skin and following perturbation of their environment by an allergen, irradiation or a viral/bacterial component, keratinocytes can express HLA-DR and secrete various immunomodulatory mediators such as cytokines and chemokines (Chu and Morris, 1997; Luger *et al*, 1997; Burbach *et al*, 2001).

Indeed, UV irradiation, allergens, irritants or pathogens can activate toll-like receptors (TLRs) on/in keratinocytes, inducing production of pro-inflammatory cytokines like TNF- $\alpha$  and IL-1 $\beta$  (Mempel *et al*, 2003; Lebel *et al*, 2007; Barker, 1997; Burbach *et al*, 2001). Other cytokines which can be secreted by keratinocytes following stimulation with IFN- $\gamma$  (produced by LCs and infiltrating leucocytes) include IL12, IL-15, IL-18, IL-8 and GRO- $\alpha$  (as

well as IL-1 and TNF- $\alpha$ ) (Chu and Morris, 1997; Luger *et al*, 1997; Burbach *et al*, 2001; Cruz *et al*, 2001). Furthermore, under IFN- $\gamma$  influence keratinocytes can act as antigen presenting cells by presenting the pertinent antigen to naive and effector cutaneous T-cells (Chu and Morris, 1997; Black *et al*, 2007; Kim *et al*, 2009). When encountered with a pathogen, keratinocytes secrete antimicrobial peptides (e.g. LL37, defensins) that kill pathogens, recruit immune cells and modulate cytokines secreted by local T-cells (e.g. IL-17A, IL-22 by T<sub>H</sub>17) (Weaver *et al*, 2007; Gallo and Nizet, 2009).

Keratinocytes can act as non-professional APC as they have been reported to interact with primed T-cells and induce anergy and tolerance, for example, upon superantigen stimulation, keratinocytes have been shown to promote T-cell clonal expansion (Nickoloff *et al*, 1993). Absence of transcription factors AP-1 and NF- $\kappa$ B as well as overexpression of STAT3 in keratinocytes can result in a chronic inflammation phenotype, thus supporting the hypothesis that the keratinocyte is an active member of the skin immune system (Pasparakis *et al*, 2002; Sano *et al*, 2005; Zenz *et al*, 2005).

#### 1.2.4. Skin dendritic cells

Dendritic cells are immune cells whose main function is to internalise, process and present antigens to T-cells. They reside in every organ of the human body forming network that scans the tissues for foreign antigens or danger signals. Skin DCs are very important in skin immunity and can be divided in 3 categories: epidermal DCs, dermal DCs and lymph node (LN) resident DCs. Different DC subtypes have been identified for each category.

Epidermal dendritic cells are comprised of a single DC population called Langerhans cells (LCs) and inflammatory dendritic epidermal cells (IDECs) have also been reported in the human inflamed epidermis (Wollenberg *et al*, 2002). LCs reside in the basal and suprabasal layers of the epidermis, express E-cadherin to attach to keratinocytes and they can be distinguished from the other skin DC types by their unique expression of the C-lectin langerin (CD207), high expression of CD1a, CD103 and Ep-CAM (epithelial cell adhesion molecule) (Jaksits *et al* 1999; Cumberbatch *et al*, 2000; Jacob *et al*, 2001; del Rio *et al*, 2010). Another morphological feature of LCs are the Birbeck granules. They are tennis

racket shaped organelles whose function remains elusive; it has been postulated that they assist in the transport of langerin from the cytosol to the cell surface, or that they are involved in the processing of antigens presented by CD1a such as *Mycobacterium leprae* (Dermott *et al*, 2002; Asahina and Tamaki, 2006). LC myeloid CX<sub>3</sub>R1<sup>+</sup> CD45<sup>+</sup> CD115<sup>+</sup> CD11b<sup>+</sup> precursors have been shown to migrate to the epidermis in early weeks of embryogenesis in mice and form a network of LC precursor cells that proliferate and replenish LCs in a natural turnover cycle in mice and humans (Merrad *et al* 2003; Kanitakis *et al*, 2004; Chorro *et al*, 2009). Upon inflammatory condition migratory LCs are replaced by blood-derived monocytic CD1a<sup>+</sup> precursors in a CCR2 dependent pathway (Larregina *et al*, 2001; Ginhoux *et al*, 2006; Koch *et al*, 2006).

It is thought that, under normal physiological conditions, Langerhans cells play an important role in the initiation of immune responses to exogenous antigens. During this process, Langerhans cells can produce IL-1 $\beta$  and IFN- $\gamma$  (which activates the surrounding keratinocytes), bind protein antigens or haptens, process antigens (by internalizing, degrading and associating them with MHC molecules) and migrate from the skin to the local lymph nodes to present these antigens (Cumberbatch *et al* 2000). LCs migrate by morphing long pseudopodia which they use to travel through keratinocytes, until they reach the epidermal-dermal junction where they secrete matrix metalloproteinases (MMPs) in order to degrade the thick network of the junctions fibres so that they can move through it (Ratzinger *et al*, 2002; Stoitzner *et al*, 2002; Oakford *et al*, 2011). LCs change shape again within the epidermis in order to move along the collagen fibres and then they enter into the lymphatic vessels to present the antigen to T-cells in the lymph node (Stoitzner *et al*, 2002). The migration process coincides with LC maturation and upregulation of cell surface markers (CD40, HLA-DR, CD83, CD86) that facilitate T-cell stimulation (de Gruijl, 2006).

LCs also associate closely with intra-epidermal T-cells upon antigen exposure (Kissenpfennig *et al*, 2005). LCs have been shown to be quite inert in the absence of keratinocytic signals; the close interaction between the LC and the keratinocyte is indispensable when it comes to immune responses, for instance CpG was successfully presented by LCs only in the presence of keratinocytes (Asahina and Tamaki, 2006; Sugita *et al*, 2006; Chorro *et al*, 2009). LC activation and migration from the skin is dependent on the

secretion of pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  by local keratinocytes (and LCs in an autocrine fashion) and is suppressed by IL-10 (Wang *et al*, 1999; Cumberbatch *et al* 2000; Jakob *et al*, 2001). In addition, it has been reported in a mouse model that ligation of the platelet activating factor receptor (PAFR) on keratinocytes but not on LCs, by PAF is essential for LC migration (Fukunaga *et al*, 2008).

IDECs originate from a monocytic precursor and are only present in highly inflamed epidermis in conditions such as atopic eczema (Bieber, 2007). IDECs differ from LCs as they express the mannose receptor (CD206) and high affinity Fc $\epsilon$ RI (Wollenberg *et al*, 2002). Also they are known to switch the T-cell response from T<sub>H</sub>2 to T<sub>H</sub>1 by secreting IL-12 and IL-18 (Novak *et al*, 2005).

Dermal dendritic cells have attracted a lot of interest in the past few years since Fukunaga *et al* reported that in PAFR(-/-) mice, there was no detectable LC migration but nevertheless there was CHS (contact hypersensitivity reaction) manifestation (Fukunaga *et al*, 2008). The notion of the LCs being the most important and determinative DC type in the skin has been revisited several times and many different LC ablated mice models have been employed to explore the functions of the other DC types. However, it has also been reported that dermal DCs will compensate as the main DC type when LCs are absent (Romani *et al*, 2006; Kimber *et al*, 2009). Dermal DCs are different between mice and human and their phenotypes can be briefly summarised in Table 1.2 (Mathers and Larregina, 2006; Merrad *et al*, 2008).

Table 1-2 Dermal DCs phenotypes in mice and humans.

Organism	Dermal Dendritic Cells Subtypes Phenotype	
Mice	Dermal DCs Langerin <sup>+</sup>	MHC II <sup>+</sup> , EpCAM <sup>+</sup> , CCR7 <sup>+</sup> , CD45 <sup>+</sup> , CD11C <sup>+</sup> , CD205 <sup>+</sup> , CD11b <sup>+</sup> , E-cadherin <sup>+</sup>
	Dermal DCs Langerin <sup>-</sup>	MHC II <sup>+</sup> , CD45 <sup>+</sup> , CD11c <sup>+</sup> , CD205 <sup>+</sup> , CD11b <sup>+</sup>
	LN DCs	MHC II <sup>+</sup> , CD8 <sup>+</sup> , CD45 <sup>+</sup> , CD11c <sup>+</sup> , CD103 <sup>+</sup> , CCR7 <sup>+</sup>
Humans	Dermal DCs	MHC II <sup>+</sup> , CD1a <sup>+</sup> , CD1d <sup>+</sup> , CD45 <sup>+</sup> , CD11c <sup>+</sup>

Plasmatooid DCs (pDCs) and Tip-DCs (TNF and inducible nitric oxide synthase producing DCs) have also been found in inflamed human dermis, particularly in conditions such as psoriasis and atopic dermatitis in which these DCs confer to the inflammatory character of these conditions by secreting LL37 (pDCs) and TNF- $\alpha$  and iNOS (Tip-DCs) (Romani *et al*, 2006).

Investigating the function of dermal DC populations has given new insight into the role of LCs in the homeostasis of the skin tissue and in the fate of an immune response. In mice, it has been shown that LCs comprise only 50% of the Langerin<sup>+</sup> population in draining LNs (Ginhoux *et al*, 2007). Also, Carbone *et al* report that migratory DCs give away their antigen to local LN DCs to present; LCs failed to present HSV *in vitro*, but *in vivo* LCs and dermal DCs transferred the HSV-derived antigen to LN DCs that primed a specific CD8<sup>+</sup> response (Carbone *et al*, 2004; Merrad *et al*, 2008). RANKL is a receptor that activates NF- $\kappa$ B pathway and RANKL (-/-) mice have decreased LC numbers and proliferation but an inflammatory phenotype whereas RANKL overexpression induced LC activation and Treg formation and maintenance (Barbaroux *et al*, 2008). TLR7 and TLR8 are involved in the aggravation of psoriasis, however they are absent from LCs, therefore dermal DCs and pDCs were immediately considered as the main DC type involved in the pathogenesis of psoriasis (Gilliet *et al*, 2004; Flacher *et al*, 2006). Additional data support that LCs exhibit weaker expressions of co-stimulatory molecules and maturation markers than DC counterparts from other tissues (e.g. splenic DCs) when encountered with TLR 3, -4, -9 ligands (Sugita *et al*, 2006; Peiser *et al*, 2008; Hari *et al*, 2010). Combining this data together and taking into

account that LC ablation lead to enhanced or similar CHS and graft versus host disease (GVHD) reactions, it can be assumed that LCs have a tolerogenic role and dermal DCs are responsible for the orchestration of crucial inflammatory responses (Grabbe *et al*, 1995; Steinman *et al*, 2003; Kissenpfennig *et al*, 2005; Hongmei *et al*, 2011).

### 1.2.5. Hypersensitivity reactions

Hypersensitivity reactions are abnormal immune responses that result in tissue damage and disease. Classically there are four types of hypersensitivity reactions as classified by Gell and Coombs in 1963, all summarised in Table 1-3 (Gell and Coombs, 1963; Descotes and Choquet Kastylevsky, 2001; Rajan, 2003).

Table 1-3 Classification of hypersensitivity reactions as per Gell and Coombes.

Type of hypersensitivity	Description
<b>Type I</b> (Also known as allergy)	Allergen triggered release IgE that ligates Fc $\epsilon$ receptor on mast cells. Mast cells degranulate and induce vascular permeability, neutrophil recruitment and pro-inflammatory cytokine release from other cell types. Exaggerated inflammation causes tissue damage and morbidity. Usually characterised by a T <sub>H</sub> 2 response, e.g. asthma.
<b>Type II</b>	The immune system produces IgG antibodies against a benign antigen that is bound on self-cells, which leads to granulocyte (eosinophils, neutrophils, basophils) recruitment and hydrolytic enzyme release. Extensive tissue damage is caused e.g. pemphigus vulgaris (a blistering autoimmune skin disease).
<b>Type III</b>	IgG antibodies are released against a benign soluble antigen. Antibody-antigen complexes accumulate in the blood vessel walls, lymphoid organs or lungs causing disturbance of the local environment that leads to complement activation and granuloma formation. The granulomas cannot be eliminated from the body and they become toxic affecting the normal function of the affected organs e.g. serum disease.
<b>Type IV</b> (or delayed type hypersensitivity)	Benign antigen is ingested and processed by APCs that present it as an immunogen to T-cells in lymph nodes. Memory T-cells are deposited in the site of exposure. Upon re-exposure, T-cells propagate and are attracted to the site causing tissue damage and inflicting increased inflammation. Reaction occurs 24-72hrs following exposure e.g. allergic contact dermatitis. (See following section.)



### 1.3. ALLERGIC CONTACT DERMATITIS

#### 1.3.1. Prevalence and pathophysiology

On a daily basis, the skin is exposed to synthetic or natural chemicals which are present in the home (e.g. cosmetic formulations, jewellery, metal buttons) or the occupational environment (e.g. chemical solvents, paints). Some people develop allergic reactions to a wide range of these chemicals. One of the most significant skin diseases caused by chemical allergens is contact dermatitis. Contact dermatitis can be irritant or allergic. Both diseases can cause severe morbidity.

Occupational contact dermatitis accounts for 30% of all occupational diseases and 95% of all occupational skin diseases; out of which the incidence of allergic contact dermatitis (ACD) is 20% (Lushniak *et al*, 2004; Diepgen and Weisshaar, 2007). ACD can be confirmed with patch testing, a technique where chemicals are suspended in volatile medium and applied on an occluded small skin surface. If an obvious reaction is observed (see description of signs below) then the patch test is considered positive and the patient is allergic to the applied chemical. In population epidemiological studies, it has been found that a range of 12.5-40.6% of people is allergic to at least one allergen as confirmed by multiple patch testing (Thyssen *et al*, 2007). It has also been shown that approximately 40% of people with positive patch tests are allergic to more than one chemical (Schnuch *et al*, 2008). It is generally accepted that an ACD sufferer is more susceptible to becoming allergic to a new allergen than a non-allergic individual (Moss *et al*, 1985; Schnuch *et al*, 2008). Gender does not seem to affect the manifestation of ACD, however there are studies that report women are more susceptible to develop a reaction during PMS and that there are more nickel allergic women, probably due to ear piercing (Thyssen *et al*, 2007; Morrissey *et al*, 2008). ACD prevalence is the same for individuals aged 15-65 years old (Thyssen *et al*, 2007). With no cure available and ACD being a recurrent disease, avoidance of the chemical allergen is essential; a skin site that has developed an ACD eczema is more likely to flare up to the allergen it had initially responded, creating a chronic inflammation (Kimber *et al*, 1999). ACD sufferers are subject to a severe impact on their quality of life, not only because the affected areas are localised on exposed skin affecting their social activities but also

because they could be obliged to change jobs in order to avoid the chemical (Kadyk *et al*, 2003).

ACD is a type 4 hypersensitivity. It manifests on skin regions that have come in contact with various chemicals; most common symptoms are pruritus, stinging redness, erupted vesicles, papules, scaling and fissures (Vein, 2006). Histological changes in ACD, include a) fluid accumulation between the keratinocytes (spongiosis) that moves from the basal layer to the stratum corneum where they rupture, b) dilated vessels surrounded by a mononuclear cell infiltrate that spreads towards and sometimes invades the epidermis, c) dermis appears oedematous (Lachapell and Marot, 2006). In chronic ACD lesions, skin becomes lichenified, keratinisation occurs by retention of nuclei in the *stratum corneum* (parakeratosis) and keratinocytes proliferate faster (Krasteva *et al*, 1999).

### 1.3.2. Allergens and ACD sensitization phase

Allergic contact dermatitis is a delayed-type hypersensitivity (cell mediated response) which starts by the invasion of the contact sensitizer into the epidermis and from there, via three pathways it is taken up by the skin DCs; a) it is internalised and haptenated (see next paragraph) with intracellular proteins, b) it is haptenated by extracellular proteins and c) it directly interacts with the MHC molecules of the skin DCs (Friedmann *et al*, 2003; Karlberg *et al*, 2007; Pickard *et al*, 2007; Saint-Mezard *et al*, 2007). Concomitantly, the skin DCs migrate from the epidermis (LCs) or the dermis (dDCs) to the local lymph nodes, undergoing maturation, to finally present the antigen to naive T-cells, from which a specific memory T-cell population arises that is deposited at the site of exposure (Kripke *et al*, 1990; Cumberbatch *et al*, 2000; Friedmann *et al*, 2003).

Contact sensitizers can be found anywhere; from the household to the workplace. Some very common examples are metals (e.g. nickel), cosmetic recipients (e.g. geraniol, parabens) and industrial solvents (e.g. DNCB). The sensitizing potency of chemicals depends on their antigenicity, which is basically their ability to penetrate the hydrophobic epidermal barrier and to bind covalently or co-ordinately to skin proteins forming complexes that are potent sensitizers. A low molecular weight compound that has the

capacity to create bonds with peptides is called a hapten. The target of these haptens can be a functional group on an amino-acid residue such as the disulphide group in cysteines, the imidazole group in histidines or the  $\epsilon$ -amino group of lysins (Divcovic et al, 2005). It is essential for a low molecular weight to bind to a local protein in order to induce an allergic response, otherwise due to the small size it wouldn't be perceived by the immune system. Antigenicity can be predicted in computer models that combine the nucleophilicity, molecular shape and size, hydrophobicity and selectivity for specific amino acid residues (Roberts and Lepoittevin, 1998; Smith Pease *et al*, 2002). Some of these substances (e.g. p-phenylenediamine (PPD) and eugenol) are chemically inert but become active (able to cause ACD) after bioactivation processes taking place in keratinocytes or phagocytic cells intracellularly (e.g. cinnamic alcohol) or by air oxidation (e.g. limonene) (Merk, 1998; Rustemeyer *et al*, 2006). The skin detoxification mechanism entails two phases: phase 1) cytochrome P450 isoenzymes (e.g. alcohol dehydrogenase) create a highly oxidative reactive derivative, phase 2) conjugating enzymes (e.g. glutathione) react with the previous product to yield a hydrophilic product that is very easy to be eliminated from the body (Merk, 1998; Karlberg *et al*, 2008). For the chemicals that become antigenic during this process, haptenation can occur after phase 1, when there is an excess of the reactive intermediate derivative that fails to be further processed by phase 2 enzymes and interacts with amino acids of intracellular proteins (Merk, 1998; Karlberg *et al*, 2008). Innate metabolic processes are crucial for the sensitization phase of ACD. For example, in sensitization human in vitro studies of potent sensitizer 2,4-dinitrochlorobenzene (DNCB), Pickard *et al* found that DNCB requires glutathione depletion in viable APC to be effectively presented to T-cells (Pickard *et al*, 2007).

Besides the chemical reactivity of contact allergens or their detoxified derivatives, another factor has been added to a contact allergen's antigenicity potency; the activation of members of the innate immune system such as the inflammasome and TLRs. The inflammasome is a protein complex involved in innate immunity as it contains an NLR (see section 1.2.1) and caspase-1 that is activated upon ligation of the NLR and exerts its action by cleaving pro-IL-1 $\beta$  and IL-18 (Yazdi *et al*, 2007). The latter two pro-inflammatory cytokines are upregulated epidermally in ACD and are required for activation and migration

of Langerhans cells from the epidermis in the sensitization phase of ACD (Shornick *et al*, 1996; Ryan *et al*, 2005). It has been observed that failure of inflammasome activation leads to tolerance, while activation always results in sensitization when it comes to contact allergens (Watanabe *et al*, 2008). As shown by previous work, ACD is mediated by a danger signal caused by the activation of the TLR signalling pathway, which is triggered by the perturbation of the cutaneous environment once the contact allergen has been applied to the skin (Takeda and Akira, 2005). In order for delayed type hypersensitivities such as ACD to manifest, IL-12 secretion by APCs is required; Martin *et al*, used an IL-12 knockout mouse model to prove that TLR4 is also required for an ACD response to a contact allergen (Martin *et al*, 2008).

The sensitization phase of ACD is a process that depends on many factors despite the antigenicity of the chemical in question. Very potent contact allergens fail to sensitize human or mouse subjects unless an adequate concentration is applied to an appropriate area of the skin (Kimber *et al*, 1999; Scott *et al*, 2002). In an extensive DNCB sensitization study, Friedmann concludes that effective sensitization occurs when enough Langerhans cells present as many DNCB haptens as possible: a dose  $\geq 16.4 \mu\text{g}/\text{cm}^2$  of DNCB applied in an area  $\geq 3.5 \text{cm}^2$  is enough to sensitize 70% of volunteers (Friedmann, 2006). Repeated exposures of low doses in a small skin area of a potent sensitizer can also sensitize individuals potentially due to the accumulation of oxidative damage caused by a single hapten (Paramasivan *et al*, 2009). The latter finding triggers the question whether repeated exposure could render weak sensitizers into potent and that there is no safe exposure level after all contradicting Scott *et al* that supports irritants and even potent sensitizers can be used in low levels (Scott *et al*, 2002).

A very important step in the sensitization phase is LC and dDC maturation and migration to local lymph nodes. The specific activation of the innate immune system at the site of exposure to the contact allergen described above is a cascade of events that lead to the presentation of the haptenated allergen to T-cells; Mizuashi *et al*, as well as Katagani *et al* report that glutathione depletion and DC cell surface thiol groups oxidation induces p38 phosphorylation and concomitant DC maturation (Mizuashi *et al*, 2005; Kagatani *et al*, 2009). In LC depletion mouse models, dDCs were detected carrying and presenting haptens

in the lymph nodes while LC derived IL-10 was considered essential in order for the hypersensitivity response to be restricted (Fukunaga *et al*, 2008; Igyarto *et al*, 2009). Reines *et al*, used a chemical that abrogates DC migration in a mouse ACD model and they observed an attenuation of the hypersensitivity reaction proving how important DC migration is to the pathogenesis of ACD (Reines *et al*, 2009). DCs are important players in the manifestation of ACD. Perhaps the answer to the onset of this disease lies within the DC reaction to contact allergens.

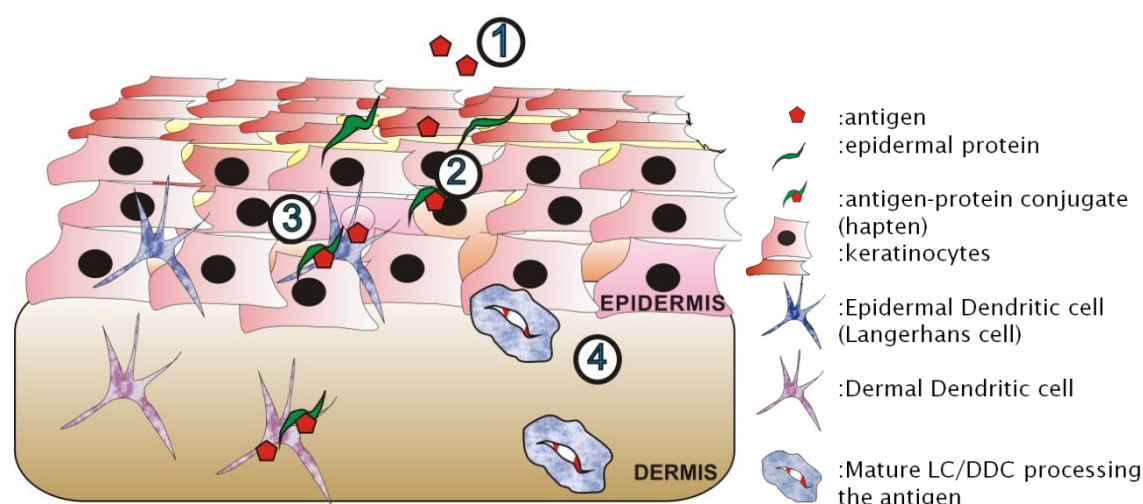


Figure 1-2 **Schematic representation of the sensitization phase of ACD.**

1. Chemicals come in contact with skin 2. Chemicals form conjugates with epidermal proteins perturbing the micro-environment of the epidermis, activating the keratinocytes that start sending danger signals 3. LCs and dDCs uptake the antigen 4. ...process it and migrate to the local lymph nodes

### 1.3.3. Elicitation phase of ACD

Upon further exposure to the chemical, memory T-cells are activated and T-cell clone expansion takes place while freshly migrated DCs have induced the recruitment of more effector cells, resulting in skin lesions (Kimber *et al*, 2002; Friedmann *et al*, 2006; Saint-Mezard *et al*, 2007). Different contact allergens elicit different  $T_H$ ,  $T_C$  (see table 1.1) responses that are determined by different kinetics of DC migration, as IL-12 cytokine that promotes  $T_H1$  responses ceases to be expressed very early in the maturation process (Krasteva *et al*, 1999; Cumberbatch *et al*, 2003). For example both  $CD4^+$  and  $CD8^+$  T-cells

respond to DNCB *in vitro* with a  $T_H1/T_C1$  profile (Toebak *et al*, 2006; Pickard *et al*, 2007). It has also been reported that there is a persistent neutrophil infiltrate in ACD elicitation sites (Zhang and Tinkle, 2000). NKT cells also gather and proliferate in ACD elicitation sites as they have been activated by CD1d LC marker (Gober *et al*, 2008). Nevertheless, repeated elicitation of ACD converts the  $T_H1$  profile to  $T_H2$  (Kitagaki *et al*, 1997).

#### 1.4. CELL CYCLE AND DNA DAMAGE

The contents of this section are inspired by and can be thoroughly examined in 'The molecular biology of the cell' by Alberts *et al* and 'Genes' by Lewin.

##### 1.4.1. Understanding the cell cycle

Life in cellular organisms is perpetuated by the maintenance and functions of DNA. DNA is present in the nuclei of cells, where it exists in pairs of chromosomes (elaborately packed long molecules of DNA-chromatins). DNA holds essentially all the information a cell needs to survive and function as it was destined to, therefore it is understood that DNA's integrity will have a major impact on the cellular fate. The cell cycle is a cascade of consecutive events governed by the state of DNA at a given moment; cell growth (DNA chromatins are loosely packed to allow gene transcription), DNA replication and division in two daughter cells (propagation of replicated DNA). Conserving these pathways is of crucial importance to cell survival, proliferation and death, that is why they are very closely regulated and intertwined in a fine tuned balance in order for the cell to be protected against DNA insults that could result in genomic mutations.

The normal cell cycle is divided in four phases in order of occurrence:  $G_1$ , S,  $G_2$  and M.  $G_1$  stands for Gap 1 and represents the time the cell spends between mitosis and DNA synthesis. Depending on the cell type and tissue condition this phase can last from 10 to 400hrs, for example  $G_1$  is shorter in wound healing due to high proliferation rate of the cells compared to a resting condition (Fairley and Zinovy, 2001). S stands for synthesis and it represents the time spent in DNA replication. It can last from 8 to 12hrs (Fairley and Zinovy, 2001). Following S phase, the cell enters in  $G_2$  phase that stands for Gap 2 and it is the time

the cell prepares for cell division (mitosis) which is the next phase called M.  $G_2$  lasts for 8hrs and cell division takes place in 1-2hrs only. There are three alternative routes the new cells can take: a) to engage in  $G_1$  phase and continue the cell cycle, b) to exit the cell cycle and commit to a terminal differentiation and c) enter the  $G_0$  phase during which the cell is considered quiescent (Johnson and Walker, 1999).

For DNA replication to occur chromatins unwind and the double helix of the DNA opens amidst protein complexes and enzymes allowing the two complementary strands to become templates in order to give rise to new identical DNA strands. The replicated DNA is recoiled in M phase to its highest folded form, the chromosome, joined by a spindle that connects the two copies and polarises them to opposite directions. The cytoskeleton then attaches to DNA promoting the parting of the two identical chromosomes. Cytokinesis (cell movement) takes place, the cell divides and two cells with identical genetic material are formed.

As previously described the cell cycle is a sensitive set of events that is highly controlled by a perplexed network of checkpoints. There are certain criteria to be met for the cell to enter each phase of the cycle. There is a crucial checkpoint at  $G_1$  that a cell has to pass in order to engage in DNA replication (Hartwell *et al*, 1974; Nurse, 1975) and at  $G_2$ , where the cell monitors the quality of the replicated DNA that has to be immaculate in order for the cell to proceed in mitosis. Cell proliferation depends on the function of cyclins and cyclin-dependent kinases (CDKs). Cyclins are proteins that are expressed in an oscillating fashion during the cell cycle with a peak at  $G_1$  and  $G_2$  phases and a dip in mitosis. The cyclin proteins share a conserved region in their amino-acid sequences called the 'cyclin box' that is the substrate on which the CDKs bind to be activated. Their function is to mediate the progression of the cell cycle through a series of phosphorylations of CDKs that can either activate or inhibit cyclins. There are cyclins that act in  $G_1$  phase (D-type cyclins) which undergo very quick turnover and cyclins that act in  $G_2$  phase which have a longer half life and are degraded prior to mitosis (King *et al*, 1996; Pagano, 1997). Growth factors are detected by  $G_1$  cyclins that induce the expression of more cyclins and promote the cell cycle progression. In absence of growth factors  $G_1$  cyclins are ubiquitinated (degraded by a ubiquitin-dependent proteolysis). Each cyclin has a very specific function, for example  $G_1$

cyclin CDK4 phosphorylates the retinoblastoma tumour suppressor protein (Rb) thus inducing the activation of E2F, a protein required for DNA synthesis (Hinds *et al*, 1992; Lukas *et al*, 1995).

#### 1.4.2. How DNA damage affects the cell cycle

DNA damage is essentially any modification on DNA sequence that affects replication and transcription processes. It can take the form of DNA adducts (aberrant nucleotides), single strand breaks, double strand breaks, DNA cross-links (nucleotides covalently bound to other parts of DNA/RNA or even proteins) and insertion deletion mismatches (Pecorino, 2008). These DNA lesions can occur after cell exposure to various genotoxic stimuli such as: reactive oxygen species (ROS), ultraviolet radiation (UV), ionising radiation (IR), chemicals with a high binding affinity to nucleotides (e.g. chemotherapy drugs or benzene) and heavy metals.

DNA damage is detected by a protein complex called MRN (Mre11/Rad50/Nbs1) which scans the DNA for possible strand breaks; once it detects a lesion, MRN attracts the signal transducer kinases DNA-dependent protein kinase (DNA-PK), phosphoinositide 3 kinase (PI3K), ataxia-telangiectasia mutated (ATM) and ATM/Rad3-related kinase (ATR) (Lowndes *et al*, 2000; Cortez *et al*, 2001). These kinases link the DNA replication apparatus with the DNA damage/repair apparatus.

The reason for cyclins to exist is to convey the message that it is safe for the cell cycle to progress. They ensure the fidelity of the cell cycle phases in every checkpoint. DNA damage initiates a pathway which results in the temporary inactivation of CDKs (Nigg, 2001). DNA damage is detected by proteins ATM/ATR which in turn activate CDKs CHK1 and CHK2 that induce the ubiquitination of cell-cycle regulatory phosphatases cdc25 and the hindrance of other cyclin complexes. These events lead to the blockage of the transition of the cell from G<sub>1</sub> to S phase or from G<sub>2</sub> to M phase in order to facilitate DNA repair (Falck *et al*, 2001; Schmitt *et al*, 2006). Another set of proteins is also activated following DNA damage contributing to cell cycle arrest, the CDK inhibitors (cdkIs) (e.g. p21, p27, p16) (Tanaka *et al*, 1996).



#### 1.4.3. Cell fate following DNA damage response and the role of p53

One very important member of the DNA damage response pathway is p53. Protein 53 was first discovered in 1979 and is now described as a tumour suppressor protein and its inactivation is associated with 50% of human cancers (DeLeo *et al.* 1979; Levine 1990; Hollstein *et al.*, 1991). p53 acts as a transcription factor (TF) by binding to promoters of several genes initiating their transcription process. p53 belongs to a family of proteins that include p63 and p73 which exhibit similar DNA binding abilities to p53 in the genome and can affect p53 function, sometimes considered required for proper p53 responses (Flores *et al.*, 2002).

Depending on the type of DNA damage ATM, ATR or PARP proteins phosphorylate p53 thus activating it (Tong *et al.*, 2001). With p53 being in the centre of a vast network of proteins that respond to DNA damage, ATM, ATR or PARP proteins are not the only ones participating in the post-translational modifications (PTMs) (mainly phosphorylation and acetylation) of p53. Different proteins phosphorylate p53 on different amino-acid residues resulting in different conformations of p53 having a remarkable impact on p53's downstream gene expression (Lakin *et al.*, 1999). Therefore, different phosphorylations lead to a different transcriptional affinity and p53 concentration that determines the cell fate: i) cell death, ii) survival, iii) senescence summarised in Table 1-4 (Lockshin and Zakeri, 2004; Okada *et al.*, 2004; Sumpter and Levine, 2010).

Table 1-4 Cell fate.

Outcome		Morphological or other changes	Effector Pathways	Causes
Cell death	Apoptosis	Dynamic process happening to an individual cell requiring RNA and protein synthesis. Cytoplasm shrinks, chromatin condenses and nucleus is destroyed. Cells are detected by the innate immune system and phagocytosed.	Cysteine protease family of <u>caspases</u> pathway. Pro-apoptotic factors released from mitochondria.	DNA damage, activation of Fas receptor, elimination of unnecessary cells (developmentally or because of cancerous features), defence against viral infections.
	Necrosis	Swelling of the cell, influx of water and ions, disruptions of cell organelles and plasma membrane, release of intracellular contents in the extracellular space. Cells not phagocytosed-necrotic accumulation. Irreversible process.	RIP kinase, mediated $\text{Ca}^{2+}$ influx, JNK/p38, PARP activation	Depletion of ATP and nutrients.
	Autophagy	Components of the cytoplasm are engulfed in multiple membrane phagosomes that mature into lysosomes for degradation. It is part of the normal function of cells; excessive autophagy though leads to cell death.	Bcl-2 family.	Restriction of growth factors, defence against infections as part of the innate immune response.
Survival		Cell returns to the normal cell cycle.	MDM-2, m-TOR pathway, SIRT1.	Successful DNA repair, restoration of growth and developmental factors concentrations.
		Cell hyper-proliferates, becomes cancerous.	Defective cell death pathways.	DNA mutation eludes the DNA lesion detection scavengers, viral genome interfering with host genome etc.
Senescence		The cell is now programmed to undergo a set number of divisions and then apoptose.	Negative regulators of apoptosis, cyclins and telomerases.	Natural process of aging, tissue atrophy and accumulation of DNA lesions.

The action of p53 is essential in the progression of all the outcomes described in Table 1-5 and in p53's absence p63 and p73 are thought to act in a compensation mechanism (Prives and Hall, 1999; Flores *et al*, 2002; Schavolt and Pietsenpol, 2007). The most conserved role of p53 is the induction of apoptosis and cell cycle arrest upon cellular injury. One of the direct effects of PTMs on p53 is the disassociation of the p53-MDM2 complex. MDM2 is a p53 negative regulator and once disassociated from it, p53 is stabilised and transferred to the nucleus or the mitochondrial region of the cell (Alarcon-Vargas and Ronai, 2002). Upon translocation to the nucleus, p53 induces cell-cycle arrest by activating the transcription of cdkl p21. Then, p53 engages in the transcriptional activation of many pro-apoptotic genes such as Bax, Apaf1, PUMA and Noxa (Zuckerman *et al*, 2009). In addition, p53 represses anti-apoptotic genes such as survivin and Bcl-2. The Bcl-2 family members Bax, PUMA and Noxa facilitate along with p53-induced protein OKL38 release of cytochrome c from mitochondria that is responsible for the caspase cascade activation (Yao *et al*, 2008). Apart from its transcriptional activity, the mere concentration level of p53 is determinant on cell fate: repeated transient moderate upregulation of p53 leads to senescence while an acute upregulation leads to death (Santoro and Blandino, 2010). p53 protein functions are summarised in Figure 1-3.

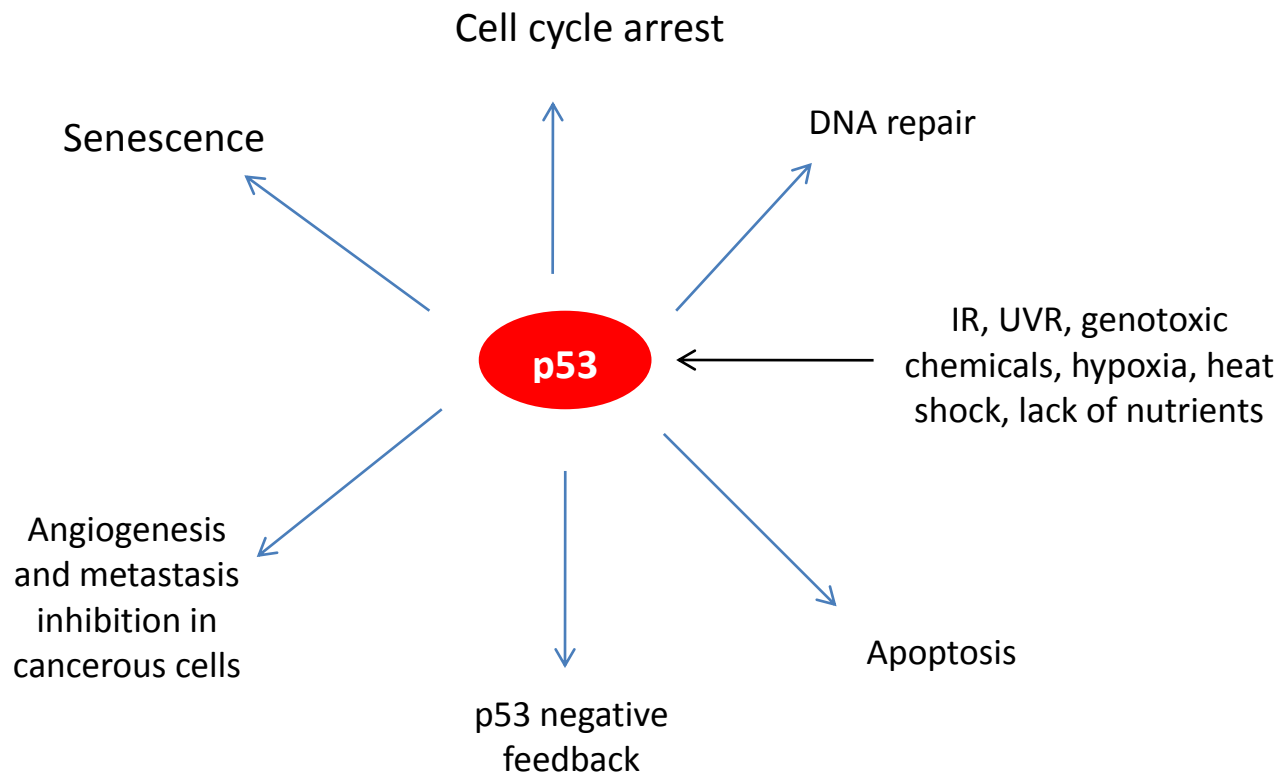


Figure 1-3 **p53 functions.**

p53 is namely the 'guardian' of the genome. Any process that involves the integrity and propagation of cellular DNA involves p53 in the first line of action.

## 1.5. ULTRAVIOLET IRRADIATION AND THE SKIN

### 1.5.1. UVR and its acute effects to the skin

Ultraviolet radiation (UVR) is the part of the electromagnetic spectrum between x-rays and visible light. UVR can be divided in three spectrums: i) UVA 320-400nm, ii) UVB 280-320nm and iii) UVC 100-280nm. UVA is 95% of the solar UVR that reaches the earth surface. UVB is highly absorbed by the ozone layer; however it carries enough energy to have detrimental effects on biomolecules (see below). UVC is scattered and absorbed by the ozone layer and only a miniscule amount reaches the biosphere.

UVR (especially UVA and UVB) is a very important modulator of the skin functions. Ultraviolet light is responsible for the conversion of 7-dehydrocholesterol into vitamin D<sub>3</sub> which is needed in the maintenance of calcium and phosphorus levels in the osteic and

other tissues of the human body. Furthermore, upon UVR the local skin melanocytes produce the neuropeptide  $\alpha$ -MSH (Schauer *et al*, 1994; Chakraborty *et al*, 1996) and the pigment melanin (Carsberg *et al*, 1994; Rees *et al*, 2006; Miyamura *et al*, 2007) that play a role in human cutaneous pigmentation, with  $\alpha$ -MSH being a behavioural, cardiovascular and weight regulator (Versteeg *et al*, 1998; Abdel-malek *et al*, 2000; MacNeil *et al*, 2002; Bertolini *et al*, 2009). Admittedly UVR is needed for a healthy living, in moderation. Melanin and the *stratum corneum* only protect the skin up to a certain degree (mean erythema dose of UVR is different for each individual) from the DNA and reactive oxygen species (ROS) damage (Rees *et al*, 2004; Yamaguchi *et al*, 2006).

UVR reacts with cutaneous intra- and intercellular proteins that absorb photons, creating reactive oxygen species that lead to lipid peroxidation, matrix components' degradation, protein-protein or DNA-protein cross-linking and mutagenesis (Trautinger *et al*, 2001; Jenkins, 2002; Ichihashi *et al*, 2003; Tsourelis-Nikita *et al*, 2005). UVB in particular, directly interacts with DNA resulting in cyclobutane pyrimidine dimers and (6-4) photoproduct formation (Chadwick *et al*, 1994; Norval 2001; Trautinger *et al*, 2001). All this DNA damage, triggers stress signals within the cell and RNA transcription is blocked by activation of protein p53. Once p53 is activated, entry into the S and M phases of the cell cycle is inhibited, in order for the amplification of genetic mutations to be suppressed (May *et al*, 1999; Sionov *et al*, 1999; Latonen *et al*, 2005). From there, nucleotide excision repair takes place, notwithstanding, if the damage done is extensive and the cell's organelles have been detrimentally altered, growth arrest stops and p53 induces apoptosis (Bates *et al*, 1999; Sionov *et al*, 1999; Latonen *et al*, 2005). However, due to possible defective checkpoints in the apoptotic mechanism, mutations can elude the repair process, and can be passed on to daughter cells, ultimately leading to cancer.

### 1.5.2. UVR induced immunosuppression

Along with the DNA damage and concomitants events, another phenomenon that favours the formation of a tumour, takes place in the irradiated skin; photo-immunosuppression. The photochemical isomerism of urocanic acid, the cytokines secreted by the surrounding keratinocytes and fibroblasts combined with topical infiltration of T-

suppressor lymphocytes, have been reported to attribute to deficient antigen presentation and UV-induced tolerance to haptens after solar mimicked irradiation (Reeve *et al*, 1999; Beissert *et al*, 2001; Schwarz, 2002).

The proposed mechanism of UVR induced immunosuppression starts from the pyrimidine dimers and general DNA damage inflicted upon the epidermal keratinocytes that leads to MAP kinase p38 activation and concomitant PAF release. PAF then ligates PAFR on keratinocytes of close proximity that induces prostaglandin-2 (PGE2) release. PGE2 inhibits IL-12 production from LCs thus abrogating a T<sub>H</sub>1 response to UVR-induced inflammation. A skewed regulatory T<sub>H</sub>2 response in combination with a complement cascade and keratinocyte derived neurogenic peptides promote tolerance (Walterscheid *et al*, 2002). Keratinocyte derived IL-10 is a key mediator in photo-immunosuppression, as its knockdown or antibody mediated blockage restores delayed type hypersensitivity responses in mouse models (Rivas *et al*, 1992; Beissert *et al*, 1996).

#### 1.5.3. Chronic cutaneous effects of UVR on skin

An obvious effect of chronic UVR skin exposure is photoageing. The keratinocytes hyper-proliferate thus amplifying epidermal thickness. Keratinocytes lose their typical characteristics (atypia). Melanocytes produce more melanosomes but due to the compromised melanosome transfer in photoaged skin, freckly features of irregular pigmentation accumulate in sun exposed areas (Gilchrest *et al*, 1983). LCs decrease in numbers thus enhancing the immunosuppressive effect of repeated UVR (Toyoda *et al*, 1997; Wulf *et al*, 2004).

In the dermis, matrix metalloproteinases and collagenases levels increase, aggravating the natural turnover of collagen and other matrix fibers (Bernstein *et al*, 1996). By consequence, this phenomenon in combination with UVR induced fibril cross-linking leads to an impaired mechanical support of the skin by the dermis and as the epidermis is thickened, ridges are formed, skin becomes relaxed surrendering to gravity and epidermis demonstrates a leathery appearance (elastosis) (Yamauchi *et al*, 1991; Craven *et al*, 1997).

Chronic exposure to UVR is the main cause of non-melanoma skin cancer. Non-melanoma skin cancers are not commonly lethal but due to their high occurrence it costs England around £71 million to treat it (Morris *et al*, 2005). The most prevalent types of non-melanoma skin cancers are squamous cell carcinomas (SSCs) and basal cell carcinomas (BCC). Repeated UVR exposures lead to an accumulation of DNA mutations and hyper-proliferating cells that thrive under the prolonged immunosuppressive conditions in the skin and cancer appears despite the UVR protection that melanin offers (Agar and Young, 2005). Different skin phototypes though have a different risk factor of developing skin cancer; skin phototypes I and II (low ability to produce melanin and the main melanin type is pheomelanin) are more susceptible to SSCs and BCCs (Jansen *et al*, 1989).

AMPs (antimicrobial peptides) are induced in the outermost layers of the epidermis of UVR exposed skin which proves to be beneficial as bacterial encounters occur in this area (Glaser *et al*, 2009). So, despite the phenomenon of immunosuppression, the skin is still competent against a bacterial infection. However this is not true in the case of HSV (herpes simplex virus) that is more prone to exacerbate during UVR exposure (Norval, 2006).

## 1.6. MICRORNAS

### 1.6.1. RNA interference history

In 1990, Napoli *et al* observed that multiple copies of a pigment transgene lead to colorless petunias (Napoli *et al*, 1990). A few years later Ambros's group discovered a gene in *C. Elegans* that did not code for a protein but a small transcript (Lee *et al*, 1993). This would be the first microRNA ever identified: lin-4. In 1995 Guo and Kemphues defined the notion of RNAi (RNA interference, protein expression control by RNA) and in 1998 Fire and Mello (that won the Nobel Prize for RNAi in 2006) gave an answer to the RNAi mystery, revealing that it is mediated by dsRNA (Guo and Kemphues, 1995; Fire and Mello, 1998).

### 1.6.2. microRNAs' biogenesis and silencing mechanisms

microRNAs consist of approximately ~22 nucleotides and are the mature forms of non-coding RNA transcripts deriving from independent genes or introns of protein-coding genes (Bartel 2004). They are transcribed by RNA polymerase II, as long polyadenylated transcripts called pri-microRNAs, which fold in multiple complementary regions (Lee *et al*, 2003). Then they are spliced in the nucleus by the RNase III type endonuclease Drosha, resulting in a double-stranded hairpin structure, which is exported to the cytoplasm by exportin-5 (Zeng *et al*, 2004). In the cytoplasm they will be spliced again by another RNase III type endonuclease, Dicer, forming a mature microRNA-in some cases from both branches of the hairpin (Filipowicz *et al*, 2008). Then, the single stranded miRNA will then form a ribonucleoprotein complex with AGO proteins called RISC (RNA induced silencing complex) and will commence translation repression (Lee *et al*, 2004, Bartel 2004; Valencia-Sanchez *et al*, 2006; Filipowicz *et al*, 2008; Wu *et al*, 2008).

Translation repression by microRNAs occurs in many different ways. Each mRNA has one or multiple sites semi-complementarily to one or more microRNAs (Brennecke *et al*, 2005; Wu *et al*, 2008). A binding site is determined by both sequence and tertiary structure (Brennecke *et al*, 2005; Wu *et al*, 2008; Filipowicz *et al*, 2008). Generally the microRNA bound to RISC pairs with the 3'untranslated region (UTR) of the mRNA. The binding site for a microRNA (seeding region) on an mRNA presents the following features: i) an AU-rich nucleotide sequence close to the site, ii) usually the binding site for one microRNA are next to the binding sites for other microRNAs that can act as a synergy factor, iii) the regions around the binding site allow a stable microRNA-mRNA interaction, iv) the binding site is far from the stop codon and v) the centre of the UTR (Grimson *et al*, 2007).

It has been suggested that translation may be interrupted by microRNAs in 4 ways: a) by blocking the initiation step, b) by deadenylating the mRNA to be translated (both these ways result in transfer of the mRNA to P-bodies, which co-operate with RNA interference) c) by destroying the newly synthesized peptide and d) by blocking the elongation step (Brennecke *et al*, 2005; Valencia-Sanchez *et al*, 2007; Wu *et al*, 2008; Filipowicz *et al*, 2008).



The fact that 30% of the protein-encoding genes of the genome seem to be post-transcriptionally regulated by microRNAs and their high conservation between different species implies that they possess a significant role in development, disease and other biological events (Bartel 2004).

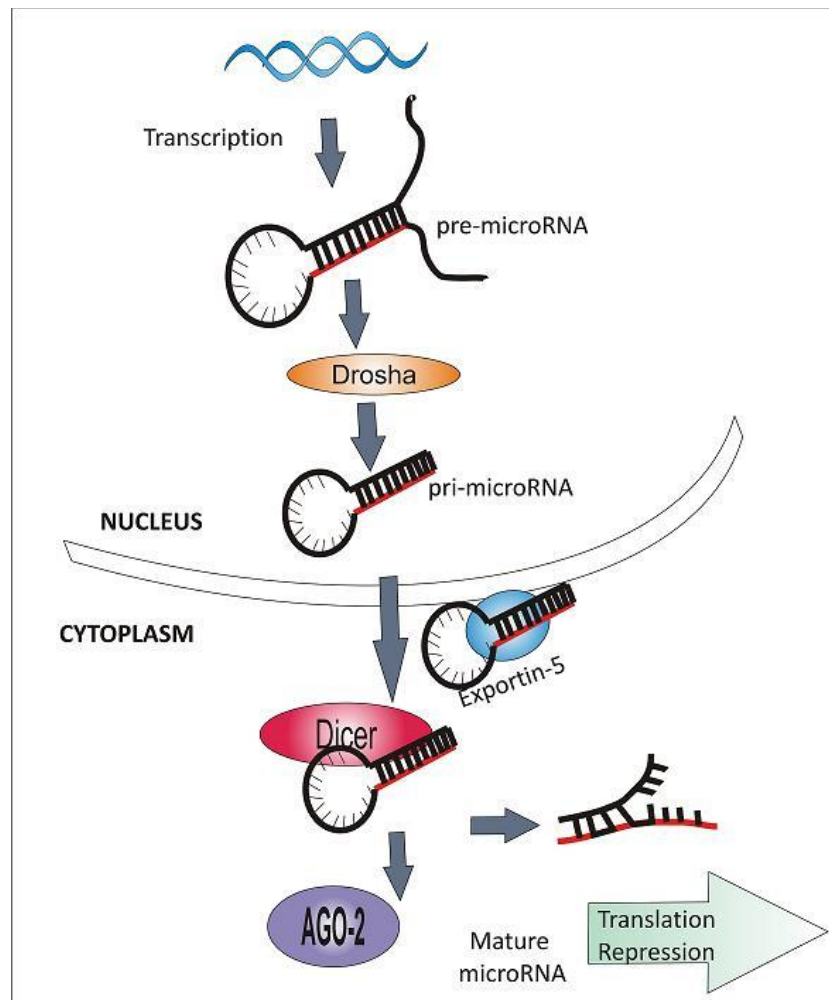


Figure 1-4 **Biogenesis of microRNAs.**

### 1.6.3. microRNAs in skin

The silencing of Dicer in mice had detrimental effects on the development and morphogenesis of skin (Yi *et al*, 2006). This accentuated the fact that microRNAs are important for skin biology. In 2007, Sonkoly *et al*, compared the microRNA profiles from psoriatic patients to healthy subjects (Sonkoly *et al*, 2007). What they found was that there

was a distinct upregulated set of microRNAs in psoriatics miR-21, miR-203, miR-146a and a downregulated one, miR-125b, with miR-203 being a skin specific microRNA.

#### 1.6.4. microRNAs in immune disorders

microRNA expression presents a specific pattern for every human tissue. For instance, lymphoid tissues and immune cells show an interesting unique microRNA expression profile, which changes depending on the differentiation or maturation stage of the pertinent immune cell (Chen *et al*, 2004; Sonkoly *et al*, 2008).

microRNAs are indispensable for the immune system. T-cells devoid of Dicer protein failed to differentiate successfully to physiological numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, T-reg<sup>s</sup> were severely decreased in the thymus and general T-cell proliferation was impaired due to early apoptosis (Muljo *et al*, 2005; Cobb *et al*, 2006). Antibodies against P-bodies and RNAi components were found in systemic lupus erythematosus (SLE) and scleroderma (two grave autoimmune diseases) thus implicating the microRNA machinery in the pathogenesis of immune disorders even deeper (Pauley and Chan, 2008).

microRNAs expression levels exhibit a very delicate balance that once disturbed (especially in epithelial cells and immunocytes) it promotes inflammatory disorders (Sonkoly and Pivarcsi, 2009). For example, miR-155 knockout mice had impaired antigen presentation abilities and high affinity antibody production and a general inflammatory phenotype markedly pronounced by extensive airway remodelling (Rodriguez *et al*, 2007; Thai *et al*, 2007). Deregulation of expression levels of other microRNAs (such as miR-15a, miR-16.1, miR-17-92) leads to leukaemia, autoimmunity and inflammation (Calin *et al*, 2002; Costinean *et al*, 2006; Xiao *et al*, 2008).

#### 1.6.5. p53 associated microRNAs

It is widely known that activated p53 binds to specific binding sites in the genome promoting the transcription of various genes involved in cell growth-arrest, apoptosis and DNA repair (Vogelstein *et al*, 2000; Balint and Vousden, 2001; Wei *et al*, 2006; Aylon *et al*,

2007). However there is evidence showing that p53 also participates directly or indirectly, in gene repression (Spurgers *et al*, 2006).

Tarasov *et al* found out microRNA 34 family (miR-34a, -b and -c) was completely absent after microRNA screening in several p53 mutated cancers (Tarasov *et al*, 2007). A p53 binding site has been recognized on the first exon of the genes coding for the miR-34 family, proving that the latter is a transcriptional target of p53 (Bommer *et al*, 2007; Corney *et al*, 2007; He *et al*, 2007). In order to investigate miR-34a's modulation certain research groups used ionising radiation (IR) and adriamycin amongst other DNA damaging agents to induce DNA lesions that would elicit a p53 driven growth arrest. miR-34a was found to be significantly upregulated in the treated samples (Tarasov *et al*, 2007; Zenz *et al*, 2009). Further work, indicated that the miR-34s repress the translation of proteins involved in the cell cycle and DNA damage response e.g. BCL2 (He *et al*, 2007; Raver-Shapira *et al*, 2007). miR-34a represses the translation of silent mating type information regulation 2 homolog 1 (SIRT-1) (Yamakuchi *et al*, 2008; Luan *et al*, 2010). SIRT1 is a NAD-dependent deacetylase that affects apoptosis in response to oxidative and genotoxic stress stimuli (Haigis *et al*, 2006; Longo and Kennedy, 2006). It has been postulated that SIRT1 acts as a negative feedback loop factor in the p53 pathway by deacetylating p53, thus inhibiting its function (Cheng *et al*, 2003; Solomon *et al*, 2006).

### 1.7. OBJECTIVES OF THIS PHD

- Set up an *ex vivo* sensitization and UV irradiation *ex vivo* model
- Depict which microRNAs have an important role in ACD
- Investigate the behaviour of miR-34 family in UV responses in skin

## 2. Materials and Methods

### 2.1. SOURCE OF HUMAN CELLS AND TISSUE

This project is included in a greater study of investigation of immunological responses in skin, which has been approved by the Ethics committees of the Southampton University Hospitals Trust and all subjects have given their written consents. (Study numbers: 07/Q1704/59, 07/Q1704/46)

Redundant skin was obtained from patients undergoing mastectomies at the Princess Anne Hospital. Foreskin skin tissue was obtained from the Southampton General Hospital. In all instances the individuals were naïve to our model sensitiser DNCB, a chemical that is not encountered outside of the research setting. Skin was excluded if the individual had an existing skin disorder.

Peripheral blood was obtained by venupuncture from healthy volunteers (3 male, 2 female; mean age 27) from within the Laboratory. The following were excluded from the study.

- Subjects with an existing infection
- Pregnant women
- Atopic subjects (Atopic subjects did not respond to our positive control treatment with lipopolysaccharide so they were omitted.)

## 2.2. TABLE OF MATERIALS

Table 2-1 **Table of the materials used in this PhD.** The item description is shown on the left column and the commercial supplier of the item is listed on the right column.

<b>Materials</b>	<b>Supplier</b>
<u>Cell culture/Blood processing</u>	
RPMI 1640	Invitrogen, UK
Defined Keratinocyte-SFM (1X), liquid	Invitrogen, UK
FBS	Life Technologies, UK
Penicillin/Streptomycin	Invitrogen, UK
IL-4, GM-CSF	Miltenyi Biotec, UK
PBS pills	Oxoid, UK
Dispase	Invitrogen, UK
Trypsin-EDTA·4Na	Invitrogen, UK
Surgical Scalpel Blade No.22A	Swann Morton
Lymphoprep	Axis Shield, Norway
DMSO	Sigma-Aldrich, UK
DNCB	Sigma-Aldrich, UK
Flat PTFE coated rings	Chromacol, UK
Acetone	Fisher Scientific, UK
PPD	Sigma-Aldrich, UK
O rings	RS Components, UK
MS Columns	Miltenyi Biotec, UK
CD14 magnetic beads	Miltenyi Biotec, UK
Vacutainer tubes	BD Biosciences, UK
Franz Diffusion Chambers	Department Of Chemistry/UoS
<u>Immunohistochemistry/Immunofluorescence/Flow cytometry</u>	
DAB Kit	Biogenex, UK
Vectashield	Vector Laboratories, UK
Vectastain ABC Kit	Vector Laboratories, UK
Hydrogen Peroxide	Sigma-Aldrich, UK
Xylene	Fisher Scientific, UK
DPX mounting media	Fisher Scientific, UK
Goat-antimouse FITC labelled antibody IgG (H+L)	Jackson ImmunoResearch, UK
Biotinylated rabbit-antimouse	Dako, UK
Cell strainers 100um	BD Biosciences, UK
Parafilm M	SPI supplies, UK
IgG2b isotype control Ab [MPC-11]	Abcam, UK
EDTA	BDH Laboratory Supplies, UK
NaN <sub>3</sub>	Sigma-Aldrich, UK
Paraformaldehyde	Sigma-Aldrich, UK
Tween 20	Affymetrix/USB, Germany

CD-1a antibody[NA 1/34]	Dako, UK
IgG1,kappa/FITC Isotype Control	Mytenyi Biotec, UK
P53 antibody [DO-7]	Novocastra, UK
HLA-DR/PE Antibody	BD Bioscience Pharmingen, UK
IgG1a/PE Isotype Control	eBiosciences, UK
BSA	Sigma-Aldrich, UK
Microscope Gridded Graticule	Leica, UK
Glass coverslips	BDH Laboratory Supplies, UK
<u>Molecular Biology</u>	
Nuclease free water	Invitrogen, UK
Chloroform	Sigma-Aldrich, UK
Isopropanol	Sigma-Aldrich, UK
Taqman Universal PCR No UNG Master Mix	Applied Biosystems, UK
microRNA RT+qPCR primers	Applied Biosystems, UK
TRIreagent	Ambion, UK
Glycogen	Roche, UK
microRNA RT kit	Applied Biosystems, UK
Lysing matrix D	Anachem, UK
RNU48, has-miR-125b, has-miR-146a, hsa-miR-155, hsa-miR-342-5p, hsa-miR-138, hsa-miR-597, hsa-miR-10a microRNA assays	Applied Biosystems, UK
Dicer and GAPDH qPCR primers	Applied Biosystems, UK
<u>Protein Analysis and Immunoblotting</u>	
BCA assay	Sigma, UK
NuPAGE Antioxidant	Invitrogen, UK
NuPAGE Sample Reducing Agent (10X)	Invitrogen, UK
NuPAGE LDS Sample Buffer (4X)	Invitrogen, UK
NuPAGE Novex 10% Bis-Tris Gel 1.0 mm, 10 well	Invitrogen, UK
iBlot Transfer Stack, PVDF Mini	Invitrogen, UK
NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X)	Invitrogen, UK
SIRT1 antibody (H-300)	Insight Biotechnology Ltd, UK
Rabbit IgG Isotype Control	Santa Cruz Biotechnology Inc.
Complete Protease Inhibitor Cocktail tablets	Roche, UK
Triton X-100	Sigma, UK
Sodium Chloride	Sigma, UK
SDS	Gibco BRL, UK
Tris	Fisher Bioreagents, UK

### 2.3. BUFFERS AND SOLUTIONS PREPARATION

#### 2.3.1. Citrate Buffer

0.2% (w/v) of citric acid in sterile PBS, was adjusted to pH 6 with 2 M NaOH. Buffer was made fresh before each experiment.

#### 2.3.2. Avidin-biotin complexes

26.7 $\mu$ l of solution A and 26.7 $\mu$ l of solution B (Vectastain ABC Kit) were added to 2mL of 1X PBS for 30min in RT after a vigorous agitation. It is used fresh every time.

#### 2.3.3. 3, 3'-diaminobenzidine (DAB) solution

250 $\mu$ l of substrate buffer, 2 drops of DAB solution and 1 drop of provided H<sub>2</sub>O<sub>2</sub> solution from the DAB Kit, are added in 2.25mL of H<sub>2</sub>O. This solution must be used immediately and must be prepared fresh every time.

#### 2.3.4. EDTA solution

0.5M of ethylenediaminetetraacetic acid diluted in column purified water is placed on a magnetic stirrer. 2M NaOH was gradually added until pH 8 is reached. The volume was then adjusted to 200mL with distilled H<sub>2</sub>O, and can be kept in room temperature for 2-3 months.

#### 2.3.5. 4% paraformaldehyde solution

100mL of warm 1XPBS is added on top of 4g of paraformaldehyde. The mixture is heat up to ~70°C on a magnetic stirrer in a fume hood. When the paraformaldehyde is effectively diluted the dilution is cooled to room temperature. 2M NaOH is gradually added until pH 7.0-7.6 is reached. The buffer is then filtered with filter paper and stored at -20°C. If stored at 4°C, it can be used within 1 month.

### 2.3.6. Blocking Solution for Immunofluorescence and immunohistochemistry

Block solution consists of 20% FBS and 1% BSA diluted in sterile PBS. Stock solution is stored at -20°C. Once thawed it can be stored at 4°C and used within 2 days.

### 2.3.7. Blocking solution for western blotting

Block solution is prepared with PBS supplied with 0.1 % Tween and 5% dried milk. It can be kept at 4°C for a week.

### 2.3.8. Keratinocyte Growth Media

Defined keratinocyte serum free media growth supplement is thawed and added to defined keratinocyte serum free media. This media is then kept at 4°C in the dark and has a 10 week shelf life. No antibiotics were added for this project.

### 2.3.9. MACS Buffer

MACS buffer consists of 0.5% BSA and 2mM EDTA in sterile PBS. The buffer can be stored at 4°C for 6 weeks.

### 2.3.10. FACS Buffer

3% FBS and 0.2% NaN<sub>3</sub> were diluted in sterile PBS. The solution can be stored at 4°C for 6 weeks.

### 2.3.11. RIPA buffer

1.0% Triton X-100, 0.1% SDS, 50nM NaCl, 50nM Tris-HCl and complete protease inhibitor tablets from Roche in milliQ H<sub>2</sub>O. The solution can be stored aliquoted at -20°C up to a year.



## 2.4. EX VIVO TREATMENTS

### 2.4.1. *Ex vivo* sensitization protocol

Fresh skin was incubated in media (RPMI 1640, 10% FBS, 1% P/S) for one hour. Excess subcutaneous fat was removed. The skin was washed with sterile PBS and was cut in ~1cm diameter round biopsies. The *ex vivo* sensitisation protocol was implemented using a Franz Diffusion (FD) chamber system (Figure 2.1), which is composed of two main glass compartments that are held together by two springs; the donor and the receptor compartment. The skin is placed between 2 sets of silicon 'O' rings and flat rubber rings that are coated with a special polymer from one side to prevent leakage of the applied chemicals to the rest of the apparatus. The receptor compartment is filled with media (RPMI 1640, 10% FBS, 1% P/S). In the upper chamber, the skin's epidermis is at the air interface, where the treatment is administered from. Following application of the chemical, the top compartment covered with parafilm to prevent drying and enhance penetration into the skin. Skin mounted in the FD chamber apparatus is placed in a humidified incubator at 37°C with CO<sub>2</sub> during the treatment period. On removal, the skin explants are trimmed and washed in room temperature sterile PBS and are ready for further processing.

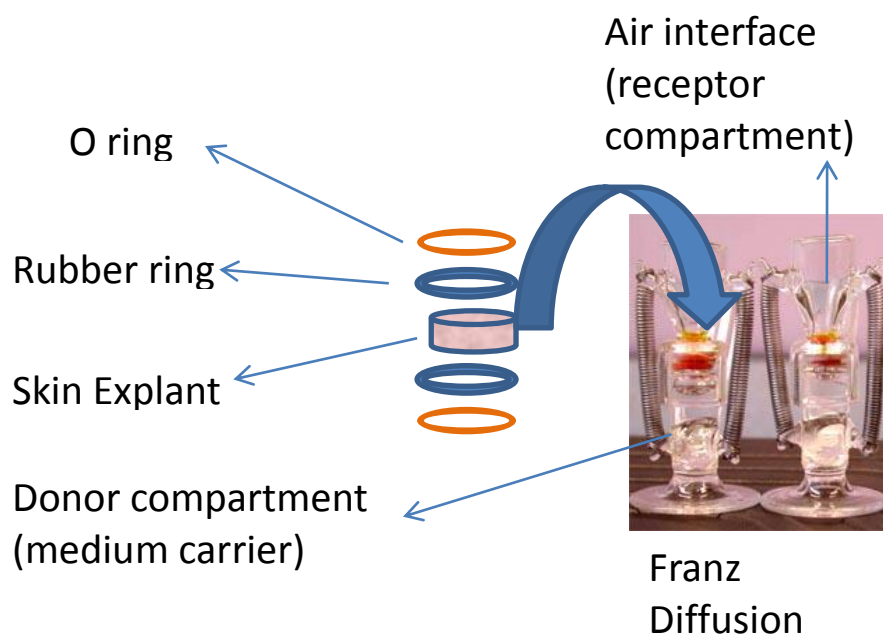


Figure 2-1 **Schematic representation of the Franz Diffusion Chambers' assembly.** The chambers are illustrated on the right and the stacking arrangement of the rings and skin explants are shown on the left.

#### 2.4.2. *Ex vivo* skin UVR treatment

Fresh skin was incubated in media (RPMI 1640, 10% FBS, 1% P/S) for one hour. Excess subcutaneous fat was removed. The skin was washed with sterile PBS and was cut in  $\sim 1\text{cm}^2$  square biopsies. After, the skin was transferred into 60x20mm petri-dishes containing 1mL sterile PBS and were irradiated for 5, 10, 20, 40 and 60min in a UV chamber (the UV chamber is a white box with LT12 UVB lamps on the top side and an opening covered with a heavy black garment). The UV emitting lamp was a LT12 Philips lamp. The sham controls were covered with aluminium foil. After irradiation, all samples were washed with sterile PBS and cultured overnight in media (RPMI 1640, 10% FBS, 1% P/S), in a humidified incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .

#### 2.4.3. *Ex vivo* skin IR treatment

Fresh human skin was processed as described in the previously (2.4.2). Skin explants were allocated in plastic sealable tubes containing RPMI 1640+ 10% FBS, on ice, until they were deposited in the irradiation compartment of a Gammacell irradiator. Sham IR control samples were left in RT during treatment. The given doses were 5, 10 and 20Gy at a dose

rate of 2.12Gy/min. The samples were immediately placed on ice post treatment, washed in room temperature sterile PBS and cultured overnight as described above (2.4.2).

## 2.5. PREPARATION OF SPLIT SKIN

### 2.5.1. For isolation of epidermal cells

For keratinocyte or other epidermal populations extraction the skin is cut in very small pieces and placed in dispase 1X overnight at 4°C. The tissue is washed in sterile PBS and the epidermis is separated from the dermis using forceps.

### 2.5.2. For immunohistochemistry

For staining procedures, excess dermis is trimmed and the skin is transferred in 3mL of pre-warmed 20mM EDTA in PBS for one hour at 37°C. Fresh EDTA solution is added for a further hour. This step is repeated if the epidermis fails to detach from the dermis. Once the skin was split, the epidermis was washed 3 times with PBS and fixed in ice cold acetone for 20min. Fixed samples were stored at 4°C in PBS until stained.

## 2.6. IMMUNOSTAINING

### 2.6.1. Immunofluorescence staining of epidermal sheets and counting of Langerhans cells (LC).

Fixed epidermal sheets were blocked with 100μM blocking solution at room temperature for 30min, on a plate shaker. Blocking solution is replaced by CD1a conjugated antibody used at 1:50 dilution in blocking solution for 45min in room temperature. After the incubation time is complete the samples are washed 3 times in PBS and mounted (with the dermal side of the epidermis looking up) on microscope slides with 10μl of Vectashield (to prevent photobleaching). Stained LCs were counted using a gridded graticule with a square grid that consists of 100 squares. Each grid is 250μm×250μm=62500μm<sup>2</sup>. To convert cells/grid numbers to cells/mm<sup>2</sup>, the cells/grid numbers were multiplied by 16. The gridded

graticule was adjusted to the eyepiece of a fluorescence microscope and 10 grids from different fields of every CD1a stained sheet were used to count LC.

#### 2.6.2. Immunohistochemistry of epidermal sheets for p53

Fixed epidermal sheets were microwaved for 10min in 0.01M citrate buffer. The samples were transferred quickly after microwaving in PBS/0.05% Tween and onto a plate shaker for 5min at room temperature. Endogenous peroxidase activity was quenched in 0.5% H<sub>2</sub>O<sub>2</sub> in methanol for 20min and washed 3 times with PBS/0.05% Tween. Then blocked using blocking solution (PBS+20%FBS+1%BSA) for one hour. The epidermal sheets were incubated with p53 antibody (1:100 dilution in blocking solution) for one hour at room temperature, then washed 3 times in PBS/0.05% Tween. The samples were then incubated with anti-mouse biotinylated antibody (1:200 in blocking solution for one hour) at room temperature then washed 3 times in PBS/0.05% Tween. Avidin-biotin (ABC) complexes were prepared according to manufacturer's instructions. Samples were incubated in ABC complexes for 45min in room temperature. The samples were immersed in a 3, 3'-diaminobenzidine (DAB) solution that was prepared according to manufacturer's instructions for 5min. The samples were washed in PBS/0.05% Tween. To avoid contamination and facilitate processing, the samples were dehydrated in 70% ethanol for 10min followed by 100% ethanol for a further 10min. The epidermal sheets were then washed in xylene for 10min x 2 and finally were mounted with DPX (Di-n-butylPhthalate in Xylene) onto microscope slides in a fume hood.

#### 2.6.3. Immunofluorescence staining of HPK cells

250,000 HPK cells/mL were pipetted onto glass cover slips (source) placed in 12 well plates. On reaching ~80% confluence, the cover slips were washed x1 with sterile PBS and transferred in 60x20mm petri-dishes and immersed in 1mL of sterile PBS. The adhered HPK cells were irradiated as described in section 2.13.3.1. Following irradiation, the cover slips were then washed with sterile PBS and cultured overnight in a humidified incubator at 37°C with 5% CO<sub>2</sub>. The following day, the cover slips were washed with 3 times with sterile PBS and fixed with 500µl of 4% Paraformaldehyde for 10min at room temperature. After 3

washes with sterile PBS, the cells were permeabilised with 500µl of ice cold methanol for 6min on ice and washed 3 times with PBS then incubated with blocking solution for 30min at room temperature. Following this, the cover slips were inverted (cell side down) onto 100µl of p53 antibody (1:100 dilution in blocking buffer) spotted on to parafilm, for 1 hour at room temperature. They were then returned to a fresh 12 well plate and washed thoroughly 3 times with PBS. The same procedure was followed for goat antimouse FITC conjugated antibody, used at 1:50 in blocking solution. On completion, the cover slips were mounted onto 10µl of Vectashield, cell side down, on microscope slides. The edges were sealed with nail varnish and the slides stored at 4°C in the dark to await visualisation.

#### 2.6.4. Indirect flow cytometry of p53 stained HPK cells

HPK cells were exposed to UV and cultured overnight at 37°C. The following days the cells trypsinised from the x and transferred into labelled FACS tubes where they were fixed with 1mL of 2% paraformaldehyde for 10min on ice. Following fixation, the cells were washed x1 with 1mL of MACS buffer containing 0.5% Triton X-100, and centrifuged at 200g for 7min at 4°C. As p53 is localized in the nucleus, the cells were permeabilised in methanol at -20°C for 15-20min, washed x 1 with 1mL of MACS buffer (0.5% Triton X-100) and pelleted at 200g for 7min at 4°C. The cells were blocked for 15-20min in FACS buffer supplemented with 10% of human serum. The cells were washed x1 in MACS buffer+0.5% Triton X-100 and incubated for 30min in 100µl of p53 antibody (1:100) or IgG2b isotype control antibody at the same concentration in FACS buffer+10% of human serum on ice. The cells were washed x2 in MACS buffer+0.5% Triton X-100 and incubated for 20min with anti-mouse FITC conjugated secondary antibody (1:100 in FACS buffer+10% of human serum) on ice. Following the incubation the cells were washed x2 with 1mL of MACS buffer+0.5% Triton X-100 and pelleted at 200g for 7min at 4°C. Cells were then re-suspended in FACS flow solution and acquired/analysed on a FACS calibur. Instrument setting were optimised and used for all the donors' samples. The criteria of the gating were based on forward and side scatter, excluding cell debris and enlarged apoptotic cells. The keratinocyte culture conditions favour the propagation of keratinocyte cells only; occasionally the cell cultures are contaminated with fibroblasts, which deplete the nutrients in the culture and the actual HPK cells eventually die. All antibodies were titrated for

primary HPK cells, using 500,000 cells/ tube. Tubes were vortexed before blocking and antibody staining steps. Data was analysed using CellQuest Pro based on main fluorescence intensity of the gated staining cells.

## 2.7. DC MARKERS FLOW CYTOMETRY

MoDCs were collected and blocked in FACS buffer supplemented with 10% human serum for 15 to 30min at 4°C. Cells were then incubated with either 0.3ng/mL anti-CD86/PE and anti-CD1a/FITC or anti-HLA-DR/FITC or IgG<sub>2a</sub>/PE and IgG<sub>1</sub>/FITC for 30min at 4°C. Cells were washed with cold PBS at least twice and spun at 200g for 5min at 4°C. Cells were re-suspended in FACS solution in order to be processed through the FACS calibur. Detection of PE fluorescence was performed in FL2 channel and FITC in FL1 channel. Compensation was conducted prior to data collection. The cells were gated for each volunteer separately based on their side and forward scatter plot.

## 2.8. KERATINOCYTE EXTRACTION PROTOCOL

Skin was split with dispase as described in section 2.5.1. Epidermis was cut in small pieces using surgical blades and forceps. It was then transferred to 3mls of Trypsin 0.05%-EDTA·4Na 0.2 g/L solution. Incubation times in trypsin varied accordingly to the type of skin sample. Mastectomised skin was incubated for 10-15 minutes at 37°C on a heated plate shaker and circumcised skin was incubated in trypsin solution for 30 minutes at the same conditions. The sample was then shaken vigorously and sieved through a 100µm strainer. Trypsin was inactivated by the addition of 300µl of FBS. The cell suspension was then diluted in calcium-free PBS and was centrifuged at 65g for 7 minutes in room temperature. The pellet was re-suspended in 6mls of keratinocyte defined medium and transferred in a T25 culture flask.

Cells are fed every 2 days and before every second feeding, they are washed twice with PBS. The HPK cells were split when they reach 80% confluence, using 1ml Trypsin-EDTA for 1-3 minutes at 37°C. Trypsin is inactivated with 100µl FBS and cells are centrifuged at 179g for 7minutes at 4°C and re-suspended in pre-warmed fresh medium.

## 2.9. ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) FROM WHOLE BLOOD

Peripheral venous blood was collected into Vacutainer tubes containing EDTA to prevent coagulation. PBMCs were separated from the blood by gradient centrifugation by layering 10mL of blood over 10mL of Lymphoprep. The samples were centrifuged at 1000 x g for 20min at room temperature, with the rotor brakes deactivated to prevent movement of the lymphocytes phase during deceleration. The PBMCs, visualized as a cloudy interphase between the Lymphoprep and the serum, were aspirated using a pasteur pipette, pooled into a single tube and diluted up to 50mL with ice cold sterile PBS. Cells were pelleted at 200 X g for 7min at 4°C, re-suspended in 10 mL of sterile PBS and counted using a Neubauer 0.1 mm haemocytometer. The tube was replenished with sterile PBS up to 50mL and was centrifuged at 200 x g for 7min at 4°C

## 2.10. MONOCYTE ISOLATION FROM PBMCs

After determining the number of PBMCs using an improved Neubauer 0.1 mm haemocytometer, monocytes were isolated from PBMCs by positive selection with a magnetic bead conjugated CD14 antibody (monocyte marker). PBMCs were incubated in for 15-20min in  $\mu\text{L}$  of anti-CD14 conjugated to magnetic beads diluted in 80 $\mu\text{L}$  of MACS buffer per  $10^6$  of PBMCs at 4°C. The cells were then washed away with the addition of fresh MACS buffer and centrifugation at 200g for 7min at 4°C. The monocyte fraction was separated from the PBMCs using a MiniMACS system. (Figure 2.3) The MS column was irrigated with MACS buffer and the CD14-labelled PBMC suspension was applied onto it. The column was washed three times with 1mL of MACS buffer, the column removed from the magnetic and the CD14+ fraction eluted using a plunger.

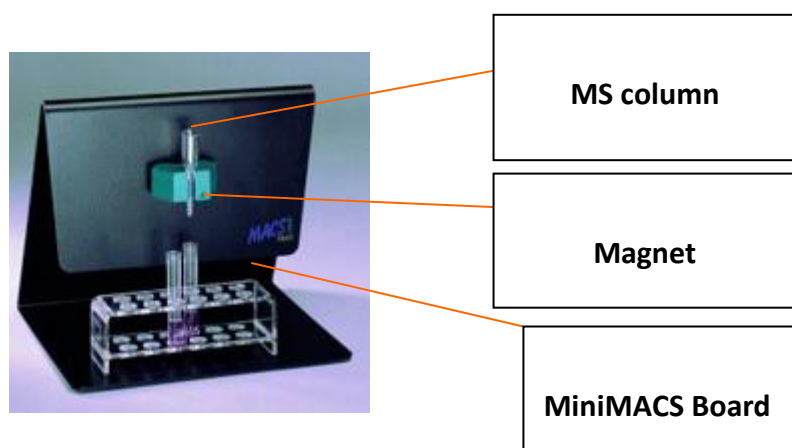


Figure 2-2 **The MiniMACS system** consists of a metal board, a magnet and an MS column. The CD14-labelled PBMCs are flushed through the MS column and the CD14<sup>-</sup> fraction is collected in a tube beneath the MS column.

### 2.11. IN VITRO DIFFERENTIATION OF MONOCYTE DERIVED DENDRITIC CELLS

Isolated monocytes were counted and plated at  $10^6$  cells/mL. Cells were supplemented with endotoxin free GM-CSF at  $1000\text{U}/10^6$  cells, IL-4 at  $500\text{U}/10^6$  cells and 10% heat inactivated FBS in RPMI 1640. At day 5, the cells are supplemented with  $500\text{U}/10^6$  cells GM-CSF and  $250\text{U}/10^6$  cells IL-4. On day six immature MoDCs were collected from the culture plates, centrifuged at  $200 \times g$  for 7min at  $4^\circ\text{C}$ , counted and plated in a fresh x well plate in order to be treated. Cell purity was checked by flow cytometry analysis; the cells were stained for CD4 T-cell marker, confirming that over 95% of the culture was CD3 negative (Data not shown). Cell differentiation was checked by flow cytometry analysis as well. MoDCs were stained for CD1a, HLA-DR and CD14 before in vitro differentiation and after. CD1a and HLA-DR were found upregulated and CD14 downregulated in 3 out of 3 occasions as expected for immature MoDCs (Naour et al, 2001) (Data not shown).

### 2.12. TRANSFECTION OF MONOCYTE DERIVED DENDRITIC CELLS

$4\mu\text{l}$  of  $5\mu\text{M}$  siRNA solution and  $1\mu\text{l}$  of lipofectamine are diluted in  $200\mu\text{l}$  of RPMI without added serum or antibiotics. After a vigorous agitation, the mixture is left in room temperature for 5min. These quantities correspond to one well of a 48 well plate. The number of immature dendritic cells is determined and the cells are resuspended in RPMI without added sera or antibiotics at a concentration of  $4 \times 10^6$  cells/mL.  $200\mu\text{l}$  of the cell



suspension is poured in the well containing the siRNA solution. The plate is gently rocked and is left in an incubator at 37°C with 5% CO<sub>2</sub> for 4 hours, and then the wells are supplemented with 40µl FBS. 48hours later, cell samples are lysed with TRIreagent and DICER expression is checked by qPCR to confirm that the transfection was efficient. Transfection efficiency was also confirmed by transfection of the cells with a fluorescent marker(here Cy3) and it was confirmed by FACS that |90% of the cells were fluorescent. The controls used were: a) cells without any transfection reagents added, b) cells with only Lipofectamine added, c) cells with Lipofectamine and scramble siRNA added and finally d) cells with lipofectamine and Dicer siRNA added in their sera.

## 2.13. IN VITRO TREATMENTS

### 2.13.1. *In vitro* Dendritic cell sensitization protocol

#### 2.13.1.1. *Direct application of DNCB*

Immature dendritic cells were counted and suspended at a concentration of  $1 \times 10^6$  cells/mL in fresh RPMI supplemented with 10% FBS. For the purposes of this model the contact sensitizer DNCB was employed as its use has been established in Prof Healy's group by Peter Friedmann and it is known to sensitize 100% of the population. As this chemical is not environmentally available, it was assumed all volunteers were naïve to it. Other chemicals were not considered at this point. Separate experiments with PPD were conducted but they were not included in this thesis. DNCB was used at 3µM since this was the concentration of the chemical that allowed a longer viability of the dendritic cells used which was important for microRNA analysis. The viability data belong to Dr Fethi Louafi and Dr Chris Pickard's GSH depletion/ROS formation unpublished project. The cells are plated in a 96U well plate. DMSO, DNCB and LPS are added immediately from solutions that have been prepared before the manipulation of dendritic cells and are kept in room temperature. DMSO is used at final concentration of 0.88µM ( $6.25 \times 10^{-3}\%$  v/v), DNCB at 3µM and LPS at 500ng/mL. DMSO treated dendritic cells served as control samples for DNCB treated dendritic cells and LPS treated cells were compared with media suspended cells. The plate is left in an incubator at 37°C with 5% CO<sub>2</sub> for 6hours. After the completion

of the incubation time, the cells are spun down at 200g for 7min at 4°C and are then lysed with TRIreagent. Samples-if not processed immediately-are stored at -20°C for RNA extraction.

*2.13.1.2. Application of supernatant from DNCB treated primary human HPK cells*

Primary human HPK cells were seeded at  $2 \times 10^5$  cells/mL in 24well plates in keratinocyte specific serum free media. When they reach ~80% confluence the media is replaced with fresh media containing DMSO at final concentration of 0.88μM ( $6.25 \times 10^{-3}\%$  v/v) or DNCB at 3μM or LPS at 500ng/mL. 24hours later the supernatants of the keratinocyte culture are collected. The dendritic cells are counted and suspended in the supernatants (supplemented with 10% FBS) at a concentration of  $1 \times 10^6$  cells/mL. The protocol follows as described in the previous paragraph.

*2.13.1.3. In vitro genotoxic damage of HPK cells/cell lines*

*2.13.1.3.1. UV irradiation*

Primary human HPK cells are seeded at  $2 \times 10^5$  cells/mL in 60x20mm petri-dishes suspended in keratinocyte specific serum free media. When cultures reached ~80% confluence, the cells were washed with room temperature sterile PBS and then instead of media, 1mL of room temperature sterile PBS was added. The petri-dishes were adequately labelled and two of them were wrapped in aluminium foil to be shammed from UV irradiation. The LT12 lamp was turned on 10min prior to the actual cell-irradiation. When the lamp was ready, the cells were left inside the UV irradiation chamber, in a black removable tray. The sham controls were left on the bench, close to the UV irradiation chamber so that they are exposed to similar temperatures as the irradiated samples, since the surface in the culture hoods was always cold. Irradiation exposure was timed, and all cell cultures were washed with fresh sterile PBS in room temperature and supplied with keratinocyte specific serum media. The cells were subsequently cultured in an incubator at 37°C with 5% CO<sub>2</sub>. In case of dose responses, when some samples were ejected from the

UV irradiation chamber earlier than others, they were covered in foil and left on the bench along with control samples. As far as HaCaT and HCT116 cells are concerned, the media used was DMEM+10%FBS+1%L-glu and RPMI+10%FBS+1%L-glu respectively.

#### *2.13.1.3.2. Ionising irradiation*

Cells were culture in petri-dishes as described in the previous method. The cells were supplied with fresh media and the petri-dishes were sealed using medical paper adhesive tape and they were transferred in a cylindrical box. The box was kept on ice and was directly inserted into the Gammacell Irradiator to receive IR radiation (5, 10 and 20Gy). Controls were also kept in a cylindrical box in RT during IR exposure. The cells were all washed with room temperature sterile PBS, supplied with fresh media and cultured overnight in an incubator at 37°C with 5% CO<sub>2</sub> before harvested for further analysis.

#### *2.13.1.3.3. Adriamycin application*

Cells were cultured in 6-well plates and seeded as previously described (2.11.2.1). Adriamycin was stored in -20°C in DMSO at 1mg/1mL. Using serial dilutions, adriamycin was dissolved in full culture media depending on the cell type, at a final concentration of 0.2µg/mL, 0.4µg/mL and 0.8µg/mL in culture for 24h.

### 2.14. LYSING OF CELLS AND TISSUE

#### 2.14.1. Lysis of cells

For RNA extraction: Adherent cells were washed in room temperature sterile PBS and 1mL/10<sup>6</sup>cells of TRIreagent was added to the culture using a filter pipette tip. After vigorous pipetting for 1-2min the lysate was transferred to nuclease free storage tubes and was stored at -80°C until use. Suspended cells were centrifuged at 200g for 5min at 4°C and cell pellet was then lysed with TRIreagent as described in the previous paragraph. For protein extraction: The cell monolayer was washed with room temperature sterile. 200µL/10<sup>6</sup>cells of RIPA buffer was added on the culture. Using a cell scraper, the cells were detached and

subsequently pipetted vigorously to ensure all cell membranes had been disrupted. The cell lysate was transferred to storage tubes and left at -80°C until use.

#### 2.14.2. Lysis of tissue

Skin explants were trimmed and washed in room temperature sterile PBS and were minced using a scalpel blade. The minced tissue was transferred in a nuclease free homogenising tube containing ceramic beads and 1mL of TRIreagent (for 50mg of tissue). The tubes were inserted in a mechanical homogenising instrument for 7-8 cycles of vigorous agitation for 20sec each time. In between cycles, the tubes were cooled on ice. The lysate was sieved through a cell strainer and was transferred into a nuclease free storage tube at -80°C until use.

#### 2.15. RNA EXTRACTION

Cell and tissue lysates were thawed and left at room temperature for 5min. Tissue lysates were centrifuged at 12,000g for 15mins at 4°C in order to discard cell and matrix debris. The supernatants were transferred into fresh nuclease free tubes. 0.2mL of chloroform per 1mL of TRIreagent was added to all lysates. The samples were vortexed for 15seconds and were left to settle for phase separation in room temperature for 5-10min. The samples were centrifuged at 17,000g for 30min at 4°C. The aqueous phase was transferred into fresh tubes with 0.5mL of isopropanol and 5µg of nuclease free glycogen per 1mL of TRIreagent for RNA precipitation. The tubes were vigorously agitated and incubated for 10min at room temperature. The samples were then incubated for 20min in -80°C. This step could be prolonged to an overnight incubation for microRNA extraction, as their quality and quantity is not affected by these conditions (Sanchez-Elsner *et al*, unpublished data). Following RNA precipitation, the samples were immediately centrifuged at 17,000g for 30-60mins at 4°C. (Centrifugation time varied between samples, as RNA pellet was absent.) The supernatants were discarded and the pellet was resuspended with 75% ethanol in distilled water. The samples were incubated on ice for 10-15min for all impurities to dissolve in ethanol. Following pelleting by centrifugation (17,000 for 20mins at 4°C) the supernatants were carefully aspirated and pellet was dissolved in DEPC-dH<sub>2</sub>O by

gentle pipetting. All plastic equipment used was nuclease free. All surfaces were thoroughly cleaned with 70% ethanol before each experiment to avoid contamination. PCR plates were immediately discarded after PCR reaction and never returned to RNA processing area.

## 2.16. RNA QUANTIFICATION

1.5µL of RNA samples was deposited on the pedestals of a Nanodrop 1000 spectrophotometer instrument to quantify RNA concentration. All RNA samples below 10ng/mL were discarded from analysis. RNA quality was determined by 260/280 and 260/230 wavelengths ratios. 260/280 ratio for pure RNA is ~2.0, if this ratio is lower than 1.8 then the sample is contaminated with protein. 260/230 ratio for pure RNA is ~2.0, if this ratio is lower than 1.0 then the sample contains phenol ions, guanidine salts and other ionic contaminants affecting the pH. Altered pH in RNA samples hinders the reverse transcription and PCR reactions. The samples were subjected to another RNA precipitation step in case they were heavily impure and difficult to reproduce.

## 2.17. REVERSE TRANSCRIPTION

### 2.17.1. Total RNA RT

For the reverse transcription of total mRNAs, 500ng of RNA was used per sample. Master mix of deoxyribonucleotides (dNTPs), random primers, multiscribe reverse transcriptase (MSRT), pH buffer, RNase inhibitor and nuclease free H<sub>2</sub>O was prepared according to Applied Biosystems' instructions for High-Capacity cDNA Reverse Transcription Kit. A non-template reverse transcription control sample was taken for every experiment. PCR tubes containing adequate amounts of master mix and RNA were centrifuged at 200g for 5min at 4°C and then placed in a thermal cycler in the following program:

**Table 2-2 mRNA reverse transcription temperature conditions**

	Step 1 (HOLD)	Step 2 (HOLD)	Step3 (HOLD)	Step4 (HOLD)
Temperature(°C)	25	37	85	4
Time	10min	120min	5sec	∞

The samples could be stored at -20°C or used immediately for the preparation of qPCR reaction.

### 2.17.2. microRNA RT

microRNAs were reversely transcribed in a different manner than classical total cDNA in the case of individual assays (total microRNA reverse transcription used for microRNA arrays is described in the next section). So for instance, if 4 microRNAs were to be quantified in one sample, then 4 reverse transcription reaction tubes would be prepared. A specific loop primer for the mature form of each microRNA was used in separate reactions. Master mix of deoxyribonucleotides (dNTPs), multiscribe reverse transcriptase (MSRT), pH buffer, RNase inhibitor and nuclease free H<sub>2</sub>O was prepared according to Applied Biosystems' instructions for microRNA Reverse Transcription and microRNA assay kits. A non-template reverse transcription control sample was taken for every experiment. An adequate amount of master mix and 10ng of RNA was transferred to PCR tubes. The tubes were subsequently centrifuged at 200g for 5min at 4°C and then allocated in a thermal cycler in the following program:

Table 2-3 **microRNA reverse transcription temperature conditions**

	Step 1 (HOLD)	Step 2 (HOLD)	Step3 (HOLD)	Step4 (HOLD)
Temperature(°C)	16	42	85	4
Time	30min	30min	5sec	∞

As before, the reverse transcription products could either be stored at -20°C or used immediately for the qPCR reaction.

### 2.17.3. Megaplex reverse transcription

The following protocols were followed for the simultaneous quantification of 380 microRNAs in one sample utilising a Taqman card qPCR array. The master of this reaction contains specific loop primers for 380 different microRNAs. The mixture of these primers is called a Megaplex™ RT Human Pool A. The procedure is more costly financially and energetically as well as time consuming, so it was only used for the array experiments. There are two workflows available for Megaplex reactions. When RNA amount was

exceeding 350ng, only the reverse transcription reaction was needed, while when RNA amount was below 350ng a pre-amplification reaction preceded the reverse transcription reaction.

#### 2.17.4. Megaplex reverse transcription without pre-amplification

Over 350ng of RNA was used per sample for the reverse transcription of 380 microRNAs without pre-amplification. Master mix of deoxyribonucleotides (dNTPs), Megaplex pool A, multiscribe reverse transcriptase (MSRT), pH buffer, RNase inhibitor, MgCl<sub>2</sub> and nuclease free H<sub>2</sub>O was prepared according to Applied Biosystems' instructions of the Megaplex pool protocol. The samples were centrifuged at 200g for 5min at 4°C. Reverse transcription reaction was performed in an Applied Biosystems instrument (7900HT Fast Real-Time PCR System) as described in the table below:

Table 2-4 **Megaplex reverse transcription temperature conditions**

Stage	Temperature(°C)	Time
Cycle (40 cycles)	16	2min
	42	1min
	50	1sec
Hold	85	1min
Hold	4	∞

The samples were kept overnight at -20°C and used the next day for the qPCR array.

#### 2.17.5. Megaplex reverse transcription with pre-amplification

Megaplex reverse transcription reaction was carried out as described in the previous section for initial RNA amounts below 350ng. An amplification step precedes the qPCR reaction in this case using the product from the Megaplex reverse transcription reaction, special Megaplex preamp primers and nuclease free water according to the manufacturer's instructions. The samples were centrifuged at 200g for 5min at 4°C. Pre-amplification reaction was run in a 7900HT Fast Real-Time PCR System under the conditions described in the table below:

Table 2-5 **Pre-amplification temperature conditions**

Stage	Temperature (°C)	Time
Hold	95	10min
Hold	55	2min
Hold	72	2min
Cycle (12 cycles)	95	15sec
	60	4min
Hold	99.9	10min
Hold	4	∞

The samples were kept overnight at -20°C and used the next day for the qPCR array.

The paired samples in the arrays had to be treated equally, so if one of the samples' RNA quantities was below 350ng, then both samples would be pre-amplified.

## 2.18. QUANTITATIVE PCR

### 2.18.1. Taqman qPCR

Quantitative qPCR was performed using Taqman technology. Taqman technology consists of a forward primer, a reverse primer, a Taqman probe and a DNA polymerase. The chemistry of the Taqman technology is based on the Taqman probe. This probe anneals to a part of the reversely transcribed mRNA or microRNA and is attached to a fluorescent reporter dye and a quencher. The quencher does not allow the reporter dye to fluoresce, unless the DNA polymerase cleaves it during the annealing process. So whenever an amplicon of the gene of interest is copied, a fluorescence signal is recorded. Background fluorescence is ignored and a threshold is set, over which the gene's fluorescence is recorded. The cycle of the qPCR procedure where this signal is recorded is called a  $C_T$  cycle and it represents the amount of the mRNA or microRNA assessed in the original sample.



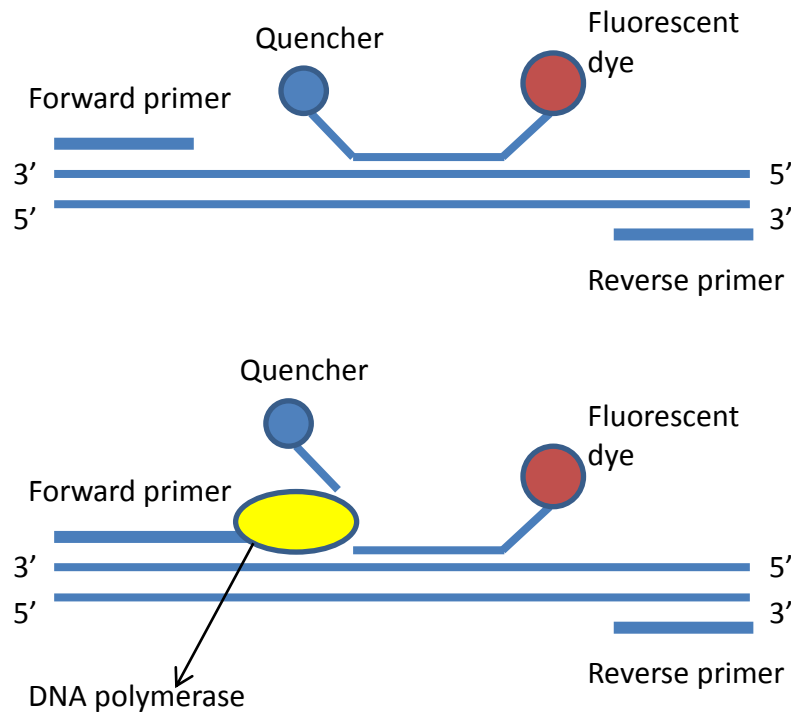


Figure 2-3 **Chemistry overview of the Taqman technology.** The DNA polymerase cleaves only the probes annealed to the target gene, allowing fluorescence to be emitted every time a new copy is generated.

The samples were prepared according to Applied Biosystems protocol of Taqman universal PCR no UNG master mix. The plates were sealed with adhesive tape, centrifuged at 200g for 2minutes at 4°C before the qPCR reaction in the 7900HT Fast Real-Time PCR System. The conditions are described in the table below:

Table 2-6 **Taqman qPCR reaction temperature conditions**

Step	DNA polymerase activation	PCR	
		Cycle (40 cycles)	
		Denature	Anneal/Extend
Temperature(°C)	95	95	60
Time	10min	15sec	1min

Data is collected as  $C_T$  values; every  $C_T$  value represents the number of cycles required for the specific signal to cross the background threshold of fluorescence. Data is plotted as relative (or fold) expressions of the gene of interest:

$$\Delta C_T = C_{T \text{ target gene}} - C_{T \text{ reference gene}}$$

$$\Delta \Delta C_T = \Delta C_{T \text{ test sample}} - \Delta C_{T \text{ calibrator sample}}$$

$$\text{Fold difference} = 2^{-\Delta \Delta C_T}$$

FED and AFED are fold expression difference and average FED respectively. It is the difference of fold expression between the control and respective test sample.

#### 2.18.2. Taqman card qPCR array

The Applied Biosystems array card is comprised by 384 microwells that contain a different qPCR primer each. The master mix containing the Megaplex reverse transcription product or the preamplification product were prepared according to the manufacturer's instructions and loaded in the wells of the array card. The cards were centrifuged in a special array holder at 331g for 1min at 4°C before inserted in 7900HT Fast Real-Time PCR System. The protocol followed was a template prepared specially for these arrays from Applied Biosystems (for more information, refer to Applied Biosystems guide PN4399721B).

Array data were collected with SDS 2.0 software and analysed using DataAssist (automatic analysis) and Excel (manual analysis). The criteria for the array data to be approved and analysed were: a) an entire data set can be discarded if the negative control primer for an arthropod microRNA was annealed; b) At least two of the total of 380 microRNAs assessed should be stable between treatments so that they can be used as housekeeping genes. The cards were already supplied with RNU44, RNU48 and RNU6 housekeeping genes' primers but they are not suitable for all tissues and cells, so data is analysed by software DataAssist and other microRNAs can be identified as housekeeping

genes. RNU44 and RNU48 were unaffected by DNCB treatment and were used as housekeeping genes in this study.

The criteria for the microRNAs to be considered were: a)  $C_T$  values must be lower than 32 in all subjects tested (N=4 for both microRNA array projects explained in section 3.4.5). A  $C_T$  value over 32 can represent biologically insignificant numbers of copies of a microRNA in the original sample. The microRNA is still accepted if the low  $C_T$  value is a result of the treatment (a microRNA induction or suppression) consistently throughout all the subjects. b) A microRNA is considered if its FED between control and treated samples is either over 1.5 or lower than 0.5.

## 2.19. PROTEIN SAMPLE PREPARATION

The cell lysates were thawed on ice and left on a hot plate at 100°C for 3min before centrifugation at 13,680g for 20min at 15°C. The supernatants were transferred in fresh storage tubes while pellets were discarded. Protein concentration in cell lysates was determined with the BCA method using Sigma's instructions. Serial dilutions of the unknown solutions and the standard solution consisting of 1.0 mg/ml bovine serum albumin in 0.15 M NaCl with 0.05% sodium azide as a preservative were prepared. All solutions were diluted in RIPA buffer containing Roche's anti-proteases cocktail. Copper(II) sulphate was appropriately diluted in reagent QB and added to the solutions prepared at 1:8 ratio. The assay was left at 37°C for 2h in a 96 well plate. Net absorbance of 562nm was read from a spectrophotometer and unknown samples concentrations were calculated using SoftMAX software that produces a standard curve of OD values and concentration values.

## 2.20. GEL ELECTROPHORESIS AND SEMI-DRY TRANSFER

NuPAGE Novex 10% Bis-Tris 10 well pre-cast gel was fixed into the Invitrogen apparatus according to manufacturer's instructions. 20X MOPS buffer was diluted in milliQ H<sub>2</sub>O to a final dilution of 1X. 500µL of Invitrogen's anti-oxidant agent was added to 220mL of the running buffer. The lower chamber of the apparatus was filled with 600mL of 1X

MOPS while the upper chamber was filled with the buffer containing the anti-oxidant. See Figure 2-4. Invitrogen's loading buffer and reducing agent was added to the appropriate amounts of cell lysates which were then heated to 70°C on a hot plate for 10min. Then the samples were loaded on the gel wells. The gel was run at 200V for 50min. After the electrophoresis completion, the gel was removed from the apparatus and placed between two iBlot transfer stacks and a PVDF membrane, in a semi-dry transfer instrument by Invitrogen. The transfer occurs in 7min. PVDF membrane is collected and directly placed in western blocking solution, while the rest of the consumables are discarded. See Figure 2-5.

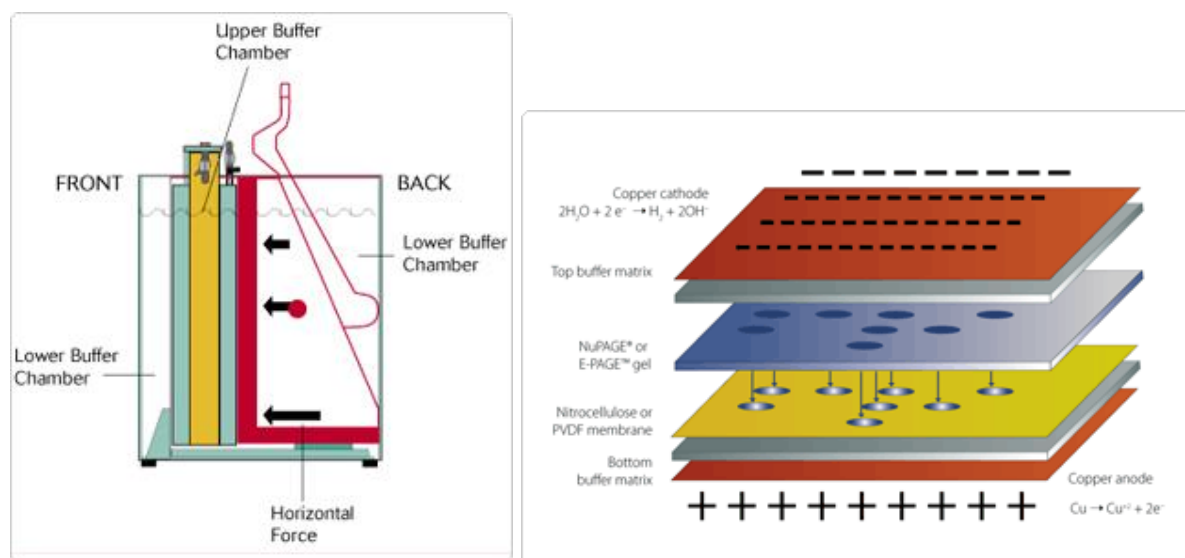


Figure 2-4 **(left) Invitrogen secure-cell electrophoresis apparatus**, consists of a cell divided in two compartments; the upper and lower chamber that don't leak into each other when the pre-cast gel is set and locked. (Picture taken from [www.invitrogen.com](http://www.invitrogen.com)) **(right) Invitrogen iBlot semi-dry transfer scheme**, consists of copper anode and cathode, stack gels (here called matrixes), the NuPage gel and a PVDF membrane. (Picture taken from [www.invitrogen.com](http://www.invitrogen.com))

## 2.21. WESTERN BLOTTING

The PVDF membrane was immersed in western blocking solution where it was incubated at room temperature with gentle agitation. Then the blocking solution was replaced with blocking solution containing anti-SIRT1 antibody at 1:500 ratio or anti- $\alpha$ -tubulin antibody at 1:1200. Membranes were washed 3 times for 5min in PBS+0.1% Tween 20 and then incubated with horseradish peroxidase conjugated anti-rabbit (for anti-SIRT1) or anti-mouse (for anti- $\alpha$ -tubulin) antibodies at 1:8,000 or 1:16000 respectively. The

membrane was laid on a flat surface and ECL plus chemiluminescence solution was applied on it. Afterwards, the membrane was flattened between two transparent plastic sheets and chemiluminescence was recorded using the Versadoc instrument and QuantityOne software. Data are plotted as intensity ratios calculated by the formula below:

$$\text{Intensity ratio} = \frac{\text{target protein intensity (SIRT-1)}}{\text{reference protein intensity (\alpha-tubulin)}}.$$

## 2.22. STATISTICAL ANALYSIS

Dose responses and time courses were analysed using one-way ANOVA test followed by Bonferroni's test. All other data was analysed by non-parametric student's *t*-test in comparison with the appropriate controls. Statistical significance was determined by a *p* value lower than 0.05. All tests were performed using Graphpad Prism 5.0 software.

### 3. The role of microRNAs in Allergic Contact Dermatitis

#### 3.1. INTRODUCTION

As discussed in 1.6.1 section, microRNAs are a family of non-coding RNA transcripts that regulate gene expression in a post-transcriptional manner. They are very important in cell development, differentiation and cell cycle as well as impacting on immune function (Lu *et al*, 2009). Deregulation in microRNA expression or the microRNA biogenesis/silencing machinery can lead to disease. In support of this, there is evidence that *Dicer1*(-/-) mice died during gestation due to impaired development (Dicer is an essential enzyme in the biogenesis of microRNAs) (Bernstein *et al*, 2003; Pauley and Chan, 2008).

ACD is a type IV hypersensitivity reaction. ACD manifests when a benign low molecular weight chemical reacts with epidermal proteins and forms a hapten that perturbs the keratinocyte environment and is phagocytosed by local DCs (mainly LCs in the epidermis and dDCs in the dermis). Keratinocytes secrete pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  that contribute in the maturation of LCs that migrate off the skin to local lymph nodes where they present the antigen to T-cells (Karlberg *et al*, 2007; Saint-Mezard *et al*, 2007; Pickard *et al*, 2007; Friedmann *et al*, 2003). This is the sensitisation phase of ACD. The ability of a chemical to cause DC maturation and migration is thought to be crucial for the initiation of this disease.

Some important microRNAs found in macrophage/DC cell types are: miR-125b, miR-146a and miR-155 (Chen *et al* 2004; Taganov *et al* 2006; Thai *et al* 2007; Rodriguez *et al* 2007; Tili *et al* 2007). Each one has an individual set of actions, but it is not elucidated yet if they are part of the same pathway (Sonkoly *et al* 2008). In addition these microRNAs are involved in Toll Like Receptor signalling pathways, (Akira *et al* 2004; Taganov *et al* 2006; Sonkoly *et al* 2008) and have been shown to be significantly deregulated in inflamed skin tissues in comparison to healthy ones (Sonkoly *et al* 2007). There is evidence that miR-146a is upregulated upon LPS and TNF- $\alpha$  stimuli and is directly activated by NF- $\kappa$ B in THP-1 cells (Taganov *et al* 2006), miR-155 is also upregulated in mature dendritic cells post LPS, TNF- $\alpha$  or poly (I:C) stimulation and it's absence is detrimental for the innate immune responses

and efficient antigen presentation (Rodriguez *et al* 2007; Tili *et al* 2007; Moffett 2007; Martinez-Nunez *et al*, 2009). miR-125b inhibits TNF- $\alpha$  translation and is reported to be suppressed post endotoxin stress in mouse splenocytes and human macrophages (Taganov *et al* 2006; Tili *et al* 2007).

### 3.2. HYPOTHESIS

Given the importance that microRNAs appear to have in inflammatory responses, this project sets out to elucidate the link between ACD and DC microRNA expression upon a hapten challenge. In order to simulate ACD sensitization *ex vivo* and *in vitro*, DNCB was utilized. DNCB (i.e. 2,4 dinitrochlorobenzene) is a compound that is known to sensitize 100% of people exposed and it is not present in the environment naturally, so sensitisation and rechallenge can only occur within the experimental system (Friedmann *et al*, 1983; Roberts *et al*, 1997; Friedmann *et al*, 2007). However, DNCB is used as a treatment for HPV induced warts, alopecia and HIV/AIDS; individuals that have received any type of these treatments were excluded from “naïve”-control groups.

Therefore it was hypothesized that since microRNAs are indispensable in the bridging of innate and adaptive immunity by affecting the maturation of DCs post LPS or TNF- $\alpha$  challenges, then potent allergens that have distinct effects on skin DCs, keratinocytes and subsequently skin tissue will modulate microRNA expression as well.

### 3.3. METHODS

#### 3.3.1. Tissue

Human skin tissue was obtained from redundant skin of mastectomies carried out in Princess Anne’s hospital. The skin tissue was processed and mounted on Franz diffusion chambers as described in section 2.4.1. All subjects have given written consent on the research use of their redundant tissue.

### 3.3.2. Isolation of primary keratinocytes and PBMCs

Primary human keratinocytes (HPK) were extracted as described in section 2.8 from foreskin and mastectomised breast tissue obtained from individuals undergoing surgery at Southampton General Hospital and Princess Anne hospital respectively.

Monocyte derived dendritic cells (MoDCs) were used as an *in vitro* DC model in replacement for LCs/dDCs. They were extracted from fresh blood isolated PBMCs and differentiated into iDCs as described in sections 2.10 and 2.11. The blood derived from volunteers that have consented on the research use of their cells.

### 3.3.3. Candidate microRNA approach

Treated skin, HPK and MoDCs were lysed for RNA extraction and specific reverse transcription for miR-155, -146a and -125b. RNU48 was used for normalisation purposes. (RNU48 is a small nuclear RNA used as an endogenous control in microRNA assays.)

### 3.3.4. Taqman qPCR arrays for microRNAs approach

DNCB or DNCB\* treated MoDCs were lysed for RNA extraction and megaplex reverse transcription reactions followed by Taqman qPCR arrays (described in 2.17.3.1,-2 and 2.18.2). DNCB\* stands for supernatant that has been collected from DNCB treated keratinocyte cultures. DNCB was left in HPK culture medium for 24h and MoDCs were then suspended in it for 6h. Supernatants were supplied with 10% FBS prior to the MoDC treatment. Controls in both cases are DMSO or DMSO\* treated MoDCs.

### 3.3.5. Dicer knockdown approach

MoDCs were transfected with Dicer siRNA as described in section 2.12. Cells were lysed 48h post transfection for Dicer and microRNA analysis.



### 3.3.6. Quantification of microRNAs and Dicer

Tissues and cells were lysed using TRI reagent as described in section 2.16. RNA was isolated, quantified and used in the total RNA reverse transcription reaction for mRNA detection or in the reverse transcription reaction where a specific stem loop primer was employed separately for each microRNA. Quantitative PCR (qPCR), was used to detect the amount of each transcript in the cDNA samples using Taqman technology primers for both mRNA and microRNA cDNA detection.

### 3.3.7. DC maturation markers flow cytometry

DCs were treated with DMSO/DNCB or DMSO\*/DNCB\* for 24h and prepared for flow cytometry as described in section 2.7. Detection of fluorescence was performed by CellQuest Pro and data were analysed in Graphpad Prism 5.0.

### 3.3.8. Statistics

The statistical difference in mean fluorescence intensity or fold expression was determined a) between different treatments using a Wilcoxon's matched paired test and b) in time courses using one-way ANOVA followed by Bonferroni's multiple test. In all cases, a probability value of  $p < 0.05$  was considered significant. All graphs and statistical analyses were prepared and performed using GraphPad Prism 5.0.

## 3.4. RESULTS

### 3.4.1. Validation of the *ex vivo* sensitisation protocol

Previous studies have shown that Langerhans cells' (LCs) migrate from the skin of mice in response to inflammatory signals including the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  accumulating in the local lymph nodes where they are able to present antigens to naïve T-cells (Kripke *et al*, 1990; Cumberbatch *et al*, 2000; Jacob *et al*, 2001; Griffiths *et al*, 2005). Similarly, in vivo studies carried out on human volunteers in this laboratory demonstrated a

significant decrease in LC numbers counted in punch biopsies taken following in vivo application of DNCB (Oakford *et al*, 2011).

To validate the *ex vivo* model of skin inflammation, we set out to determine whether LCs were able to migrate from the epidermis of the mastectomised skin. To this end, 6mm or 1cm diameter biopsies of breast skin was mounted in Franz diffusion chambers and treated topically with 18 $\mu$ g/cm<sup>2</sup> DNCB (the dose of sensitizing 100% of patch tested volunteers as previously described by Friedmann *et al* (Friedmann *et al*, 2007) or vehicle alone (acetone). The chamber placed in a humidified incubator at 37°C for 18h following exposure. The epidermis was disassociated from the dermis as described in section 2.5.2. The isolated epidermal sheets were stained for the LC surface expression marker CD1a and visualised using a fluorescence microscope.

In initial experiments using skin in a 6mm diameter diffusion chambers demonstrated that there was that there was a significant ( $p<0.05$ ) decrease in LC numbers present on the epidermal sheets, suggesting that the chemical treatment had initiated migration (Figure 3-2). At the time, the Franz diffusion chambers that were employed encompassed the skin biopsy in a 6mm diameter area, in which a rubber and an O ring had to be accommodated to support the apparatus, thus leaving a 3mm diameter area for the chemicals to be applied on. A great variability (Figure 3-1) was observed in the numbers of Langerhans cells counted in different fields of the same epidermal sheet. This was attributed to the small sensitization area and the big tension exerted by the O rings to the small biopsies being used. In some controls, the Langerhans cells appeared as activated as in the DNCB treated samples. To address this, the experiment was repeated using 1cm diameter Franz diffusion chambers that gave a 7mm diameter sensitization area.

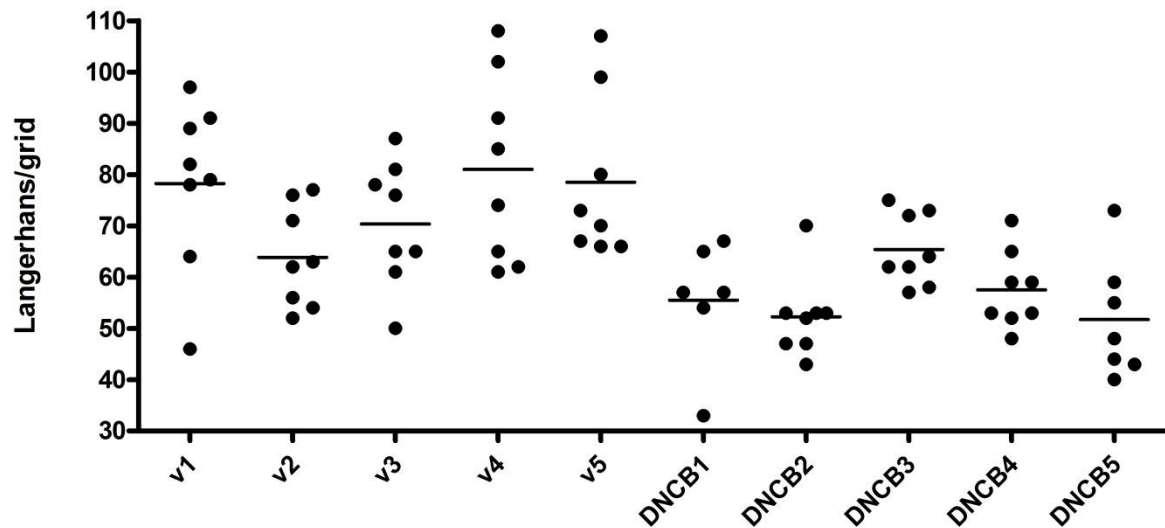


Figure 3-1 **Langerhans cells numbers in skin mounted on 6mm diameter Franz Diffusion Chambers.** Five human skin explants from the same volunteer (HS031) were treated with acetone (Control1-5) and DNCB in acetone (DNCB1-5). LCs from epidermal sheets stained with anti-CD1a were counted. Each dot represents a separate field of view (1grid=62500 $\mu\text{m}^2$ ) from a single epidermal sheet. The bars represent the arithmetic mean.

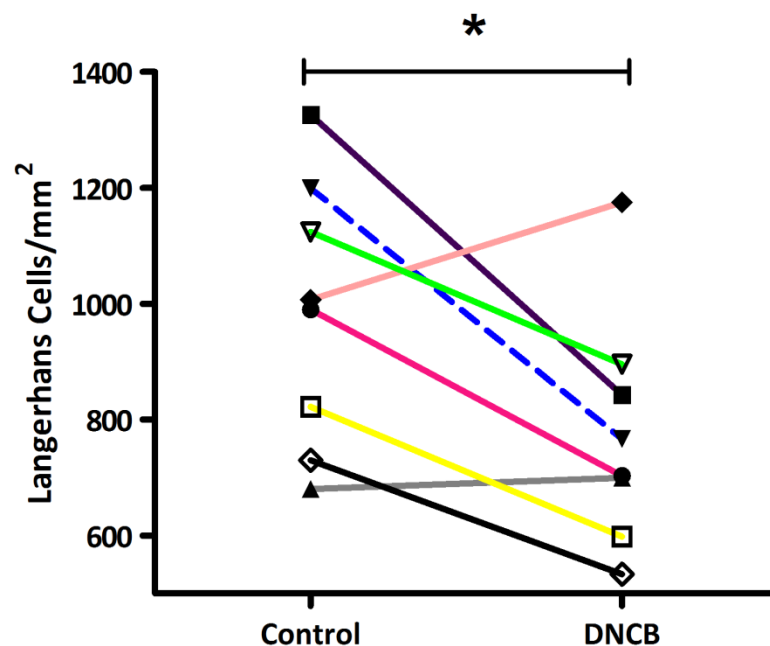


Figure 3-2 **Overview graph of LC migration in DNCB treated skin explants mounted on 6mm diameter Franz diffusion chambers.** Skin from 8 human volunteers was treated with DNCB (18 $\mu\text{g}/\text{cm}^2$ ) and CD1a positive cells were counted on isolated epidermal sheets. Each line represents a different volunteer. Each line represents a different volunteer. Error bars - not visible- (SEM) fluctuate between 26.11 and 9.01. \* $p < 0.05$

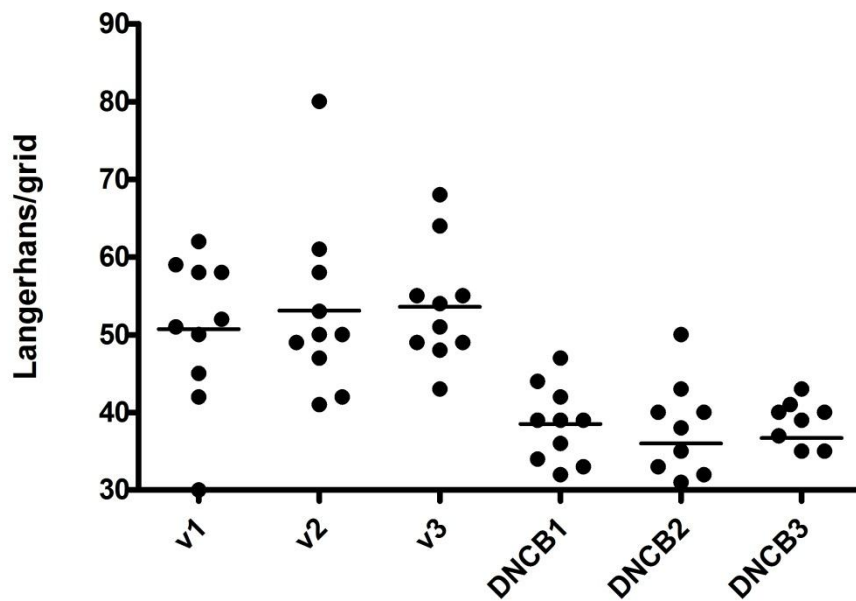


Figure 3-3 **Langerhans cells numbers in skin mounted on 1cm diameter Franz Diffusion Chambers.** Three human skin explants from the same volunteer (HS051) were treated with acetone (Control1-3) and DNCB in acetone (DNCB1-3). LCs from epidermal sheets stained with anti-CD1a were counted. Each dot represents a different field (1grid=62500 $\mu\text{m}^2$ ) on the same epidermal sheet. Lines represent the arithmetic mean.

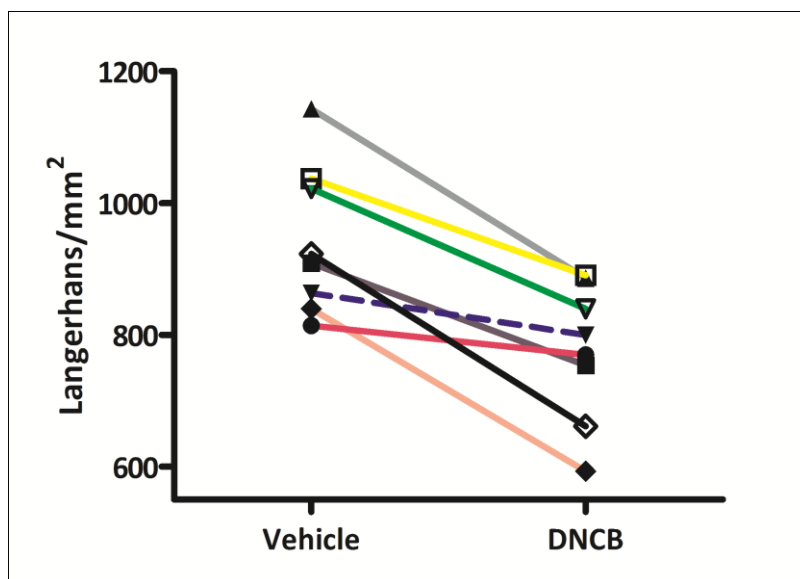


Figure 3-4 **Overview graph of LC migration in DNCB treated skin explants mounted on 1cm diameter Franz diffusion chambers.** Skin from 8 human volunteers was treated with DNCB and LC numbers were counted. Each line represents a different volunteer. LC numbers are consistently depleted in DNCB treated samples. Each dot represents the arithmetic mean. Error bars -not visible- (SEM) fluctuate between 9.16 and 3.33. \*\* $p < 0.01$

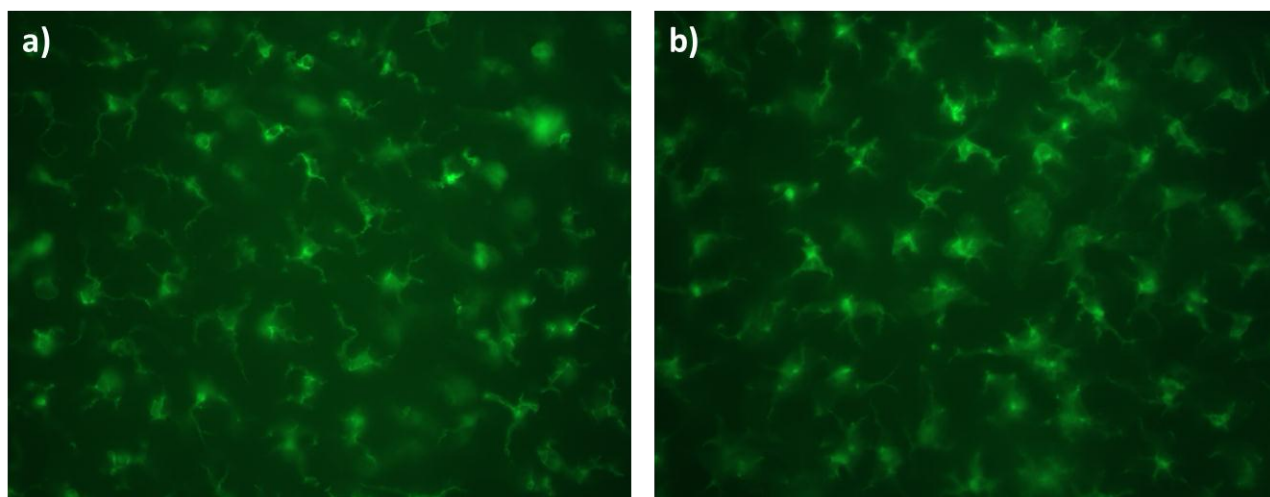


Figure 3-5 **LCs from skin mounted on 1cm Franz diffusion chambers have the same morphology as LCs from *in vivo* human controls.** *Ex vivo* (b) and *in vivo* (a) skin was treated topically with acetone. The epidermises were isolated and stained with anti-CD1a-FITC. LC morphology was examined using fluorescent microscopy.

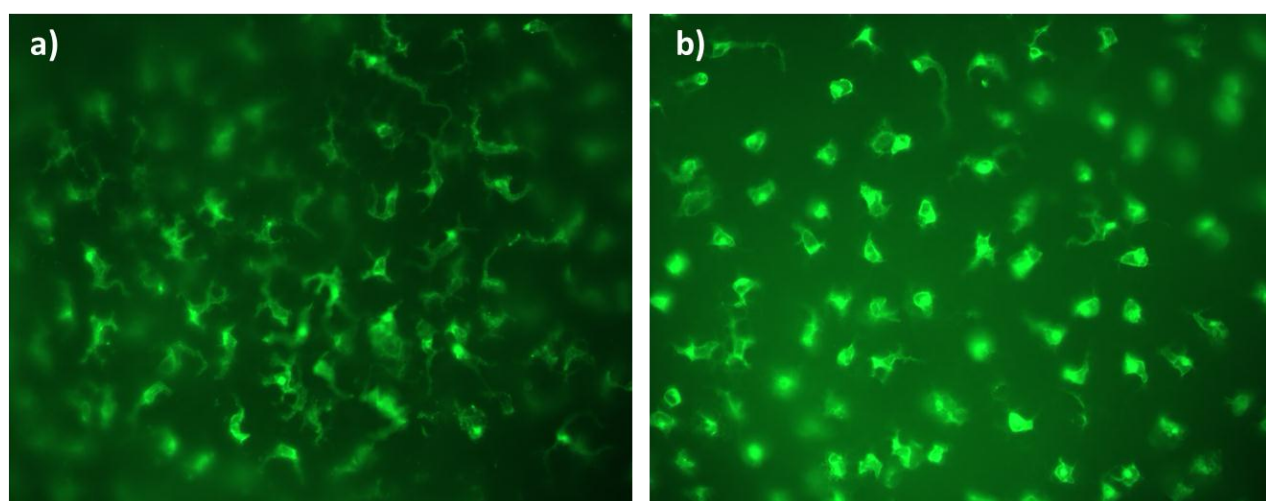


Figure 3-6 **LC migration in the *ex vivo* skin sensitization system.** *Ex vivo* skin was treated topically with acetone (a) and DNCB (b). The epidermises were isolated and stained with anti-CD1a-FITC. These epidermal sheets belong to the same volunteer.

The experiments were repeated with skin tissue from 8 volunteers. The results showed a reduced variability and overall a greater LC migration in response to the chemical sensitizer DNCB ( $P < 0.01$ ) (Figures 3-3 and 3-4). The average number of LCs per  $\text{mm}^2$  epidermal sheet was found to be decreased from 44 to 261 LCs/ $\text{mm}^2$ .

LCs in the steady state in skin have a dendritic morphology with extended dendrites that is changed upon activation into a rounder shape (Asagoe *et al*, 2001; Nishibu *et al*, 2006). The recoil of the dendrites into the cellular shape of the LCs turns the CD1a positive stained cells brighter (Figure 3-6b). To ensure that the *ex vivo* culture conditions do not affect the activation state of the LC, epidermal sheets from skin explants treated with acetone were stained with anti-CD1a-FITC and were compared with anti-CD1a-FITC stained sheets from *in vivo* acetone treated volunteers (biopsies provided by Michelle Oakford). Results depicted in Figure 3-5 show that LCs from skin explants mounted on Franz diffusion chambers have the same morphology as LCs in the steady state in *in vivo* human skin. Langerhans cells show a significantly different morphology in the DNCB treated explants. Their staining intensity is brighter and more concentrated around the nuclei, as they become more rounded and lose their protrusions indicating activation (Figure 3-6).

#### 3.4.2. Modulation of microRNAs in skin tissue

MicroRNA induction kinetics varies depending on the cell type and stimulus, for example miR-395 is significantly induced in *Populus tremula* plants 3h post salt stress (Jia *et al*, 2009) and miR-132 is significantly induced in the hippocampus of mice 45min following neuronal stimulation (Nudelman *et al*, 2010). In order to establish when miR-155 was modulated in human skin explants treated with DNCB a time course was conducted. miR-155 was used in this experiment as it is the most characterised microRNA in DC biology by this group (Martinez-Nunez *et al*, 2009; Louafi *et al*, 2010).

Using the 1cm Franz diffusion chambers, explant skin was treated topically with either acetone or DNCB 18 $\mu$ g/cm<sup>2</sup>. 1h, 6h and 18h following treatment, skin was lysed for RNA extraction and microRNA detection. Most of the dermis was trimmed prior to lysis protocol in order to avoid collagen and other matrix proteins interference with RT and qPCR sensitive reactions. RNU48 was used for normalisation.

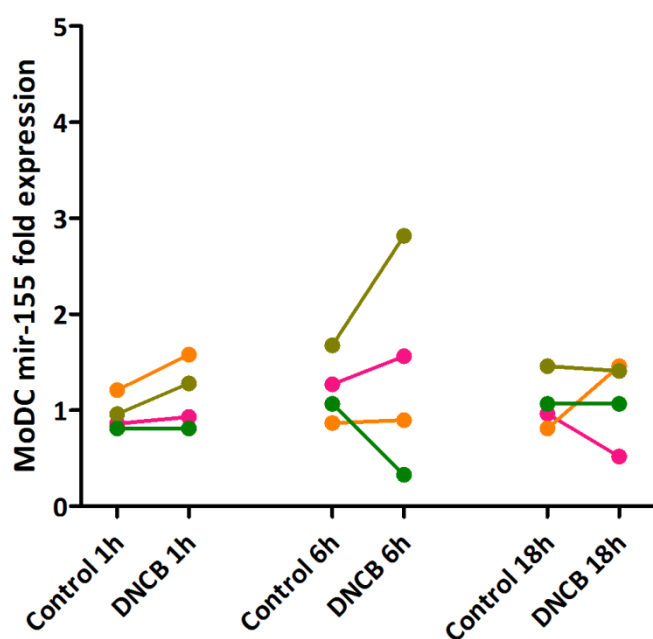


Figure 3-7 **Time course line graph of miR-155 relative expression in DNCB treated skin tissue.** Samples are taken 1, 6 and 18hours post treatment. Expression of miR-155 was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=4 independent experiments.

miR-155 was found to be highly expressed in skin tissues (between 26 and 28 qPCR cycles in acetone treated skin explants) indicating that it must be expressed by most of the epidermal and dermal cell populations and not just the dendrocytes as it was initially hypothesised. LPS couldn't be used as a positive control in this experiment, as miR-155 expression in skin in response to miR-155 is unknown as far as literature is concerned and was found variable in this system (data not shown).

Results from the time course experiment (Figure 3-7) show that miR-155 is not significantly modulated at none of the time points tested by DNCB treatment in skin cells. FED (fold expression difference) of miR-155 1h post DNCB application fluctuated between -0.17 and 0.14 cycles, meaning that the overall expression of miR-155 transcript was not affected. Similarly miR-155 FED in skin explants treated with DNCB for 18h ranged between -0.44 to 0.46. miR-155 FEDs over 0.5 were observed at 6h post DNCB treatment (from -0.74 to 1.14), although they were not significant.

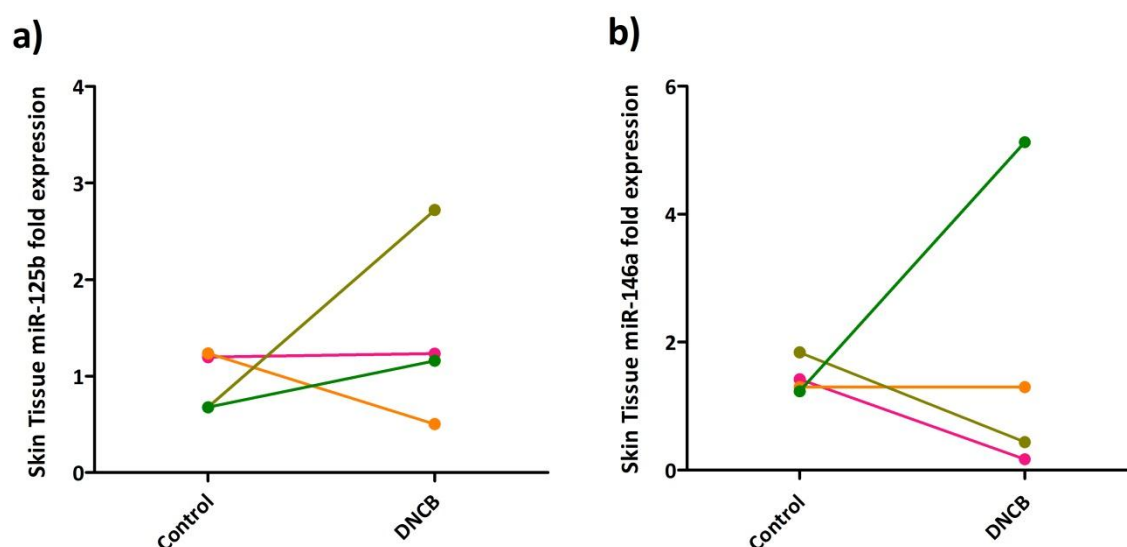


Figure 3-8 **Lack of significant modulation of miR-125b (a) and miR-146a (b) in *ex vivo* treated human skin.** Skin explants were mounted on Franz diffusion chambers and treated with DNFB or acetone for 6h. Expression of microRNAs was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=4 independent experiments. Each line represents a different volunteer. In all  $p > 0.05$ .

The effects of DNFB ( $18\mu\text{g}/\text{cm}^2$ ) treatment of *ex vivo* skin on miR-125b and miR-146a expression were examined. Skin explants were collected and lysed 6h post DNFB treatment. miR-125b expression showed high variability in DNFB treated skin explants (Fold expression  $\text{SD}=1.17$ ) with FED ranging between -0.73 to 2.04. miR-146a was downregulated in 3 out of 4 volunteers DNFB treated skin (FED from -1.4 to 4.01), however its overall expression was found non-significant.

#### 3.4.3. Modulation of microRNAs in primary human keratinocytes

In order to investigate the contribution of keratinocytes to the skin microRNA expression in response to DNFB treatment described in the previous section, HPK were treated *in vitro* with  $3.0\mu\text{M}$  DNFB (in DMSO). HPK were isolated as described in section 2.8 from fresh tissue and were treated with DMSO or DNFB in keratinocyte specific medium. The cells were incubated for 6h at  $37^\circ\text{C}$ , after which time the supernatant was discarded and the cells were lysed for subsequent microRNA analysis.



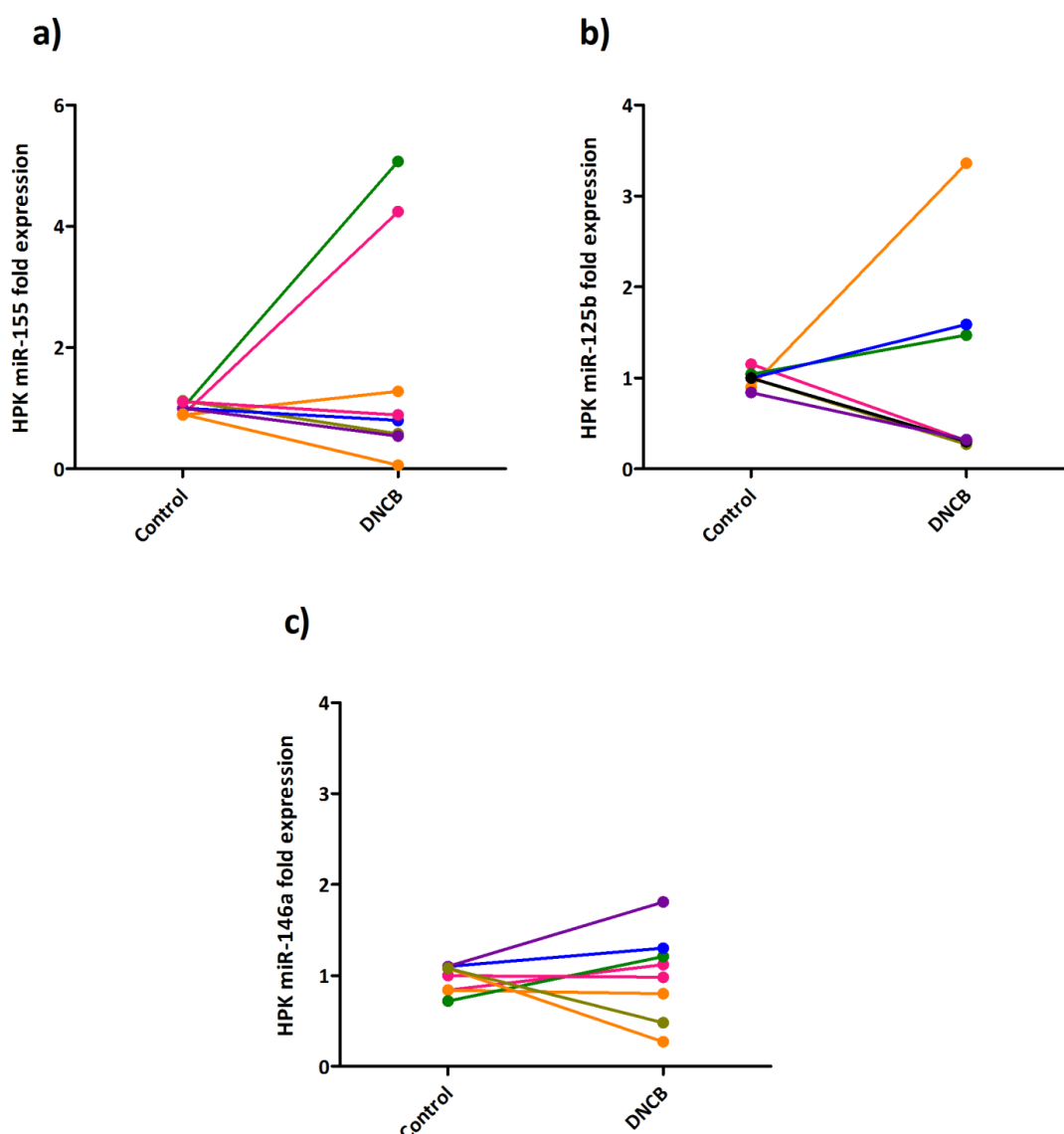


Figure 3-9 **Variable expression of miR-155 (a), miR-125b (b) and miR-146a (c) in DNCB treated HPK.** HPK cells were stimulated with DMSO or DNCB ( $3.0\mu\text{M}$ ) for 6 h. microRNAs' expression was analyzed by qPCR and normalized by using RNU48 levels.  $C_T$  values were determined by SDS 2.0 software. N=8 (a, b), N=6 (c) independent experiments. Each colour represents HPK from a different volunteer.

This experiment proves that miR-155, miR-125b and miR-146a are all constitutively expressed in HPK, with their baseline expressions in DMSO treated samples being between 28-30 qPCR cycles for miR-155, 24-26 qPCR cycles for miR-125b and 26-28 qPCR cycles for miR-146a. Following DNCB treatment there was a decrease in miR-155 expression in 6 out of 8 individuals, however it failed to reach statistical significance (FED from -0.84 to 4.07, SD 1.73). In HPK from two individuals miR-155 expression demonstrated a 5fold increase. Whether there was some underlying condition skewing miR-155 expression in the

volunteers' HPK that exhibited upregulation in response to DNCB remains elusive. Nevertheless if these values are excluded from the overall HPK miR-155 expression post DNCB treatment, the results are significant  $P < 0.05$ . miR-125b shows a variable response in HPK following DNCB treatment (FED from -0.75 to 2.46, SD=1.36). On the other hand, miR-146a tends to either remain around baseline levels or decrease, non-significantly (FED from -0.81 to 0.71, SD=0.50). The variation observed can be attributed to inter-volunteer variation in baseline expression of these microRNAs and their clinical condition that was elusive due to ethics restrictions.

#### 3.4.4. Modulation of microRNAs in MoDCs

##### 3.4.4.1. Candidate microRNAs

To model miR-155, -125b and -146a expression in skin dendritic cells following DNCB treatment, MoDCs were utilised. There is good evidence that MoDC demonstrate dendritic cell function in response to TNF- $\alpha$ , LPS or CD40 and can prime naïve T-cells (Romani *et al*, 1994; Sallusto and Lanzavecchia, 1994; Caux and Bancherau, 1996; Grassi *et al* 1998). In addition they have been suggested as models for prediction of sensitising potency of chemicals since in the presence of strong sensitizers, MoDCs increase their IL-1 $\beta$  secretion and upregulate co-stimulatory molecules (Guironnet *et al*, 2000; Kimber *et al*, 2001).

MoDCs and monocyte derived macrophages have been shown to upregulate miR-155 in the presence of LPS or poly (I:C) 3h post stimulation which culminates at 24h and then plateaus until 48h later (O'Connell *et al*, 2007; Martinez-Nunez *et al*, 2009). Therefore, a time course was conducted in order to investigate how miR-155 responds to DNCB treatment in MoDCs. In parallel, MoDCs were treated with LPS to validate the MoDC model and establish miR-155 expression.

PBMCs were extracted from fresh blood and monocytes were extracted by CD14<sup>+</sup> selection as described in sections 2.9 and 2.10. Monocytes were differentiated *in vitro* into immature DCs with endotoxin free GM-CSF at 1000U/10<sup>6</sup>cells and IL-4 at 500U/10<sup>6</sup>cells (see section

2.11). 6 days after extraction, MoDCs were stimulated with DMSO, 3.0 $\mu$ M DNCB, plain culture media or LPS 250 $\mu$ g/ml at 37°C for 3h, 6h, 24h and 48h.

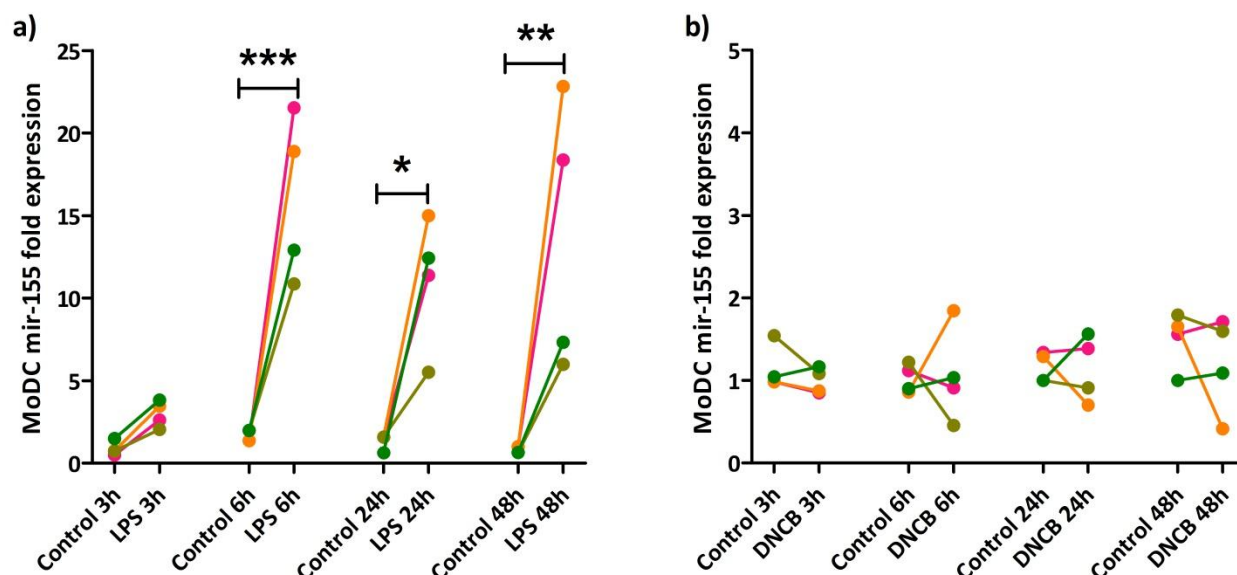


Figure 3-10 **Time course of miR-155 expression in response to LPS (a) and DNCB (b) in MoDCs.** Samples are taken 3h, 6h, 24h and 48h post treatment. Expression of miR-155 was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=4 independent experiments. Each colour represents HPK from a different volunteer. In all \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 compared with its respective untreated group.

LPS induced the transcription of miR-155 in MoDCs in all time points investigated (Figure 3-10a). In particular miR-155 was induced significantly 6 hours post LPS treatment (FED from 8.88 to 20.16,  $p$ <0.001) as well as at 24h (FED from 3.93 to 13.40,  $p$ <0.05) and 48h (FED from 5.35 to 21.82,  $p$ <0.01). This experiment confirms that the MoDC system employed here is responsive to LPS 250 $\mu$ g/ml as predicted by literature in terms of miR-155 expression.

miR-155 expression in DNCB treated MoDCs (Figure 3-10b) remained around baseline levels at 3h (FED from -0.45 to 0.35, SD=0.81) and 24h (FED from -0.68 to 0.56, SD=0.68) post DNCB treatment. DNCB was proved toxic for the MoDCs at 48h as established by trypan blue staining, only 60% of the cells were viable so this time point was discarded from further microRNA analysis. Therefore, the focus was turned to the 6h time point (FED from -

0.98 to 1.02, SD= 0.73), which is widely used for miR-155 and miR-146 research on dendritic cells and macrophages (O'Connell *et al* 2007; Martinez-Nunez *et al* 2009).

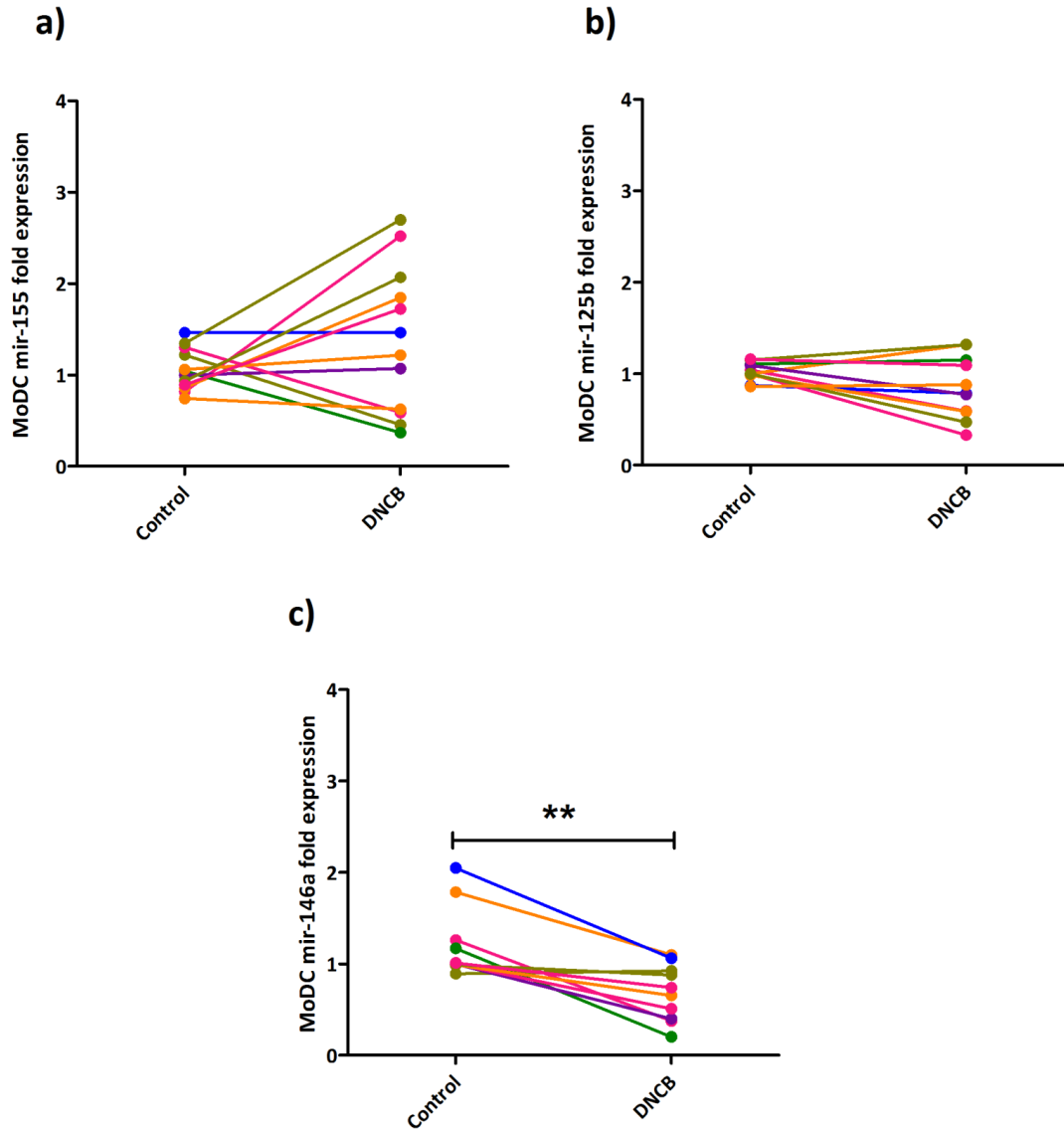


Figure 3-11 **miR-155 (a), miR-125b (b) and miR-146a (c) expression in response to DNCB in MoDCs.** MoDCs were stimulated with DNCB or DMSO for 6 h. microRNAs' expression was analyzed by qPCR and normalized by using RNU48 levels.  $C_T$  values were determined by SDS 2.0 software. N=12 (a) and N=10 (b, c) independent experiments. Each line represents MoDCs from a different volunteer. \*\*p<0.01 compared with DMSO treated samples.

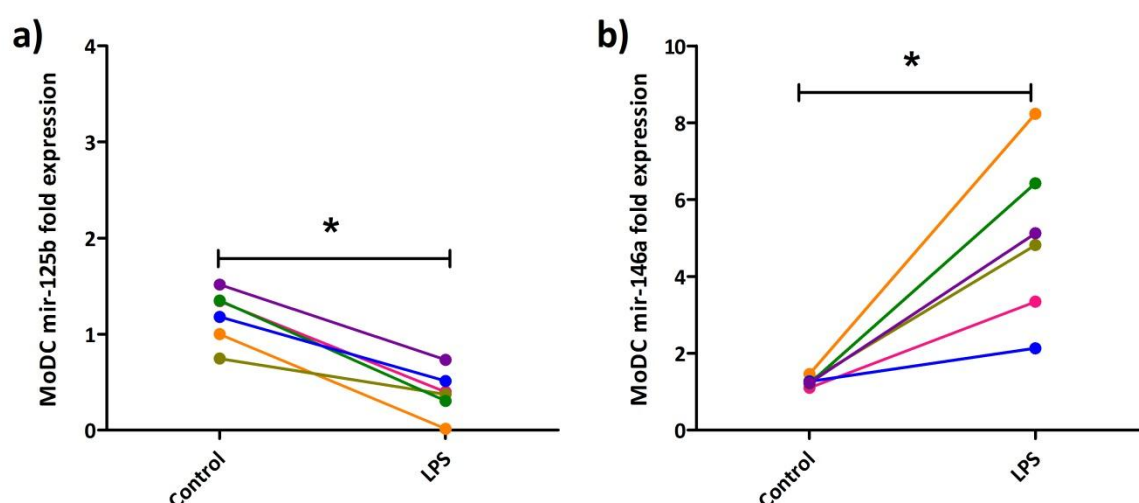


Figure 3-12 **miR-125b (a) and miR-146a (b) expression in response to LPS in MoDCs.**

MoDCs were stimulated with LPS for 6 h. microRNAs' expression was analyzed by qPCR and normalized by using RNU48 levels. CT values were determined by SDS 2.0 software.  $N \geq 6$  independent experiments. Each line represents MoDCs from a different volunteer. In all  $*p < 0.05$  compared with its respective untreated group.

DNCB induced a variable response in miR-155 (FED SD=1.07) so MoDCs from  $N=12$  volunteers was used in order to investigate whether there is a trend in miR-155 expression post DNCB treatment. DNCB suppressed miR-155 in 3 out of 12 individuals (minimum FED -0.76), induced it in 5 out of 12 individuals (maximum FED 3.74) and had no impact on miR-155 expression in 4 out of 12. miR-155 expression appears to be independent to DNCB application in this MoDC model.

In DNCB treated MoDCs, miR-125b either remained unaffected in 6 out of 10 individuals or was slightly suppressed (minimum FED -0.67) in 4 out of 10 individuals. Overall, miR-125b expression post DNCB treatment is not as variable as miR-155 (SD=0.31) but there is a miniscule trend towards downregulation. On the other hand, DNCB significantly suppresses miR-146a transcript ( $p < 0.01$ ). miR-146a is consistently downregulated in 9 out of 10 individuals and it shows no change only in one case (FED from -0.99 to 0.03, SD=0.36).

miR-125b and miR-146a are reported to be suppressed and induced respectively by LPS (Taganov *et al*, 2006), therefore their expression was also quantified in LPS treated MoDCs

in order to establish these transcripts' expression. In the present MoDC system, miR-125b was significantly suppressed (FED from -1.04 to -0.34,  $p < 0.05$ ) and miR-146a was significantly upregulated (FED from 1.86 to 5.21,  $p < 0.05$ ) in response to LPS 250 $\mu$ g/ml stimulation.

As explained in section 1.2.4, skin DCs are in constant communication with local keratinocytes. The environment the DCs reside in the skin tissue affects their responses to various stimuli; contact allergens activate the inflammasomes in keratinocytes resulting in IL-1 $\alpha$  and IL-1 $\beta$  secretion (Yazdi *et al*, 2007; Watanabe *et al*, 2007). IL-1 $\alpha$  and IL-1 $\beta$  activate surrounding keratinocytes and skin DCs, keratinocytes then release TNF- $\alpha$ , IL-6 and GM-CSF that promote inflammation, phagocytosis by macrophages and DCs and skin DC migration (Kupper, 1990; Nickoloff, 2006; Arend *et al*, 2008). LCs have been shown to be quite inert in the absence of keratinocytic signals; the close interaction between the LC and the keratinocyte is indispensable when it comes to immune responses for example CpG was successfully presented by LCs only in the presence of keratinocytes (Asahina and Tamaki, 2006; Sugita *et al*, 2006; Chorro *et al*, 2009). Therefore it was considered essential to investigate the responses of MoDC to supernatants taken from DNCB treated HPK.

HPK were treated with DNCB when they reached ~80% confluence. DNCB was left in culture for 24h. The supernatants were then collected and kept on ice until they were used to re-suspend MoDC. The MoDC cultures were supplied with 10% FBS due to the absence of serum in the HPK media. MoDC were left in HPK supernatant for 6h and then the cells were lysed for RNA extraction and microRNA analysis.

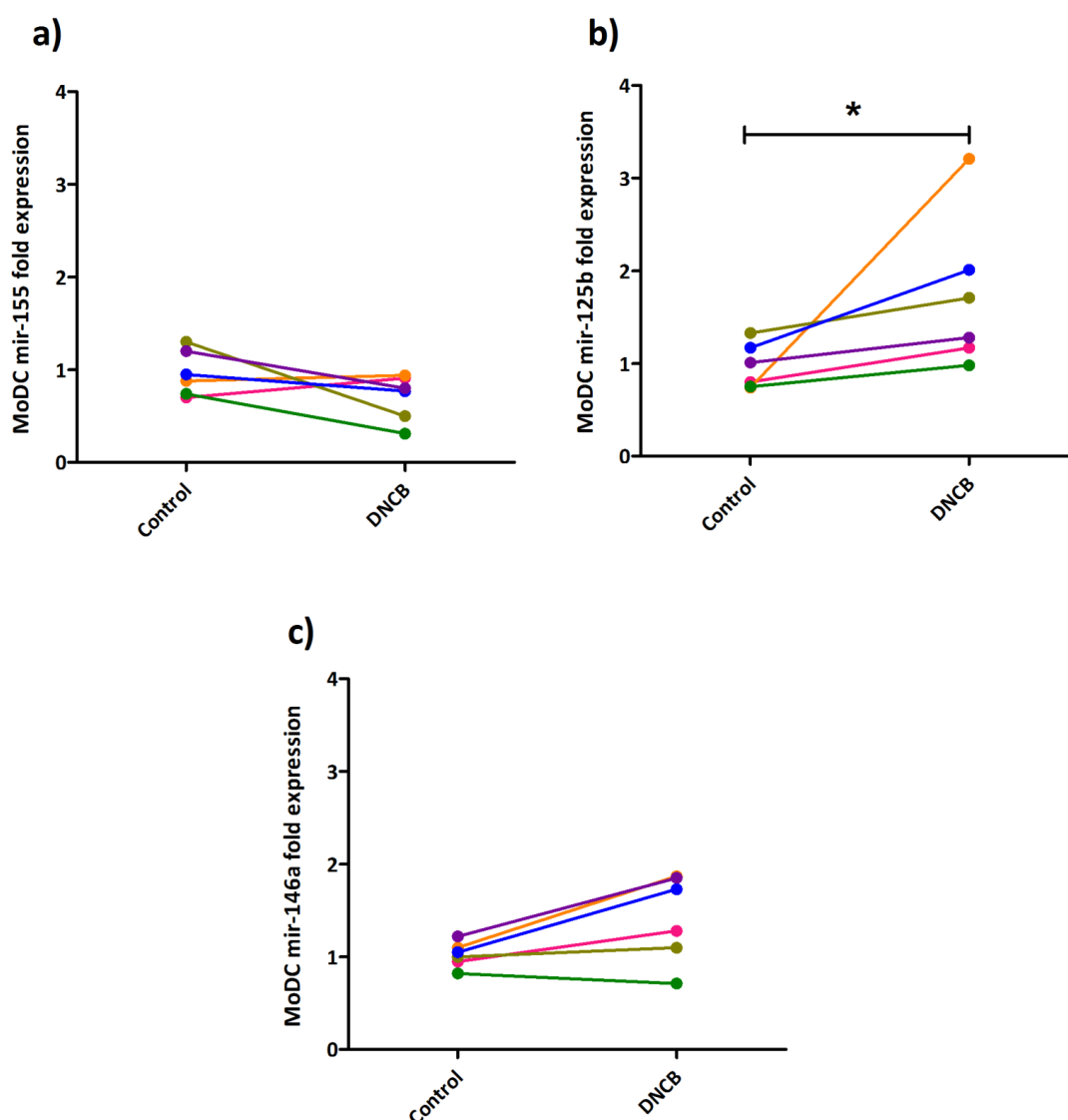


Figure 3-13 **miR-155 (a), miR-125b (b) and miR-146a (c) expression in MoDCs in response to supernatants from DNCB treated HPK (DNCB\*)**. HPK cells were stimulated with DNCB or DMSO for 24h. MoDCs were re-suspended in supernatants and supplied with 10% serum for 6h. MoDCs microRNAs' expression was analyzed by qPCR and normalized by using RNU48 levels.  $C_T$  values were determined by SDS 2.0 software. N=6 (a, b, c) independent experiments. Each line represents MoDCs from a different volunteer. \* $p < 0.05$

As seen in Figure 3-12, DNCB\* (supernatant taken from DNCB treated HPK) treatment changed the trends of all assessed microRNAs in this section compared to DNCB treatment. DNCB\* suppressed miR-155 in 4 out of 6 (minimum FED -0.8) cases and did not affect it in 2 out of 6 cases (maximum FED 0.21). DNCB\* diminished the high variability in miR-155 expression ( $SD^* = 0.37$ ) seen in DNCB treated MoDCs ( $SD = 1.07$ ). Surprisingly, DNCB\*

abrogated the suppressive effect to miR-146a observed in DNCB treated MoDCs; miR-146a expression did not change in 3 out of 6 individuals and was upregulated in the rest of the cases (FED from -0.11 to 0.77, SD=0.28). As seen in Figure 3-11, DNCB caused a decreasing trend to miR-125b in MoDCs, a phenomenon that was abrogated as well with DNCB\* treatment. DNCB\* induced a significant upregulation of miR-125b (FED from 0.37 to 2.47,  $p<0.05$ ).

In order to establish maturation status in the DNCB and DNCB\* treated MoDCs, a flow cytometry protocol was employed for the detection of DC cell surface maturation markers. MoDC were treated with DMSO/DNCB or DMSO\*/DNCB\* for 24h. The cells were harvested and stained as described in section 2.7. CD86 and HLA-DR are DC surface receptors whose expression is increased upon DC maturation (Chaplin, 2006). CD86 and HLA-DR expression was established by flow cytometry. CD1a was used as a positive staining control; however some changes were noted and are included in the following graph. Data were collected by CellQuestPro and analysed in GraphPad Prism 5.0.



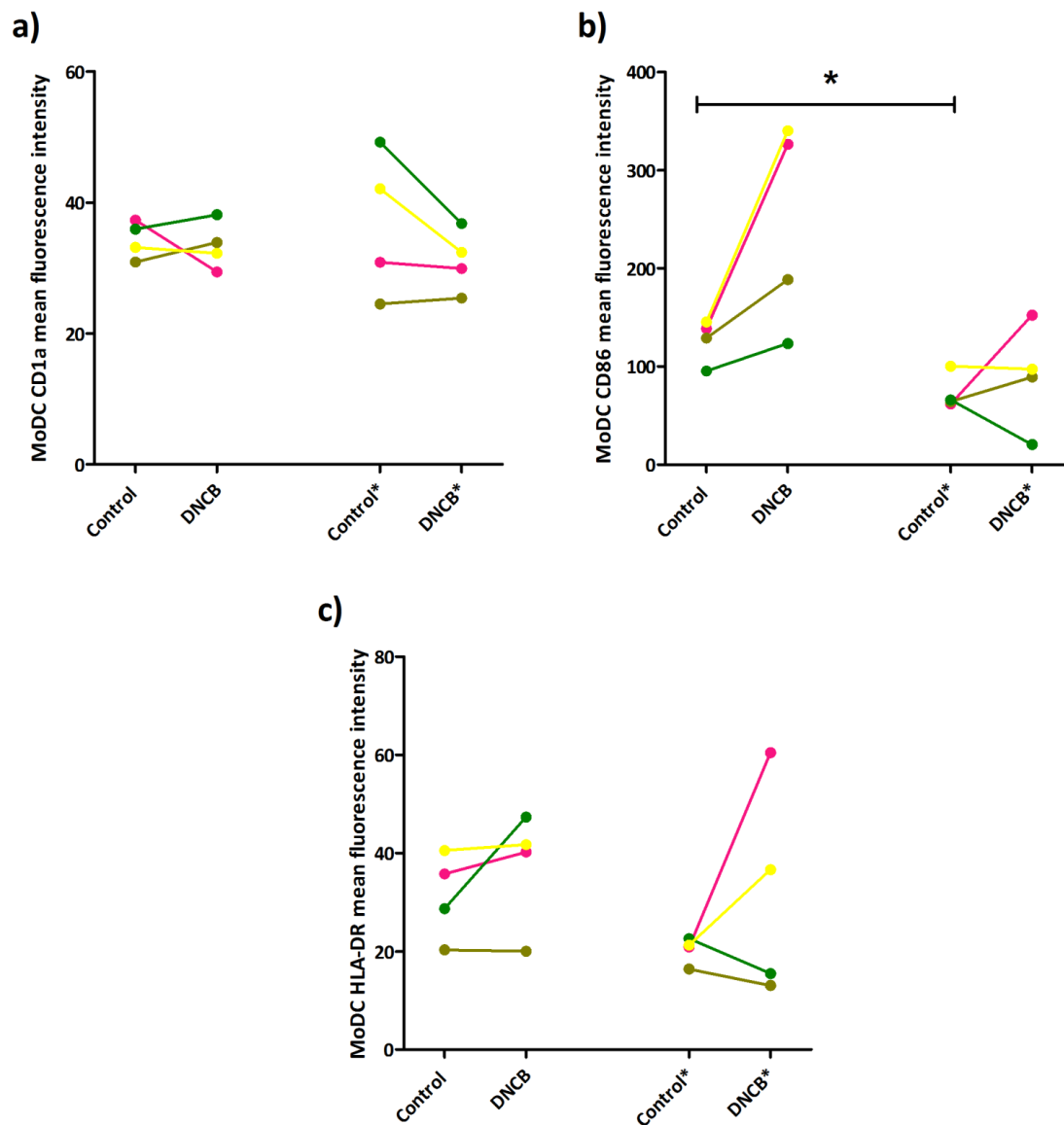


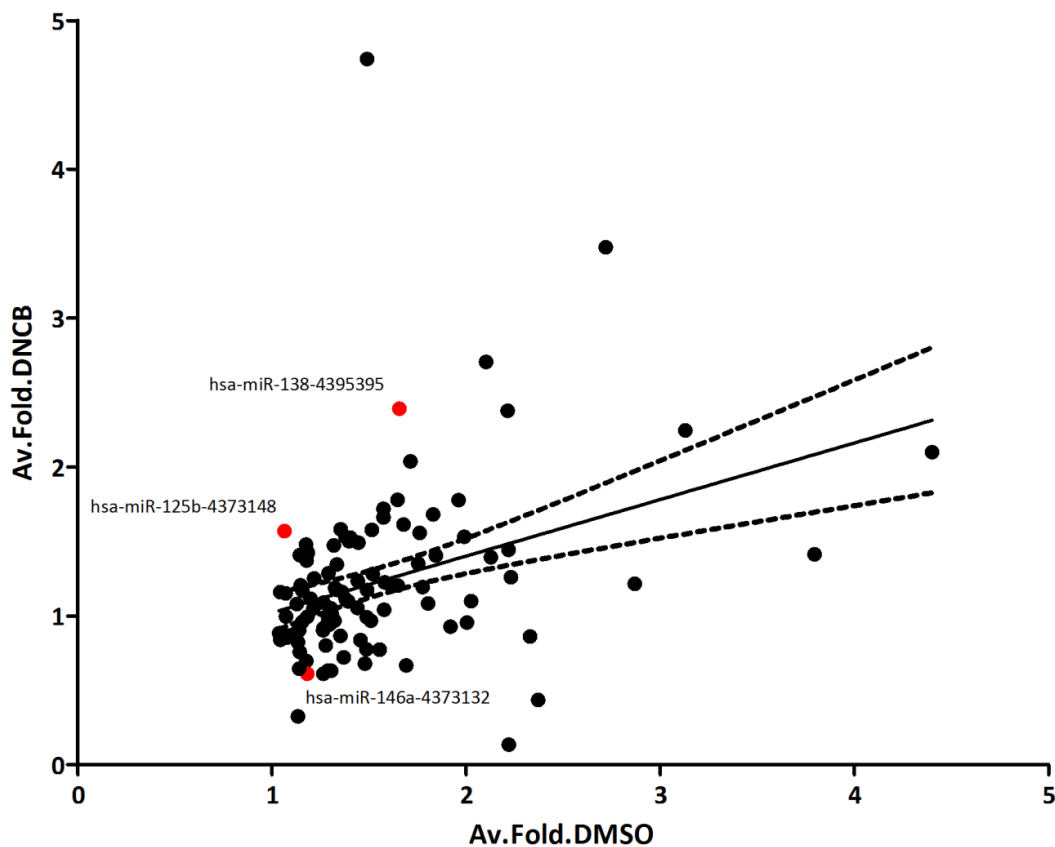
Figure 3-14 **Comparison of CD1a (a), CD86 (b) and HLA-DR (c) expression in DNCB and DNCB\* treated MoDCs.** MoDCs were collected 24hours post DNCB or DNCB\* stimulation. Cell surface expression of DC maturation markers CD1a, CD86 and HLA-DR was detected by flow cytometry. Mean fluorescence intensity of isotype controls was subtracted from the markers detected staining as read by CellQuest Pro software. N=4 independent experiments. Each colour represents a different volunteer. \* $p < 0.05$  as resulted by comparison of Control and Control\* samples in (b).

DNCB caused a variable CD1a expression change in MoDCs; CD1a was upregulated in 2 out of 4 individuals, remained stable in one case and was downregulated in 1 individual (mfi difference from -7.92 to 3,  $SD=4.97$ ). In DNCB\* treated MoDCs however CD1a is downregulated in 3 out of 4 cases (mfi difference from -9.9 to -12.44) and remains

unchanged in one individual. DNCB induced a consistent upregulation of CD86, alas non-significant (mif difference from 27.96 to 194.86, SD=86.21). HPK supernatants significantly suppressed CD86 expression in MoDCs; the average CD86 mfi for DNCB (control) samples was 127.05 and 73.03 in DNCB\* (Control\*) samples ( $p<0.05$ ). In addition, DNCB\* treatment induced a variable CD86 response (mfi difference from -45.77 to 90.41, SD=57.14); CD86 levels were increased in 2 out of 4 individuals and decreased in 1 out of 4. Similarly, HLA-DR shows a non-significant up-regulating tendency (HLA-DR is upregulated in 3 out of 4 individuals) in DNCB treated MoDCs (mif difference from -0.7 to 18.64, SD=8.76) that is replaced by a variable modulation of the maturation markers in DNCB\* treated samples (mfi difference from -7.12 to 39.58).

#### 3.4.5. DNCB\* qPCR microRNA arrays

As mentioned in section 3.2, it was hypothesised that DNCB affects the expression of microRNAs in DCs thus inducing their maturation and consecutive initiation of ACD. Apart from the candidate microRNAs approach described in section 3.4.4.1, another method was utilised. Applied Biosystems TaqMan Low Density Array cards were used following the manufacturer's instructions. 500ng of total cellular RNA was extracted as described in section 2.14.1, was used to cDNA generation. qPCR was run in microfluidic cards containing 384 primers for the most relevant microRNAs for this project. Data were analysed in DataAssist software (automatic analysis) and Excel software (manual analysis) using the  $\Delta\Delta C_T$  method. RNA content was normalised with RNU44 and RNU48 expression. Each microRNA was calibrated independently to the same sample control sample chosen arbitrarily. Results shown in Figures 3.15 and 3.16 were calibrated separately. Blood from 8 volunteers was taken and 16 qPCR array cards were used in total; 4 for each treatment: DMSO, DNCB, DMSO\* and DNCB\*. One volunteer's derived MoDCs corresponds to one DMSO or DMSO\* and one DNCB or DNCB\* treatment.



**Figure 3-15 Quantitative PCR analysis of microRNA expression in MoDCs after DNCB stimulation.** The scatter plot shows averaged (N=4) fold expressions for each probe 6-h DNCB-treated samples. Each dot represents one microRNA probe. The area between the intermittent lines indicates the microRNAs that remained unaffected by the treatments. RNA was isolated with a modified phenol extraction protocol. Reverse transcription was conducted using MegaPlex (Pool A) primers for Taqman array card A. qPCR  $C_T$  values were detected from SDS 2.0 software and analysed in Excel and Graphpad Prism 5.0.

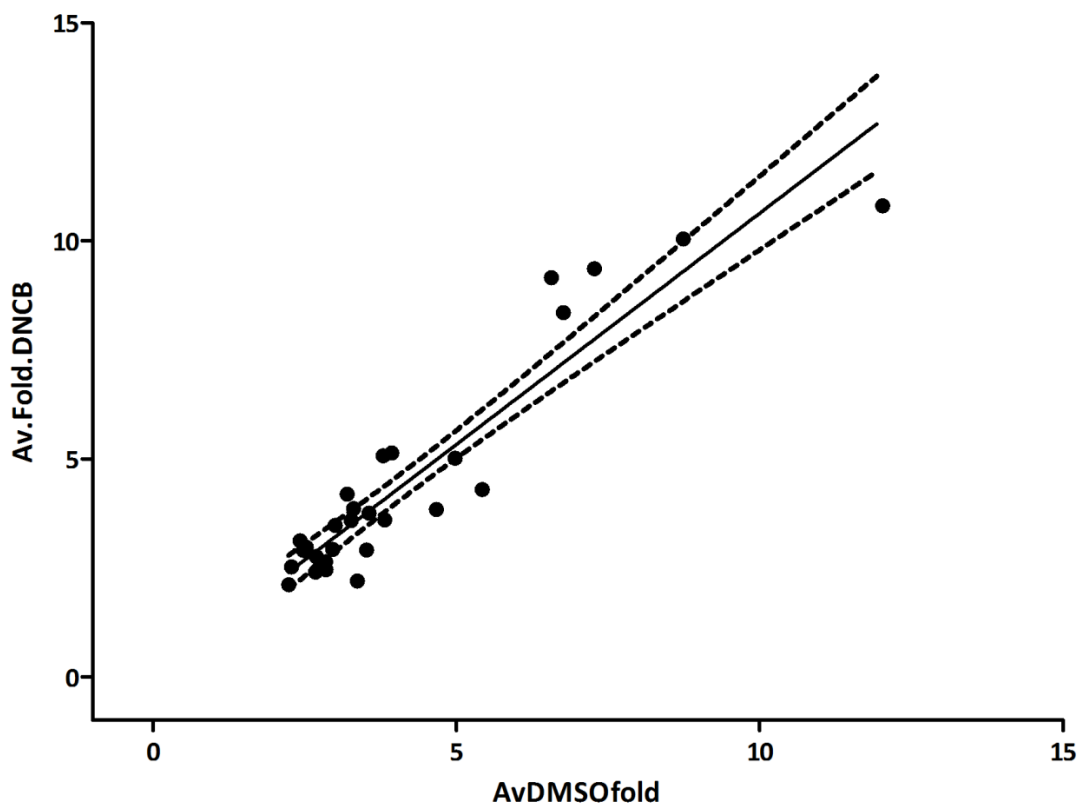


Figure 3-16 **Quantitative PCR analysis of microRNA expression in MoDCs after stimulation with supernatant from DNCB treated HPK.** The scatter plot shows averaged (N=4) fold expressions for each probe 6-h supernatant-treated samples. Each dot represents one microRNA probe. The area between the intermittent lines indicates the microRNAs that remained unaffected by the treatments. Keratinocytes were treated with DNCB or DMSO which was left in culture for 24h. Supernatants were collected and MoDCs were counted and re-suspended in them. Experimental procedures and analysis carried out as described in Graph 3-14.

The criteria of approving microRNAs in order to be included in the analysis are explained in section 2.18.2. Each dot in Figures 3-15 and 3-16 symbolises the average fold difference of a different microRNA. All microRNAs that fall on the black line show no overall modulation in the dataset. The microRNAs that fall under the black line are suppressed while the ones that are above the black line are upregulated. The space between the dotted lines contains microRNAs that have an average fold induction below 1.5. Only the microRNAs depicted in red on Figure 3-14 were found to be consistently modulated towards up- or downregulation in all volunteers' MoDCs assessed. Surprisingly miR-125b was found to be up-regulated in all array samples for DNCB experiments (FED from 0.22 to 1.23) but no modulation of miR-125b was observed in DNCB\* samples, in contradiction to

what's been demonstrated in Figures 3-11 and 3-13. miR-146a was found to be consistently up-regulated in all DNCB array samples (FED from -0.40 to -1.78), but failed to show any modulation in DNCB\* array samples. miR-138 was found to be consistently up-regulated in all DNCB array samples (FED from 0.44 to 5.01). The microRNAs presented as black spots in Graphs 3-15 and 3-16, were subsequently excluded from analysis as each dot represents the average modulation of each microRNA across all arrays which does not mean this modulation was consistent in all paired arrays; for instance the overall modulation of a microRNA might appear upregulated 4 times in DNCB treated samples but in reality it is upregulated in three cases and downregulated in one, and as such was discarded. Specific RT for miR-138 was conducted using the same RNA used for the array experiments (indicated with "valid" suffix in Figure 3-17) as well as with new RNA from 4 different volunteers. miR-138 expression upregulation was validated and was found significant (FED from 1.09 to 2.31,  $p < 0.05$ ).

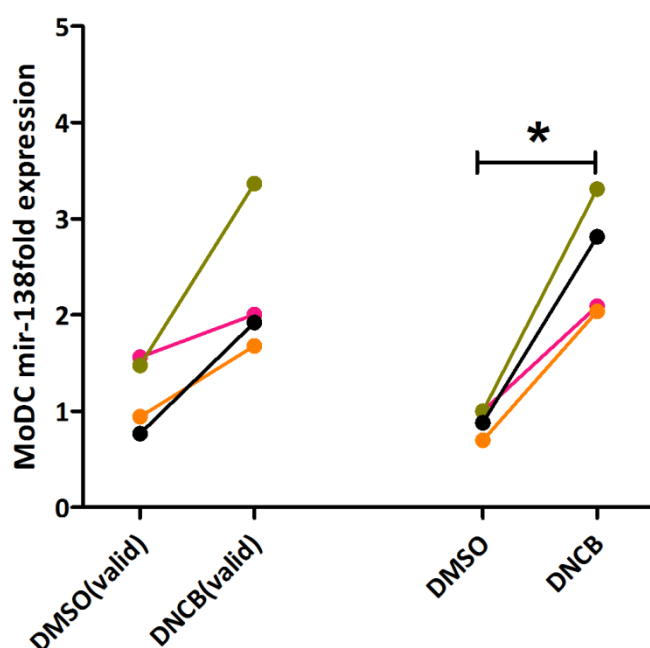


Figure 3-17 **DNCB induces miR-138 expression in MoDCs.** DMSO-DNCB(valid):RNA used for qPCR arrays was checked for miR-138 expression using single assays. DMSO-DNCB:MoDCs from different volunteers were stimulated with DNCB or DMSO for 6 h. miR-138 expression was detected by qPCR and normalized by using RNU48 levels.  $C_T$  values were determined by SDS 2.0 software. N=4 independent experiments. Each colour represents MoDCs from a different volunteer. \* $p < 0.05$  compared with DMSO treated samples.

#### 3.4.5.1. Dicer knockdown

In order to establish whether there is a microRNA element affecting the way MoDCs respond to DNCB, Dicer knockdown experiments were carried out as well. Initially Dicer was effectively knocked down (FED from -0.48 to -1.14,  $p < 0.05$ ). Two different protocols were employed for MoDC transfection, on the left of Figure 3.18 are the results of direct 0.1  $\mu$ l siRNA administration and on the right are the results of lipofectamine mediated delivery of siRNA (5  $\mu$ l of lipofectamine per ml). MoDCs were suspended in plain RPMI and transfection reagents were added. Serum was supplied 4 hours later and samples were collected for RNA extraction 48hours post transfection.

Dicer is an essential enzyme in the biogenesis of microRNAs; it splices the pre-microRNA transcripts in the cytoplasm to generate mature microRNAs before the latter combine with AGO proteins to form RISC complexes (Bernstein *et al*, 2003; Pauley and Chan, 2008). It was

therefore expected that microRNA levels would drop post Dicer silencing by siRNAs.

However, as seen in Figure 3-19 microRNA levels did not alter post Dicer knock down so this approach was abandoned.

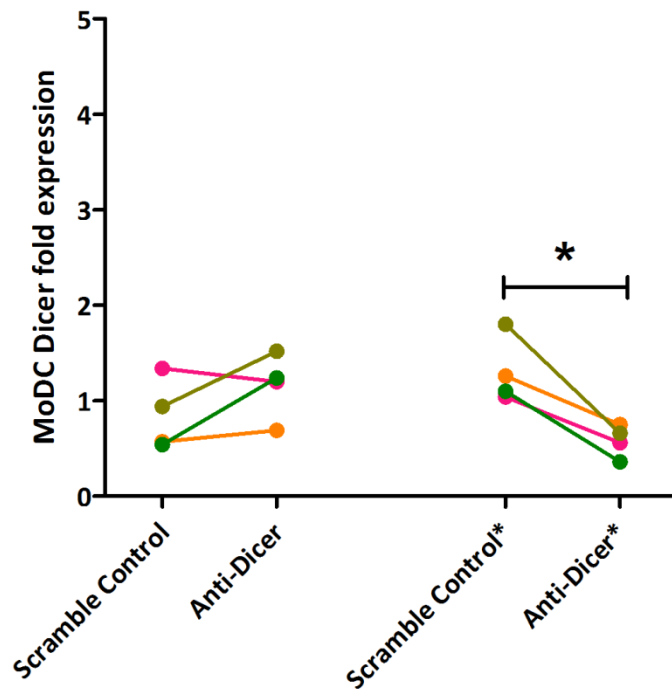


Figure 3-18 **Transfection of MoDCs with anti-Dicer siRNA.** Results on the left demonstrate Dicer expression in MoDCs transfected with scramble or anti-Dicer siRNA without lipofectamine. Samples marked with a star sign show Dicer expression in MoDCs transfected with scramble or anti-Dicer siRNA with lipofectamine. Expression of Dicer was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=4 independent experiments. GAPDH was utilised for normalisation purposes. \* $p < 0.05$  compared with scramble control\* samples.

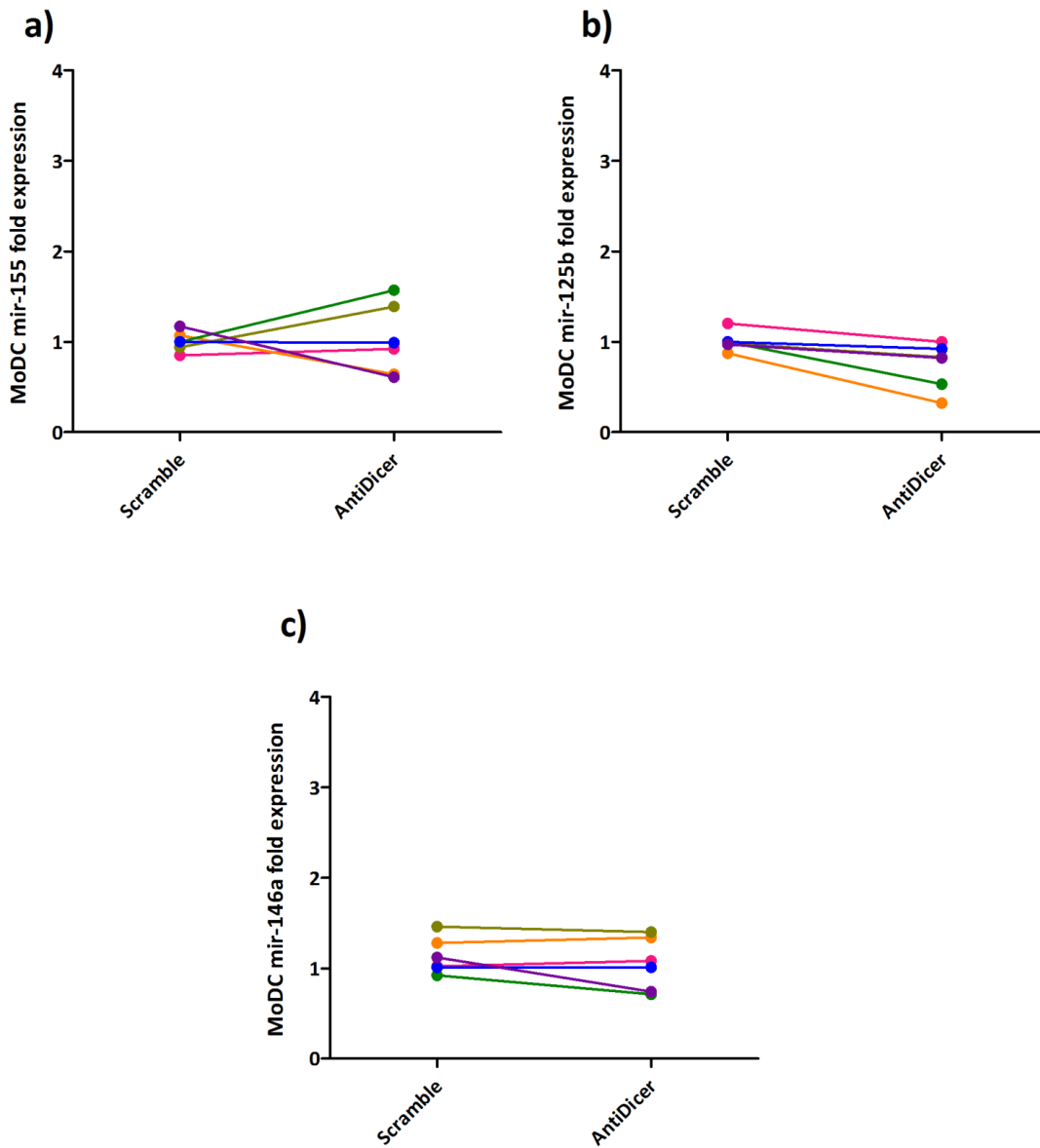


Figure 3-19 **miR-155 (a), miR-125b (b) and miR-146a (c) expression does not change in anti-Dicer transfected MoDCs.** MoDCs were transfected with either scramble siRNA or anti-Dicer siRNA. miR-155, miR-125b and miR-146a expression was investigated post knockdown. microRNAs' expression was analyzed by qPCR and normalized by using RNU48 levels.  $C_T$  values were determined by SDS 2.0 software. N=5 (a,b), N=6 (c) independent experiments. Each colour represents MoDCs from a different volunteer.



### 3.5. DISCUSSION

One of the aims of this study is to establish an *ex vivo* skin sensitization model. The tissue used for this model is redundant human skin tissue from mastectomies. The skin is then mounted on Franz diffusion chambers elaborated by Prof Healy's group. The culture conditions within the Franz diffusion chambers do not affect the LCs that retain the same morphology as steady state LCs (Figure 3-4). Essentially, this is an *ex vivo* model using human skin that closely mimics *in vivo* innate responses to chemical sensitisation.

When an area of skin is exposed to a variety of exogenous agents (infection, allergens), the Langerhans cells at that site will take up and antigens before travelling to the local draining lymph node and maturing to become fully-functional antigen-presenting cells (Udey *et al*, 2001; Janeway, 2005). LC and dDC migration is crucial for the initiation of ACD, since it is these cells that present contact allergens to T-cells in local lymph nodes and initiate an immune response against the allergens (Steven *et al*, 1987; Kissenpfennig *et al*, 2005). LC migration was consistent in all (N-8) volunteers' skin explants tested in response to a DNCB sensitization dose (Figure 3-4). Approximately 20% of the original LC population migrated from the DNCB treated skin explants, similarly to *in vivo* data from mice and humans (Weinlich *et al*, 1998; Cumberbatch *et al*, 2005; Friedmann, 2007; Ouwehand *et al*, 2008). Therefore this system could be used for further study of skin sensitization by contact allergens as it has already been used by this group (Pickard *et al*, 2009).

DNCB failed to induce a discernible pattern of miR-155, miR-125b or miR-146a expression in skin tissue or HPK cells. miR-125b exhibited the most variable expression profile for DNCB treated tissue and cells. A tendency for increased miR-155 expression was observed in DNCB treated skin which was absent in DNCB treated HPK. This could be explained by the fact that the skin is rich in other immune cell types such as memory B- and T-cells and possibly Tregs that are affluent in miR-155 expression in the steady state (Lu and Liston, 2009).

When MoDCs were treated with supernatants from DNCB stimulated HPK, miR-155 levels showed a non-significant tendency towards downregulation apart from one

exception (where miR-155 was upregulated Figure 3-13a). miR-155 upregulation facilitates DC maturation in multiple ways, especially when treated with endotoxins: a) by targeting SOCS1 mRNA. SOCS1 is a transcription factor that negatively regulates the ability of DCs to present antigens in the lymph nodes (Davey *et al*, 2006; Dimitriou *et al*, 2008) b) by targeting many components of TGF- $\beta$  pathway such as SMAD2 and SMAD5 (Louafi *et al*, 2010; Rai *et al*, 2010). The DC is then less susceptible to the immunosuppressive effects of TGF- $\beta$ . c) by decreasing further antigen binding since it silences PU.1 protein that induces a concomitant decrease of cell surface phagocytic receptor DC-SIGN (Martinez-Nunez *et al*, 2009). In addition, miR-155's absence or downregulation favours IL-1 $\beta$  expression and IL-1 $\beta$  downstream pathway as well as caspase-1 (inflammasome component) activation (Ceppi *et al*, 2009). Another target of miR-155 is IL-13R $\alpha$  which is responsible for T<sub>H</sub>1 responses; when miR-155 is knocked down MoDCs promote T<sub>H</sub>2 responses (Martinez-Nunez *et al*, 2011). Therefore this suggests that the MoDCs treated with DNCB\* are less capable of maturing but at the same time very susceptible to IL-1 $\beta$  signalling because of the decreased levels of miR-155.

Because the supernatants used came from DNCB treated HPK cultures, it is assumed that this could be the first part of the keratinocyte-skin DC communication. What is known so far is that through the activation of inflammasomes in keratinocytes, contact allergens induce IL-1 $\alpha$  and IL-1 $\beta$  secretion (Yazdi *et al*, 2007; Watanabe *et al*, 2007). The neighbouring keratinocytes and skin DCs express IL-1 receptors and in the presence of IL-1 $\alpha$  and IL-1 $\beta$ , keratinocytes release TNF- $\alpha$ , IL-6 and GM-CSF that promote inflammation, phagocytosis by macrophages or DCs as well as skin DC migration (Kupper, 1990; Nickoloff, 2006; Arend *et al*, 2008). Also, keratinocytes secrete IL-18 when challenged with pro-inflammatory cytokines or DNCB (Naik *et al*, 1999; Ohta *et al*, 2001). IL-18 induces IFN- $\gamma$  secretion by DCs or keratinocytes (McInnes *et al*, 2000; Koizumi *et al*, 2001). Keratinocyte derived IL-1 $\alpha$  and IL-1 $\beta$  induce production of IFN- $\gamma$  and TNF- $\alpha$  in resident DCs (that induce a new set of cytokine and chemokine release from keratinocytes; IL12, IL-15, IL-18, IL-8 and GRO- $\alpha$ ) (Chu *et al* 1997; Luger *et al* 1997). DNCB has been reported to induce IFN- $\gamma$  production in keratinocytes (Howie *et al*, 1996). IFN- $\gamma$  and IL-1 $\beta$  have been shown to induce miR-155, miR-146a and suppress miR-125b so it can be assumed that the presence of another

mediator in HPK supernatants abrogates these effects (Imaizumi *et al*, 2010; Kutty *et al*, 2010). IL-10 has been reported to abrogate LPS induced miR-155 upregulation in macrophages but also induced miR-146a expression (McCoy *et al*, 2010; Monk *et al*, 2010). Keratinocytes have been shown to secrete IL-10 in the absence of LCs (Enk and Katz, 1992). Perhaps DNCB treated HPK lacking the DNCB challenged DC signals secrete IL-10 because their role in DNCB sensitisation phase is immunomodulatory as IL-10 impairs LC maturation and migration in DNCB treated mouse skin (Cumberbatch *et al*, 2005).

miR-146a exhibits a very interesting expressional pattern in MoDCs treated with DNCB or DNCB\*. When DNCB is applied directly to MoDCs, miR-146a expression is significantly suppressed which has also been confirmed by the qPCR array. By contrast, miR-146a expression was not downregulated when the cells were cultured with the supernatant derived from DNCB treated HPK, instead demonstrating a slight increase in miR-146a expression, however this did not reach statistical significance. miR-146a is directly up-regulated by NF- $\kappa$ B induced by ligands of TLR-2, -4, -5, and it is also induced by TNF- $\alpha$  and IL-1 $\beta$  (Taganov *et al*, 2006). Overexpression of this microRNA in monocytes resulted in low IL-1 $\beta$  production and suppressed concomitant RANTES release (Perry *et al*, 2008). Tang *et al* observed a markedly decreased expression of miR-146a in PBMCS of systemic lupus erythematosus patients; SLE is a disease characterised by high IFN production. It was later proved that miR-146a targets several parts of the IFN pathway including STAT1 and IFN- $\alpha$  and - $\beta$  (Tang *et al*, 2009). Also Sonkoly and Pivarcsi claim that this microRNA is absent in T<sub>H</sub>1 driven diseases (LSE) and overly expressed in T<sub>H</sub>2 diseases (psoriasis) (Sonkoly and Pivarcsi, 2009). Recently, miR-146a has been implicated in endotoxin tolerance. Chassin *et al*, report that miR-146a is an important building block of a dynamic gut epithelial immune tolerance by being constitutively expressed by gut epithelial cells (Chassin *et al*, 2010). Nahid *et al* confirmed the finding in LPS treated THP-1 cells: miR-146a promoted the silencing of IL-1R-associated kinase and TNFR-associated factor6, thus inducing endotoxin induced TLR mediated tolerance (Chassin *et al*, 2011). miR-146a is a part of the innate immune system, orchestrating TLR responses as described above. Without the signals from DNCB treated HPK, DNCB stimulated skin DCs would keep miR-146a levels down thus (extrapolating to an *in vivo* system) releasing uncontrollably TNF- $\alpha$  and IL-1 $\beta$  creating

conditions for extended inflammation and injury. Perhaps the downregulation of this microRNA can be utilised as a marker for contact allergen prediction.

miR-125b is significantly upregulated in DNCB\* treated MoDCs while DNCB induced a slight non-significant downregulation if any modulation at all (Figure 3-11b and 3-13b). In contrast with the results shown in Figure 3-10 (where miR-125b was found either unaffected or downregulated by DNCB), miR-125b was found consistently upregulated in qPCR arrays. However, when the same RNA used for MegaPlex RT reactions was processed with a single RT primer for miR-125b this upregulation was not confirmed (data not shown). miR-125b has been found to be downregulated post endotoxin stress (Tili et al, 2007). Furthermore, Tili et al also confirmed TNF- $\alpha$  as miR-125b's target and postulated that this microRNA is a negative regulator of pro-inflammatory signals. Recently, another target of miR-125b was confirmed:  $\kappa$ B-Ras2 (Murphy et al, 2010).  $\kappa$ B-Ras2 is a negative regulator of NF- $\kappa$ B signalling pathway, thus its silencing enhances the downstream effects of NF- $\kappa$ B. miR-125b has been recognised as a key microRNA in skin inflammatory diseases, as it is abundant in keratinocytes and its downregulation reveals a chronic inflammation phenotype (Sonkoly et al, 2007). Sonkoly also found that miR-125b is poorly expressed in skin suffering from psoriasis or atopic dermatitis. Taken together these findings support the previous hypothesis, that the upregulation of miR-125b induced by DNCB\* suppress the maturation process of MoDCs and render them more susceptible to future danger signals. It is of course of great importance to study the signals deriving from DNCB exposed keratinocytes in detail in order to identify the factor(s) that contribute to the conditioning of the microRNA profile and maturation status of DCs. This studies could entail cytokine arrays or even HPLC analysis of the supernatants in various time points and different DNCB doses to create a full picture of what it is that comprises the keratinocyte response to DNCB.

In summary, the effects of direct DNCB application on MoDCs' miR-155, -125b and 146a expression levels contribute to a convergent inflammatory pattern which includes: increased IL-1 $\beta$  and TNF- $\alpha$  production and an increased susceptibility to NF- $\kappa$ B, IL-13 and IL-1 $\beta$  downstream effects. IL-1 $\beta$  is a key cytokine in LC migration and keratinocyte-LC communication, its up-regulation is crucial in inflammatory responses as it induces

inflammasome activation and chemokine release in keratinocytes (Griffiths *et al*, 2005; Watanabe *et al*, 2007). Therefore it is very important that on the onset of a skin inflammatory response there is enough IL-1 $\beta$  production to establish the presence of dangerous molecules in the epidermis.

DNCB\* had a suppressive effect in the expression levels of CD86 and HLA-DR in control\* samples compared to control samples. Overall there was no significant modulation of CD86 and HLA-DR by DNCB or DNCB\* treatments. However, DNCB application on MoDCs induces a trend towards upregulation for both CD86 and HLA-DR that is lost in DNCB\* samples. As discussed previously, it was expected that HPK supernatants would induce DC maturation through proinflammatory cytokine (e.g. IL-1 $\beta$ , IL-18) secretion but as CD86 and HLA-DR DC maturation markers were not consistently induced in DNCB\* samples, it is thought that there is an immunosuppressive mediator produced by DNCB treated HPK. However, further work is required to establish the cytokine profiles from DNCB treated HPK. CD1a demonstrated a trend towards downregulation post DNCB\* application. CD1a has been shown to be upregulated in MoDCs and THP-1 post DNCB and phospholipase A application respectively that coincided with DC maturation markers upregulation (Aiba *et al*, 1997; Ibeas *et al*, 2009). Taken together these findings confirm the immunosuppressive effect of the DNCB treated HPK supernatants.

Since miR-138 was found to be induced upon DNCB stimulation in MoDCs, it could be hypothesized that DNCB also confers to DC maturation and inflammation induction by inflicting DNA damage which has already been suggested; MoDCs challenged with DNCB exhibited 5% of apoptosis/necrosis 12h post application and caspase-3 upregulation at 48h which proceeded MoDC maturation and effective presentation of DNCB to T-cells (Cesarone *et al*, 1984; Manome *et al*, 1999; Ade *et al*, 2006). miR-138 has not as yet been linked to the immune system. Zhao *et al* only reported miR-138 was deregulated in leukaemia (Zhao *et al*, 2010). However, miR-138 has been recently found to regulate DNA damage response by inhibiting DNA repair and promoting apoptosis (Wang *et al*, 2011). Finally, by examining closely miR-138's targets, it can be assumed that miR-138 upregulation could have an inflammatory effect since both SOCS5 and SOCS6, PPAR-D and GTP binding protein lie amongst the predicted targets as seen in TargetScan, November 2010.

Dicer knockdown experiments did not prove sufficient to eliminate microRNA expression so that it could be evaluated how much impact the RNAi machinery has on MoDC DNCB responses. As predicted by literature Dicer knockdown is not enough to deplete microRNA transcripts in a cell for many reasons: a) there are microRNAs that mature in a Dicer independent way (Yang and Lai, 2010) b) remaining Dicer expression could be sustainable for the continuation of microRNA normal biogenesis c) microRNA half-life in the cytoplasm is longer than it was assumed since microRNAs are very stable molecules and are protected at all times by riboprotein complexes (van Rooij *et al*, 2007; Soukup *et al*, 2009; Kai *et al*, 2010). An alternative approach to this experiment is viral transduction of an anti-Dicer siRNA sequence in MoDCs to achieve longer silencing of Dicer and eventual downregulation of microRNAs.

In conclusion this study depicted three microRNAs that are important in DNCB responses in MoDCs, miR-125b, miR-146a and miR-138. By studying the expression profiles and their participation in various signalling pathways, the pieces to the ACD sensitization phase come together. All of these microRNAs could effectively be used as sensitization potency markers or possibly as therapeutic agents but of course a lot of work remains to be done.

## 4. Skin microRNA responses to UV irradiation

### 4.1. INTRODUCTION

As discussed in Chapter 1, UVR causes DNA damage through direct interaction with DNA molecules or ROS induction via various pathways. UVR interacts with adjacent bases in DNA generating cyclobutane pyrimidine dimers and (6-4) photoproducts (Chadwick *et al*, 1994; Norval, 2001; Trautinger *et al*, 2003). Upon UV-induced DNA damage, p53 protein is activated. p53 protein, is an tumour suppressor protein that promotes cell cycle arrest and DNA repair. Cell fate is dependent on the extent of DNA damage; if the damage can be repaired the cell proliferates or differentiates, if not it is lead to apoptosis predominantly by the actions of p53. However, due to defective DNA repair mechanisms, mutations can be propagated resulting in skin cancers (Hussein *et al*, 2005).

As described in section 1.6.5 of Introduction, p53 directly activates the transcription of microRNA 34A and microRNA 34B/C genes in cells treated with adriamycin, IR and other DNA damaging agents. However, the impact of ultraviolet radiation (UVR) on miR-34 family has not been investigated as yet. Therefore, in order to evaluate the role of the microRNA-34 family in UV irradiated skin and cells, ionising radiation was utilised as a comparative treatment, while adriamycin was utilised as an extra positive control only in the *in vitro* experiments. In addition miR-34a has been proved to target SIRT1 mRNA thus inhibiting its translation in cells overexpressing miR-34a (Yamakuchi *et al*, 2008; Luan *et al*, 2010).

IR has been used for years as a treatment against cancer. Normally a cancer patient is treated with single doses of low radiation over a period of time until the total dose is 45-70Gy depending on the cancer type and patient status (Hall and Giaccia, 2006). Gamma rays have wavelengths of the order of  $10^{-11}$  meters (or 10 picometers). Gamma radiation induces cellular damage by directly reacting with proteins and DNA strands forming free radicals and by consequence reactive oxygen species (ROS) like ultraviolet radiation. However, gamma photons are energetically higher than UV photons and gamma wavelengths have the ability to travel deeper through human tissues (Serway and Jewett, 2000).

Upon IR, p53, NF- $\kappa$ B and AP-1 are activated in human cells but with different mechanisms than ultraviolet radiation (Rieger and Chu, 2004). IR induces p53 via various signal transduction pathways but mainly by causing DNA strand breaks and chromosome aberrations (Lu and Lane, 1993; Hall *et al*, 2006). Cell cycle arrest takes place for DNA repair. Cell fate following IR insult is a) cell death and cell replacement with healthy cells or injury, b) normal DNA repair and cell proliferation, c) incomplete DNA repair, resulting in the generation of new lines of cells that might have minor benign changes or major malignant changes that could lead to the onset of cancer.

Adriamycin (otherwise known as doxorubicin) has been used in cancer chemotherapy since the 1950s and it upregulates p53 by forming DNA adducts (Taatjes *et al*, 1996; Blagosklonny, 2002) that are later recognised by ATR/ATM proteins that initiate a DNA damage response.

## 4.2. HYPOTHESIS

As explained in section 1.5 of Introduction, UVR is a determinant factor for skin biology. From previous work it has been shown that p53 expression in skin and HPK directly correlates in a dose dependent manner with UV irradiation. IR, UVR and adriamycin are known to cause DNA damage lesions and upregulate p53. IR and adriamycin induce the transcription of miR-34a, and decrease the protein levels of SIRT1 through a p53 dependent pathway. The aim of this project is to elucidate the behaviour of miR-34a post UVR exposure in skin tissue and HPK.

## 4.3. METHODS

### 4.3.1. Cells

HPK were extracted as described in chapter 2 from foreskin and breast tissue. The tissue derived from redundant skin from operations that took place in Southampton General Hospital and Princess Anne Hospital respectively. All subjects have given written consent on the research use of their redundant tissue.



Complementary experiments were carried out using the HCT116 cell line and HaCaT cell line. HCT116 is a human epithelial colon carcinoma cell line with a wild type p53 protein, widely used in miR-34 family research. HaCaT is a human keratinocyte cell line that was spontaneously immortalised and express a mutant, non-functional variant of p53 protein (Boukamp *et al*, 1988).

#### 4.3.2. Tissue

Human skin tissue was obtained from redundant skin of mastectomies carried out in Princess Anne Hospital. The skin tissue was processed as described in chapter 2.

#### 4.3.3. Genotoxic damage treatments

Cells and tissue treated with ultraviolet light and ionising radiation (see chapter 2) were washed with PBS and then left overnight in a humidified incubator at 37°C with 5% CO<sub>2</sub>. All samples were collected 24 hours later for p53, miR-34a, p21WAF1 or SIRT1 detection. As explained in Discussion of this chapter, p53 expression peaks 24h post UVR exposure in skin. All samples were collected 24 hours later for p53, miR-34a, p21WAF1 or SIRT1 detection. p21WAF1 is a transcriptional target of p53 protein, used in this project as a marker of p53 activation (El-Deiry *et al*, 1993).

Adriamycin was dissolved in DMSO for the stock solution at 1µg/mL. The chemical was diluted in serial dilutions of RPMI supplemented with 10% FBS and L-glu for HCT116 and keratinocyte specific serum free media for HPK. Adriamycin was left in culture media of cells for 24hours.

#### 4.3.4. p53 protein detection

p53 protein was detected via an indirect flow cytometry method described in chapter 2 in the case of *in vitro* treatments. Data were collected as mean fluorescence intensity of the secondary antibody fluorochromes used by the CellQuestPro software; FITC for UVR samples and AlexaFluor 647 for adriamycin and IR. UV irradiated samples were found to be

emitting light in the emission channel of AlexaFluor 647 so a FITC labelled secondary antibody was used instead. In contrast, Adriamycin and IR treated samples fluoresced in the emission channel of FITC so an AlexaFluor 647 labelled secondary antibody was found adequate. Dead cells were eliminated from the analysis using a dead cell marker (LIVE/DEAD fixable violet stain) as shown in Figure 4.1 below.

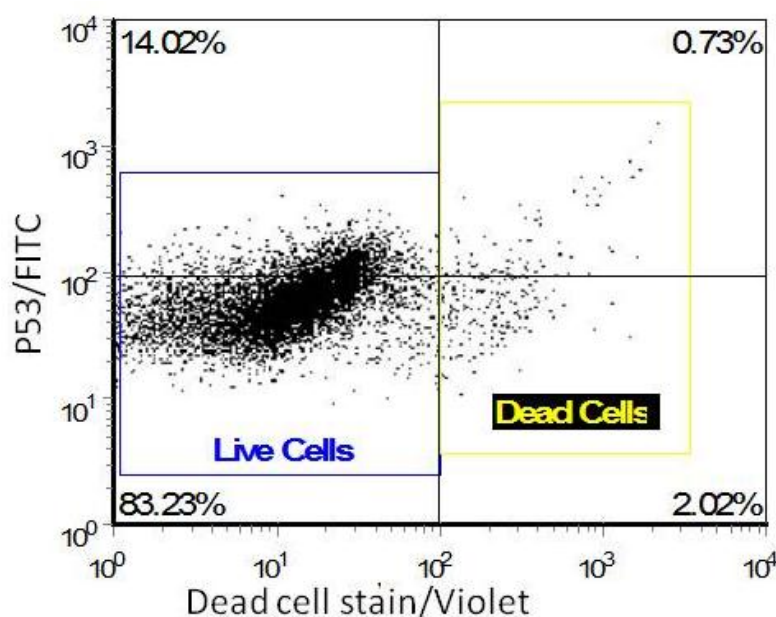


Figure 4-1 **Gating of HPK in flow cytometry.** In this dot plot, upper left quadrant shows live, p53 positive, FITC staining cells while, upper right quadrant shows dead, p53 positive violet staining cells which were excluded from the analysis.

For *ex vivo* experiments, p53 protein was detected via immunohistochemistry as described in chapter 2. The stained epidermal sheets were graded according to staining intensity and percentage of staining cells.

#### 4.3.5. Quantification of miR-34s and p21WAF1

Tissues and cells were lysed using TRI reagent as described in section 2.15. RNA was isolated, quantified and used in the total RNA reverse transcription reaction for mRNA detection or in the reverse transcription reaction where a specific stem loop primer was employed separately for each microRNA. Quantitative PCR (qPCR), was used to detect the

amount of each transcript in the cDNA samples using Taqman technology primers for both mRNA and microRNA cDNA detection. Data are collected as  $C_T$  values; every  $C_T$  value represents the number of cycles required for the specific signal to cross the background threshold of fluorescence. Data are plotted as relative (or fold) expressions of the gene of interest. FED and AFED are fold expression difference and average FED respectively. It is the difference of fold expression between the control and respective test sample.

#### 4.3.6. SIRT1 detection

The cells were lysed using RIPA buffer and acquired protein was quantified and then immunoblotted as described in chapter 2. Data are collected as pixels' intensity using the QuantityOne software.

#### 4.3.7. Statistical analysis

The statistical difference in mean fluorescence intensity, fold expression or intensity ratios was determined a) between different treatments using a Wilcoxon matched-pairs signed rank test and b) in time courses and dose responses using one-way ANOVA followed by Bonferroni's multiple test correction. In all cases, a probability value of  $p < 0.05$  was considered significant. All graphs and statistical analyses were performed using GraphPad Prism software version 5. All different line colours in graphs symbolise independent experiments unless otherwise stated.

### 4.4. RESULTS

#### 4.4.1. Ultraviolet radiation does not induce miR-34a in HPK

Previous reports have shown that p53 is induced by UVR in HPK cells, with the peak expression of p53 at 24 hours post irradiation (Gniadecki *et al*, 1997; Qin *et al*, 2002). So in order to validate the *in vitro* system, HPK were irradiated using an TL12 Philips lamp, with various UVR dosages. The TL12 lamp emits broadband UVR wavelengths (275-375nm) with a peak emission at 310-315nm (UVB region). The cell samples were trypsinised, fixed and

stained with IgG2b and p53 as described in section 2.6.4, 24 hours post treatment. The objective of this experiment was also to identify the UVR dosage required for a significant upregulation of p53 that does not result in increased cell death. As shown in Figure 4.2, the lowest UVR dose for HPK that induces a significant upregulation of p53 protein in HPK cells was found to be 151.68mJ/cm<sup>2</sup>, which is equivalent to 2 minutes under the TL12 lamp.

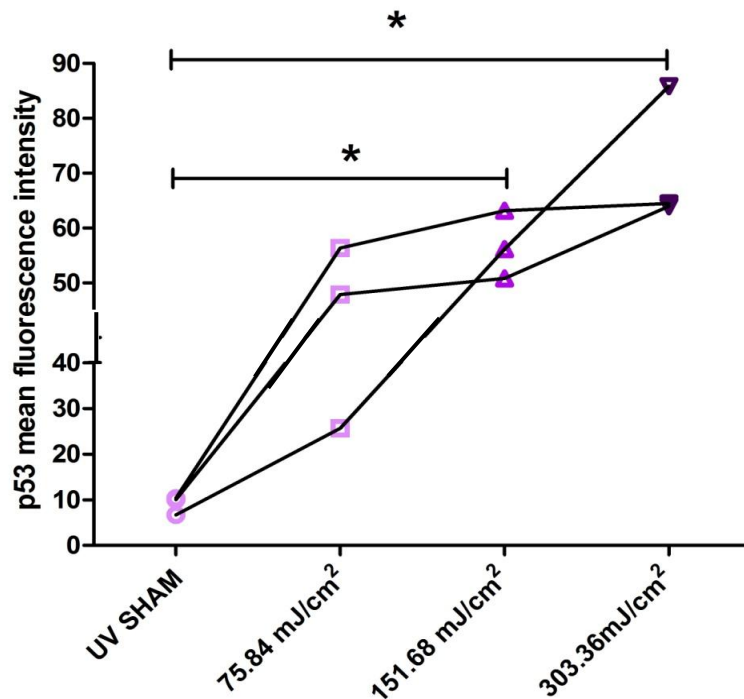


Figure 4-2 **UVR induces a dose-dependent increase of p53 expression in HPK cells.** All samples are taken 24hours post treatment. The cells were collected, fixed and stained with anti-p53/FITC labelled secondary antibodies. Fluorescence intensity was detected by FACScalibur and analyzed by CellQuestPro software. Data are means  $\pm$  SEM. \*p<0.05, compared with UV SHAM control sample.

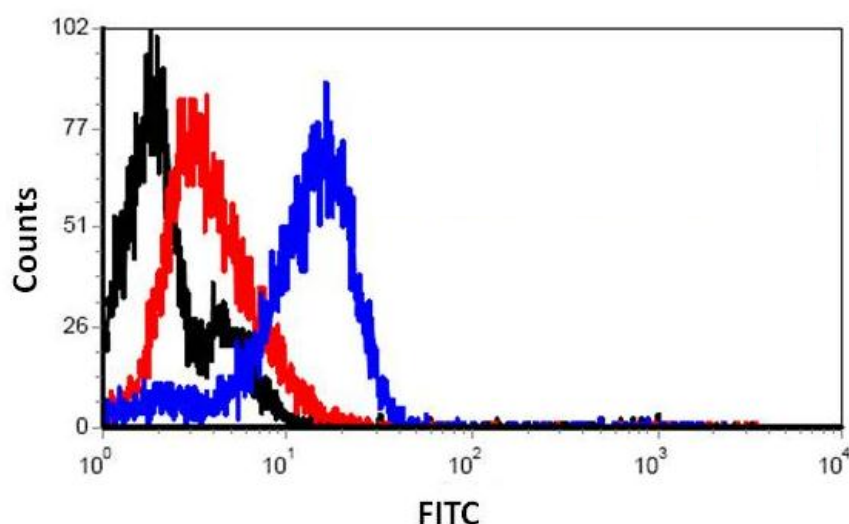


Figure 4-3 **FACS histogram of stained HPK cells.** The black peak shows the background staining of the isotype control for the DO-7 p53 antibody (IgG2b). The red and blue peaks show the recorded FITC fluorescence of p53 stained cells in the control and UV irradiated samples accordingly.

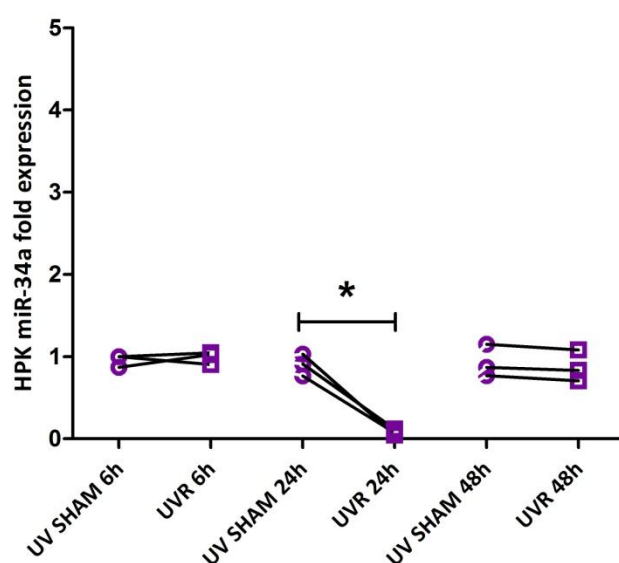
HPK were extracted from foreskin tissue and were cultured in serum free media supplemented with keratinocyte growth factors. These conditions do not allow fibroblast growth in the culture due to the lack of essential nutrients for their growth.. Hence the population of cells treated with UVR and analysed with flow cytometry were purely HPK.

Dead HPK cells were excluded from the p53 positive staining as seen in Figure 4-1. The dead cells do not represent the entire cell population that was killed by the treatment because during cell sample preparation (trypsinisation and wash steps), most of the dead cells were washed away.

151.68mJ/cm<sup>2</sup> that corresponds to 2 minutes of UVR was chosen as the optimal ultraviolet light dose to induce a significant upregulation of p53 protein. 75.84mJ/cm<sup>2</sup> increased the levels of p53 protein in all samples but not significantly. 303.36mJ/cm<sup>2</sup> also induced a significant upregulation of p53 but the cell numbers of these samples were halved 24h post irradiation in comparison to samples irradiated with 151.68mJ/cm<sup>2</sup>, so this dose was not considered suitable for microRNA and mRNA detection experiments since high cell numbers yielded enough RNA amounts for further analysis.

In order to investigate the expression of miR-34a, HPK were treated with 151.68mJ/cm<sup>2</sup> of UVR and they were lysed for miR-34a detection 6, 24 and 48 hours post irradiation in order to identify how miR-34a is modulated by UVR treatment. Results are shown in Figure 4-4.

Previous literature has only reported upregulation of miR-34a in cells treated with either genotoxic chemicals or ionising radiation. There have been no reports on the modulation of miR-34a in mammalian cells by UVR. So it was assumed that since p53 is effectively activated and upregulated in a cell after UVR treatment, miR-34a's expression would concomitantly rise. Quite surprisingly, as Figure 4-4 illustrates, miR-34a remains on baseline levels 6 hours post treatment, it is then significantly downregulated 24 hours later and returns back to baseline levels 2 days after irradiation in HPK cells. Here it has to be noted that all cells came from different volunteers and in all cases the microRNA was downregulated.



**Figure 4-4 Time course line graph of miR-34a expression in response to ultraviolet radiation in HPK cells.** Samples are taken 6, 24 and 48hours post treatment. Expression of miR-34a was detected by qPCR. C<sub>T</sub> values were determined by SDS 2.0 software. N=3 independent experiments.

#### 4.4.2. Adriamycin, ionizing radiation and ultraviolet light modulate miR-34a expression differently in HCT116 and HPK

To further characterise the effect of UV on miR-34a, HPK and HCT116 cells were treated with adriamycin and IR since previous work has shown an upregulation of miR-34a in cells treated with adriamycin (Rokhlin *et al*, 2008; Yamaguchi *et al*, 2008) and IR (Kato *et al*, 2009). p53 protein induction experiments were conducted first to depict the dose of the DNA damaging agent to use in miR-34a quantification experiments.

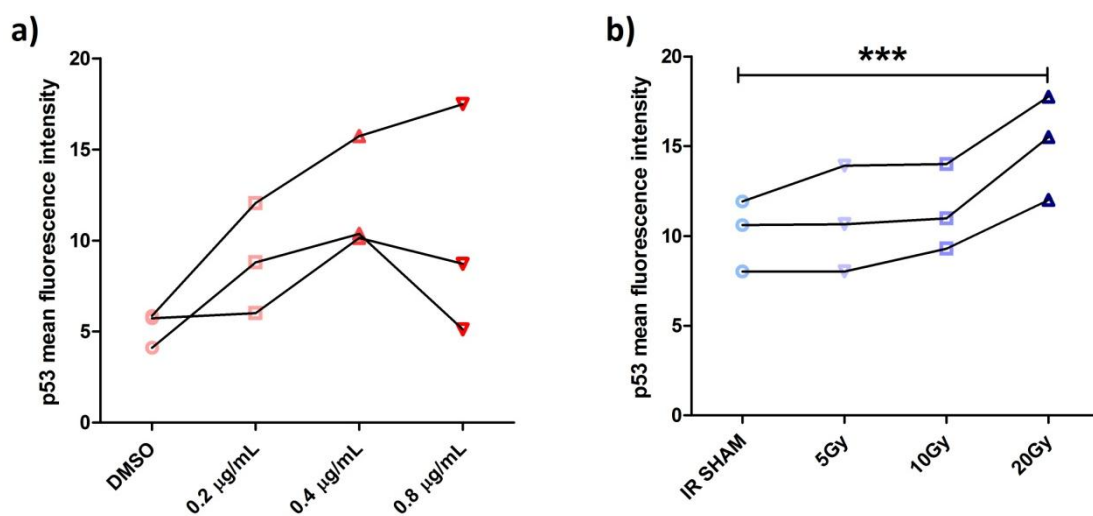
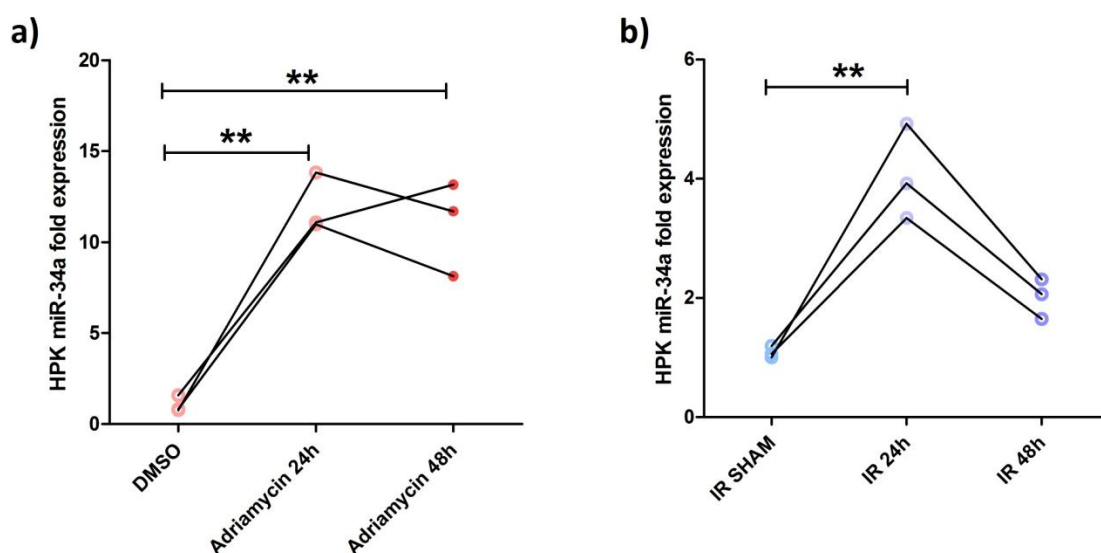


Figure 4-5 **p53 induction in response to (a) adriamycin, (b) ionising radiation in HPK cells.** All samples are taken 24hours post treatment apart from IR irradiated samples that were collected at 48h. In the case of adriamycin the chemical was left in the cells' culture medium for 24hours. The cells were collected, fixed and stained with anti-p53/FITC labelled secondary antibodies, using an indirect staining protocol for flow cytometry. Fluorescence intensity was detected by FACScalibur and analyzed by CellQuestPro software. \*\*\*p<0.001 compared with its respective untreated group. N=3

As seen in Figure 4-5, adriamycin (a) failed to produce a significant upregulation of p53 protein but shows an increasing trend in its expression until the dose of 0.4µg/ml. p53 protein was upregulated significantly 20Gy of IR (b). 5 and 10Gy of IR did not seem to upregulate p53 protein HPK cells. p53 protein was upregulated significantly 48 hours after the cells were treated with 20Gy of gamma radiation. As discussed by Zhao *et al*, p53 responses have different kinetics according to different cell types and different DNA

hazards. In this case, all assessed cell types demonstrate a p53 peak shift when analysed by flow cytometry 48 hours post gamma irradiation. The variation observed in p53 expression was expected since HPK originated from different volunteers and their tolerance to such harsh DNA damaging stimuli would vary.

Cells treated with medium supplied with adriamycin for 24h showed an increase in the detected p53 levels (Figure 4-5a) but not a statistically significant one. However, 0.4µg/mL of adriamycin was chosen for further experiments because in all cases, p53 protein levels were doubled. 0.8µg/mL of adriamycin proved to be toxic when applied to the cells for 48h.



**Figure 4-6 miR-34a induction post a) adriamycin and b) IR treatments in HPK cells.** miR-34a expression was investigated for the chosen doses: 0.4µg/mL for adriamycin and 20Gy for IR at 24h and 48h of treatment. Expression of microRNAs was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. In all  $**p < 0.01$  compared with its respective untreated group. Data are means of qPCR triplicates. N=3 independent experiments.

HPK cells were lysed for RNA extraction and miR-34a detection by qPCR 24 and 48h post IR exposure (20Gy) as well as 24 and 48h of adriamycin application (0.4µg/mL) in order to establish whether the different kinetics in p53 upregulation shown in Figure 4-5, correspond to differences in miR-34a expression too. Figure 4-6 though illustrates a statistically significant direct induction of miR-34a with both treatments in HPK cells



( $p < 0.01$ ) at 24h and then a slight decrease at 48h. miR-34a was found to be inducible by both treatments in HPK cells with a range of FED from 9 to 14 and from 2 to 4 in adriamycin and IR treated samples respectively 24h post exposure to the DNA damaging insult. Therefore, miR-34a transactivation in IR treated cells is immediate and precedes p53 upregulation; implying that small amounts of IR induced activated p53 are enough for a miR-34a response. This finding brings emphasis on the fact that miR-34a's function is possibly crucial during the first 24h post exposure to a DNA damaging stimulus for the cell. So it was decided that all samples for miR-34a detection will be taken at 24h for all treatments (adriamycin, IR, UVR).

In Figures 4-2 and 4-5, p53 levels appear to be strongly more increased in UV treated HPK cells (Figure 4-2), while they are moderately modulated in adriamycin and IR treated cells. So, in order to investigate whether miR-34a suppression was a phenomenon caused by the specific doses of the DNA stimuli (0.4 $\mu$ g/mL of adriamycin, 20Gy of IR and 151.68mJ/cm<sup>2</sup> of UVR) chosen by p53 responses, miR-34a induction dose responses were conducted with 3 doses of adriamycin, IR and UVR. HPK cells from N=3 volunteers were treated with 0.2 $\mu$ g/mL, 0.4 $\mu$ g/mL and 0.8 $\mu$ g/mL of adriamycin, 5Gy, 10Gy and 20Gy of IR and 75.84mJ/cm<sup>2</sup>, 151.68mJ/cm<sup>2</sup> and 303.36mJ/cm<sup>2</sup> of UVR.

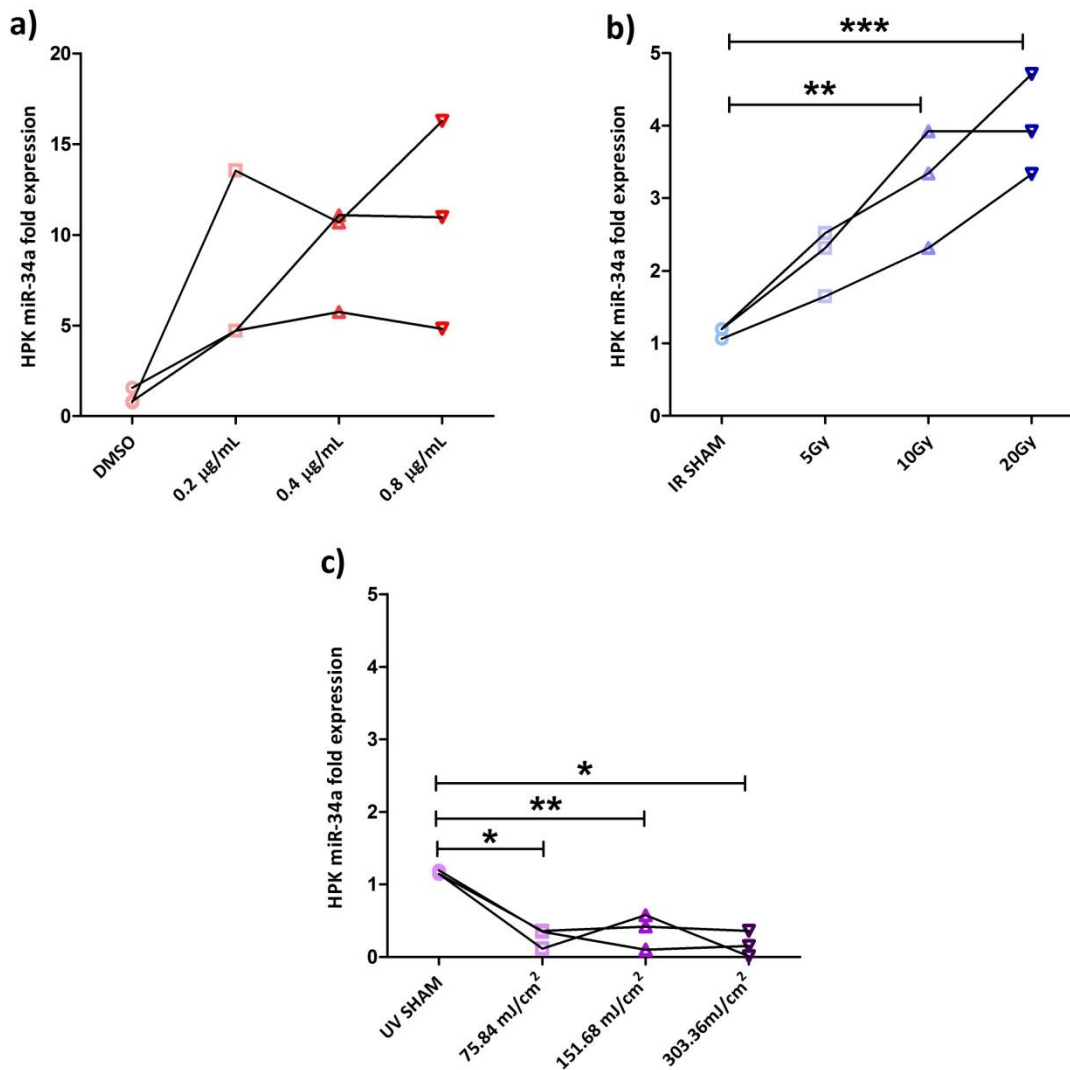


Figure 4-7 **miR-34a induction dose-response line graphs to a) adriamycin, b) IR and c) UVR in HPK cells.** N=3 independent experiments. In all \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with its respective untreated group. Data are means of qPCR triplicates.

As seen in Figure 4-7, adriamycin (a) failed to produce a significant modulation of miR-34a in HPK cells, but there is a clear trend towards upregulation (as non-significantly (AFED fluctuated between 6.5 and 7.5 for all doses)). IR induced miR-34a expression consistently amongst the HPK cells of the volunteers. Specifically, miR-34a was significantly upregulated by 10Gy and 20Gy of IR (AFED 2.5, 3.5 respectively,  $p < 0.01$  for both doses) (b). Unlike with p53 induction, 10Gy induced a significant upregulation of miR-34a (Figure 4-7b) in HPK cells. 75.84 mJ/cm<sup>2</sup>, 151.68 mJ/cm<sup>2</sup> and 303.36 mJ/cm<sup>2</sup> of UVR (c) significantly repressed the transcription of miR-34a ( $p < 0.05$ ,  $p < 0.01$  and  $p < 0.05$  respectively). The FED for all UVR doses ranged between -0.07 and -1.02.

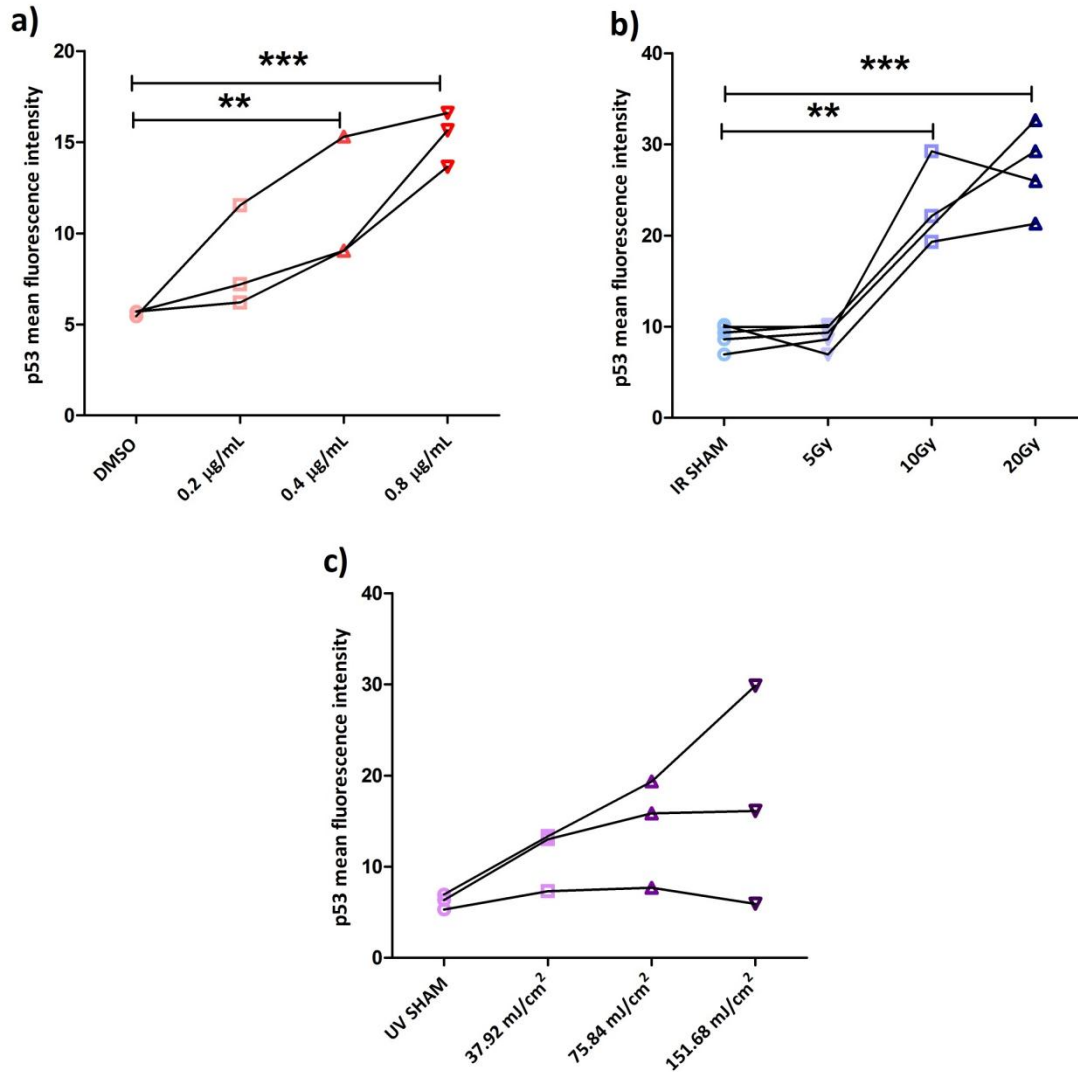


Figure 4-8 **p53 induction in response to (a) adriamycin, (b) IR and (c) UVR in HCT116 cells.** All samples are taken 24hours post treatment apart from IR irradiated samples that were collected at 48h. In the case of adriamycin the chemical was left in the cells' culture medium for 24hours. The cells were collected, fixed and stained with anti-p53/FITC labelled secondary antibodies, using an indirect staining protocol for flow cytometry. Fluorescence intensity was detected by FACScalibur and analyzed by CellQuestPro software. \*\*p<0.01, \*\*\*p<0.001 compared with its respective untreated group. N=3 for (a) and (c). N=4 for (b).

HCT116 cells were treated with 0.2 $\mu\text{g/mL}$ , 0.4 $\mu\text{g/mL}$  and 0.8 $\mu\text{g/mL}$  of adriamycin, 5Gy, 10Gy and 20Gy of IR and 37.92 $\text{mJ/cm}^2$ , 75.84 $\text{mJ/cm}^2$  and 151.68 $\text{mJ/cm}^2$  of UVR. Of course, being another cell type, HCT116 cells are reactive to different amounts of given DNA damage stimuli, so p53 induction dose responses were carried out. Dead cells were excluded from analysis with a violet dead stain as shown for HPK cells in the Methods section of this chapter.

As seen in Figure 4-8, p53 protein was upregulated significantly by 0.4 $\mu$ g/ml and 0.8 $\mu$ g/ml concentrations of adriamycin ( $p<0.01$  and  $p<0.001$ , average m.f.i. difference 5 and 7.5 respectively) (a) and 10Gy, 20Gy of IR ( $p<0.01$  and  $p<0.001$ , average m.f.i. difference 15.7 and 22 respectively) (b). UVR (c) failed to produce a significant upregulation of the protein but caused an increasing trend in its expression. The chosen doses to be used for future experiments were a) 0.4 $\mu$ g/mL of Adriamycin because it was the first dose to induce a significant upregulation of p53 protein and 80% of the cell monolayer was still alive after a 24h application of adriamycin, b) 10Gy of IR for the same reasons and c) 75.84mJ/cm<sup>2</sup> of UVR, since any dose above that was lethal for HCT116 cells and p53 levels were moderately increased. HCT116 cells proved to be more sensitive HPK to UVR because any dose beyond 151.68mJ/cm<sup>2</sup> was lethal to the cells. Thus, the doses were adjusted accordingly. In contrast to HPK cells, p53 expression levels were not as robust 24 hours post irradiation in HCT116 cells, but there is an obvious trend to upregulation. The average m.f.i. difference for 75.84mJ/cm<sup>2</sup> was 9.

Once the p53 induction doses were established, the cells were treated with the pertinent dose of the DNA damaging reagent and then were lysed for RNA extraction and miR-34a detection.

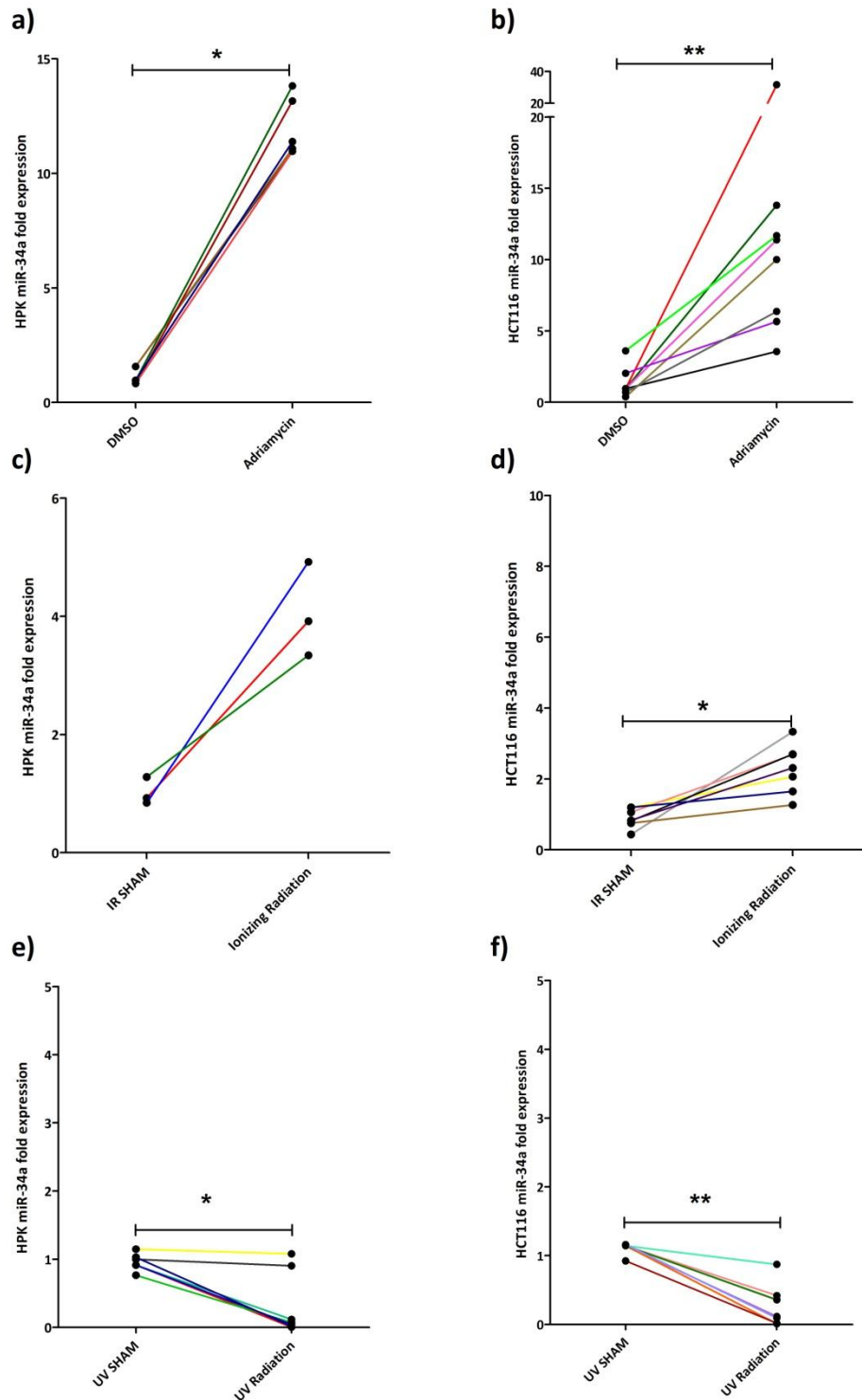


Figure 4-9 **Relative miR-34a expression in a),c),e) HPK and b),d),f) HCT116 cells treated with a),b) adriamycin, c),d) IR and e),f) UVR.** All samples are taken 24hours post treatment. In the case of adriamycin (red) the chemical was left in the cells' culture medium for 24hours. Expression of microRNAs was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=8 for b) and f). N=7 for d) and e). N=6 for a) and N=3 for c). In all \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with its respective untreated group. Data are means of qPCR triplicates.

Results (Figure 4-9) show that 0.4mg/mL of adriamycin cause a significant rise in the levels of miR-34a transcript in HPK (a) and HCT116 (b) cells ( $p<0.05$  and  $0.01$  respectively). miR-34a FED responses to adriamycin varied from 9.39 to 12.86 in HPK cells and 2.60 to 30.70 in HCT116 cells. miR34a was significantly induced in HCT116 cells (d) by 10Gy ( $p<0.05$ ) of IR. The wilcoxon test did not yield the modulation of miR-34a in HPK by 20Gy of IR significant, yet the induction of miR-34a was consistent in all 3 HPK from different volunteers' skin tested. FED varied from 2.50 to 3.64 for (c) and 0.5 to 8.63 for (d). UVR significantly suppressed the expression of miR-34a in HPK ( $p<0.05$ ) and HCT116 cells ( $p<0.01$ ).  $151.68\text{mJ}/\text{cm}^2$  decreased the levels of miR-34a transcript in HPK cells by 6 qPCR cycles that translates into a -1.02 average FED.  $75.84\text{mJ}/\text{cm}^2$  induced a FED range in HCT116 cells of -0.27 to -1.13. The question arising was whether this is an effect caused by impaired activation of p53 during UVR induced DNA damage response. To investigate that, HaCaT cells that have mutated non-functional p53 (Boukamp *et al*, 1988), were treated with IR and UVR and their miR-34a levels were quantified by qPCR.

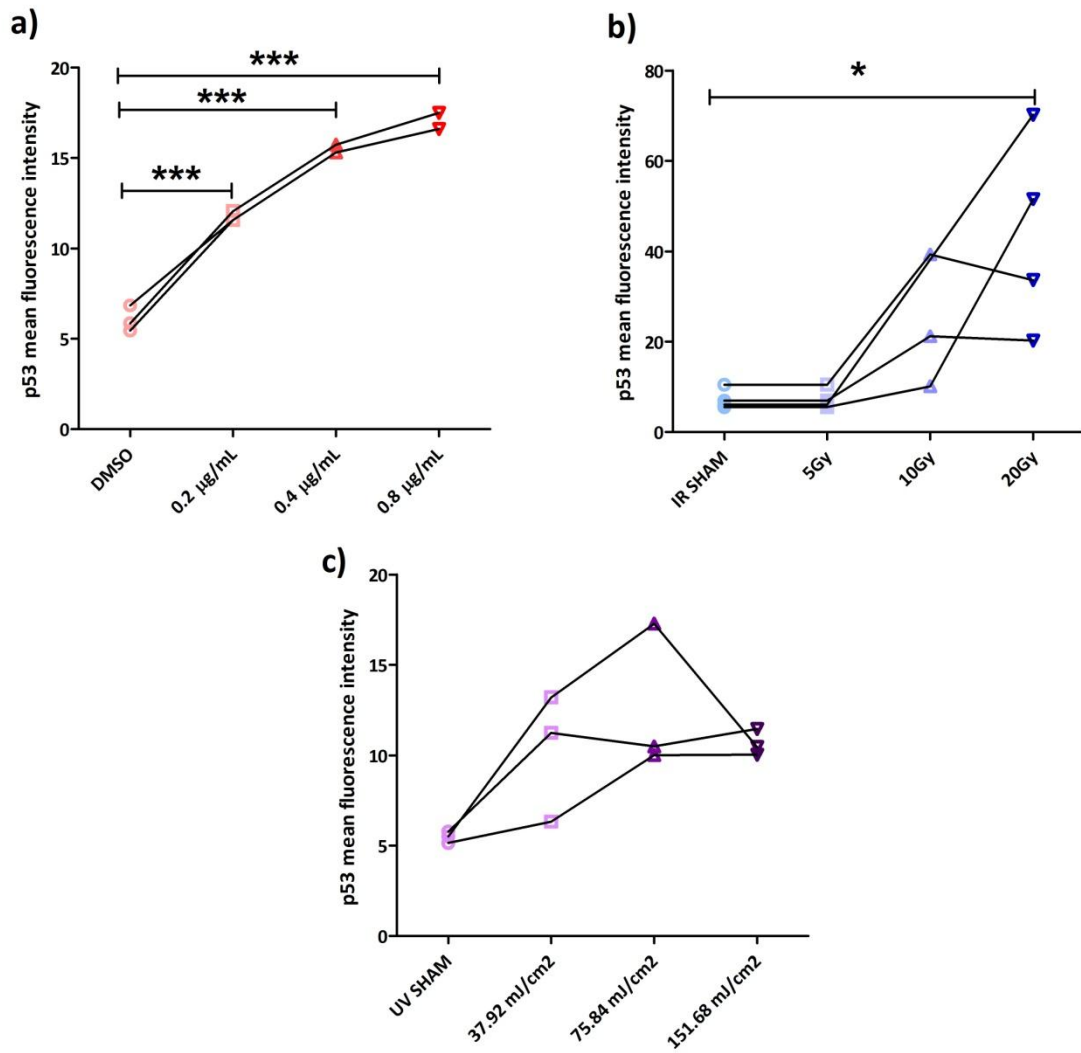


Figure 4-10 **p53 is induced by a) adriamycin, b) IR and c) UVR in HaCaT cells.** All samples are taken 24hours post treatment apart from IR irradiated samples that were collected at 48h. In the case of adriamycin the chemical was left in the cells' culture medium for 24hours. The cells were collected, fixed and stained with anti-p53/FITC labelled secondary antibodies, using an indirect staining protocol for flow cytometry. Fluorescence intensity was detected by FACScalibur and analyzed by CellQuestPro software. In all \* $p < 0.05$ , \*\*\* $p < 0.001$  compared with its respective untreated group.

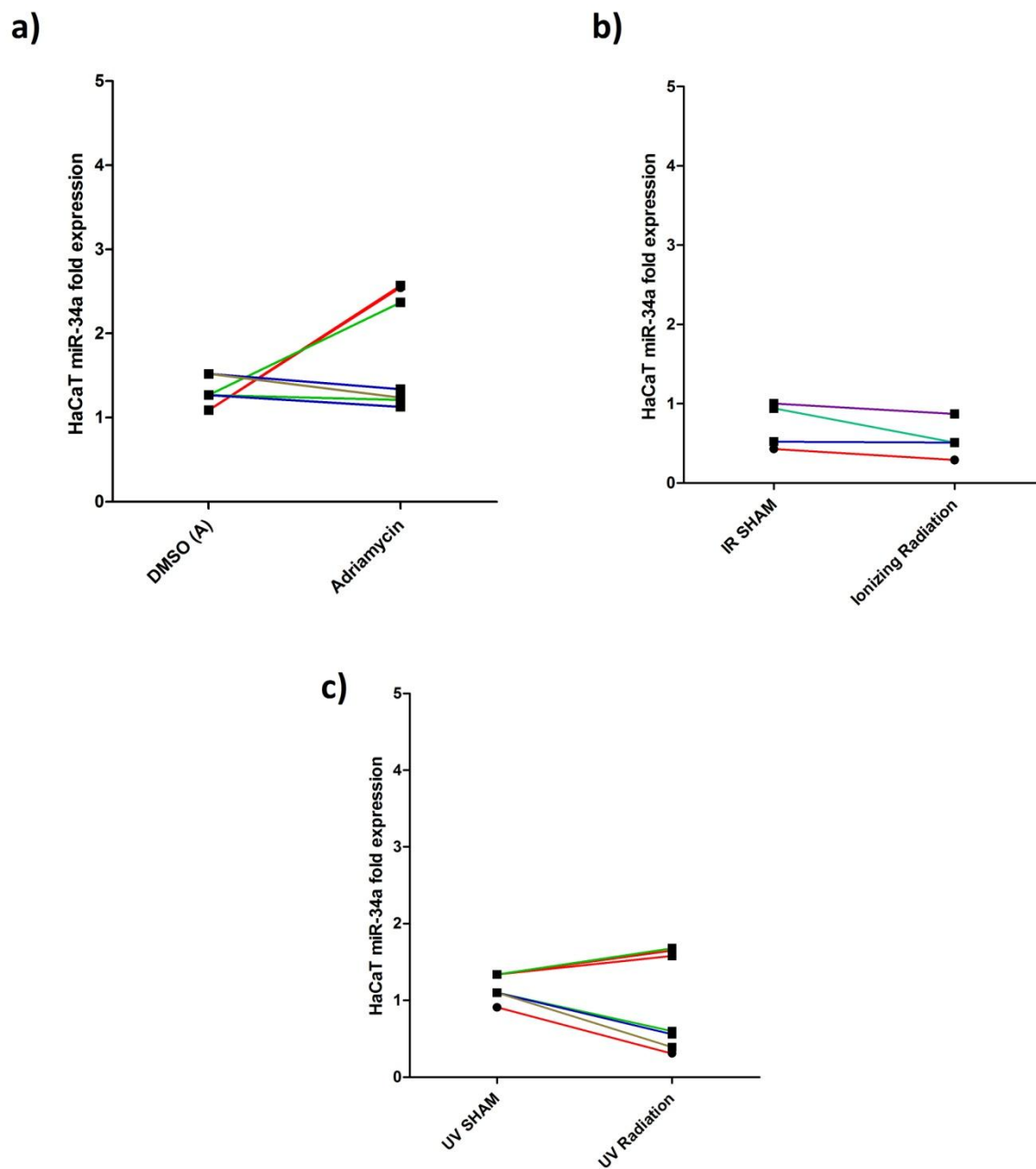


Figure 4-11 **miR-34a fails to be induced in HaCaT cells treated with a) adriamycin, b) IR and c) UVR.** All samples collected 24 hours post treatment. In the case of adriamycin the chemical was left in the cells' culture medium for 24 hours. Expression of microRNAs was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=7 for (a), (c) N=4 for (b) independent experiments. Data are means of qPCR triplicates.



Results in Figure 4-10 (a) show that p53 protein induction in HaCaT cells treated with adriamycin is dose dependent with every dose (0.2mg/mL, 0.4mg/mL and 0.8mg/mL) significantly rising the levels of p53 protein ( $p < 0.001$  for all). 5Gy of IR failed to induce any modulation of p53 protein levels in HaCaT cells, while 10Gy raised p53 levels by m.f.i. difference 4.04 to 28.82 and 20Gy significantly induced p53 protein expression ( $p < 0.05$ ) by m.f.i. difference 13.27 to 64.09. The only UVR dose that doubled p53 levels was 75.84mJ/cm<sup>2</sup> but not significantly (Figure 4-10 c). The doses that were chosen to investigate miR-34a expression in HaCaT cells were: 0.2mg/mL of adriamycin, 20Gy of IR and 75.84mJ/cm<sup>2</sup> of UVR.

Despite the upregulation of p53 protein in response to the DNA damaging stimuli shown in Figure 4-10, it was expected that miR-34a would not follow the same pattern as already shown for HCT116 cells and HPK cells (Figure 4-9) since p53 in HaCaT cells carries mutations that turn the protein transcriptionally inactive (Lehman et al, 1993; Datto et al, 1995). As seen in Figure 4-11, miR-34a is not induced by none of the DNA stimuli applied to HaCaT cells; adriamycin FED range -0.28 to 1.48, IR FED range -0.01 to -0.43 and finally UVR FED range -0.71 to 0.34. Baseline level of detection for miR-34a in control samples of HaCaT cells was found at 27 qPCR cycles which was the same amount as in HPK and HCT116 cells. However, treated samples by either adriamycin, IR or UVR did not exhibit a clear pattern of miR-34a modulation. Overall, in these experiments, miR-34a appears unaffected by the treatments (adriamycin, IR and UVR) in HaCaT cells.

In addition, further experiments were carried out using HCT116, HaCaT, and HPK cells to determine the activation status of p53 by measuring the levels of p21WAF1 mRNA; a transcriptional target of activated p53 (El-Deiry *et al*, 1993).

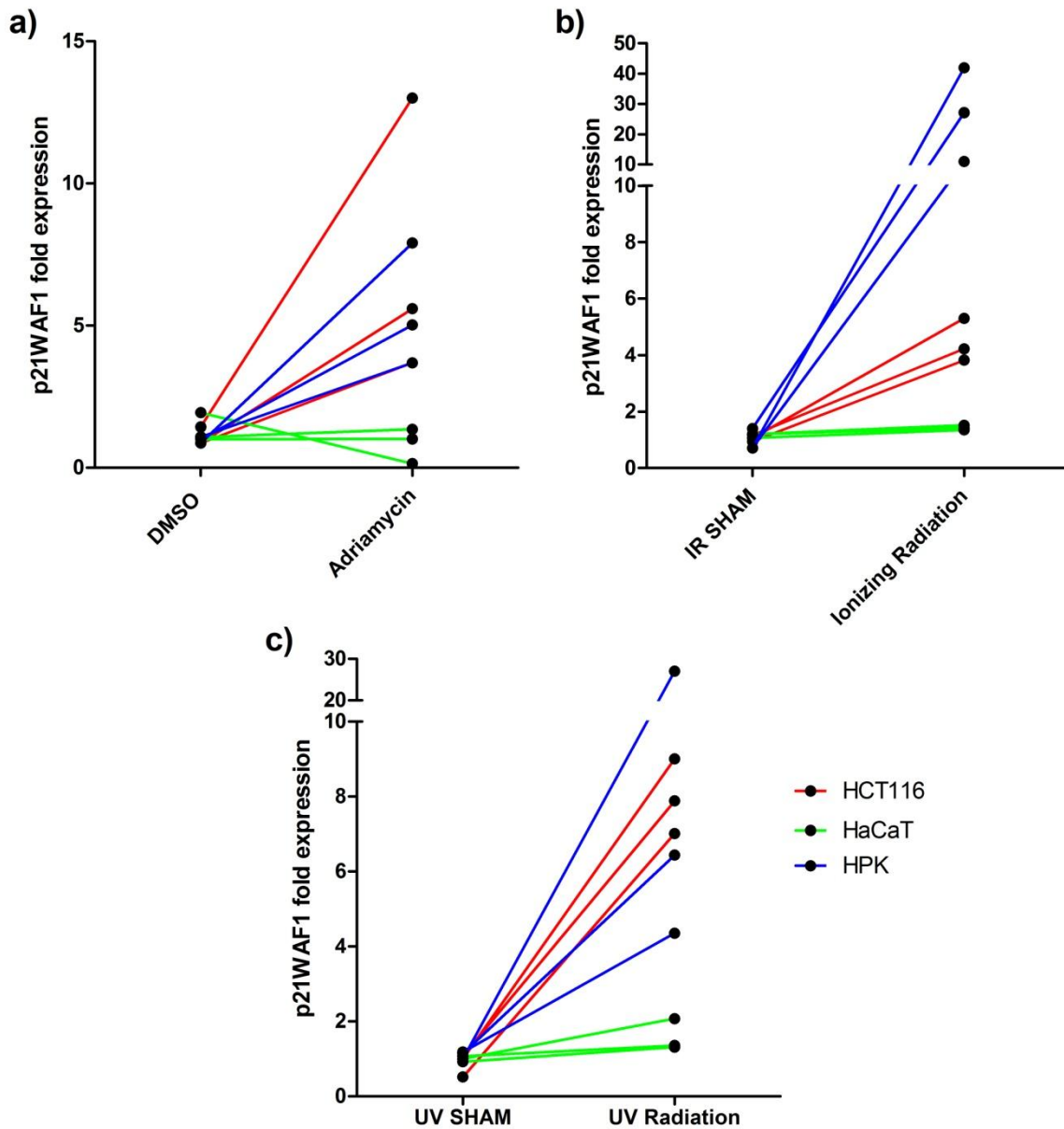


Figure 4-12 **p21WAF1 induction by DNA damage in HPK, HCT116 and HaCaT cells.** All samples are taken 24hours post treatment. In the case of adriamycin (a) the chemical was left in the cells' culture medium for 24hours. Expression of p21WAF1 was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=3 independent experiments for each cell type and treatment. Data are means of qPCR triplicates.

Results shown in Figure 4-12 p21WAF1 is induced in HPK by adriamycin (FED 2.60-7.04), IR (FED 10.49-41.19) and UVR (FED 3.17-26.05). p21WAF1 mRNA expression in

HCT116 cells was also increased by all treatments with adriamycin inducing a FED of 2.83 to 11.57, IR 2.8 to 4.24 and UVR 6.49 to 8.01. Adriamycin, IR and UVR failed to induce p21WAF1 upregulation in HaCaT cells, which was anticipated since p53 in HaCaT cells is non-functional.

#### 4.4.3. The expression of miR-34a does not change in UVR irradiated human skin tissue

Several research groups have reported that solar-simulated irradiation of human skin of healthy individuals, causes upregulation of cyclobutane pyrimidine dimers and p53 levels (Burren *et al*, 1998; de Winter *et al*, 2001; Murphy *et al*, 2002; Yamaguchi *et al*, 2008). Cyclobutane pyrimidine dimers can be detected by immunohistochemistry of the skin biopsy immediately after irradiation, while for p53; the biopsy must be taken at least 18 hours after irradiation, because that is the time point of its peak expression in human skin (O' Grady *et al*, 1998; Murphy *et al*, 2002). The arising problems are a) whether the tissue in the skin explant culture system will be a representative picture of what occurs *in vivo* and b) whether the amount of p53 detected will be due to the irradiation alone and not any other kind of stress during the explant culture. As seen in Figure 4-13, overnight incubation of *ex vivo* skin explants in culture medium does not induce p53 production in the epidermis; therefore the p53 staining cells observed in the treated samples were due to UVR. In addition, what this experiment proves, is that the skin explants respond to the UVR by expressing p53, as it happens *in vivo* which renders the skin explant system suitable for this project.

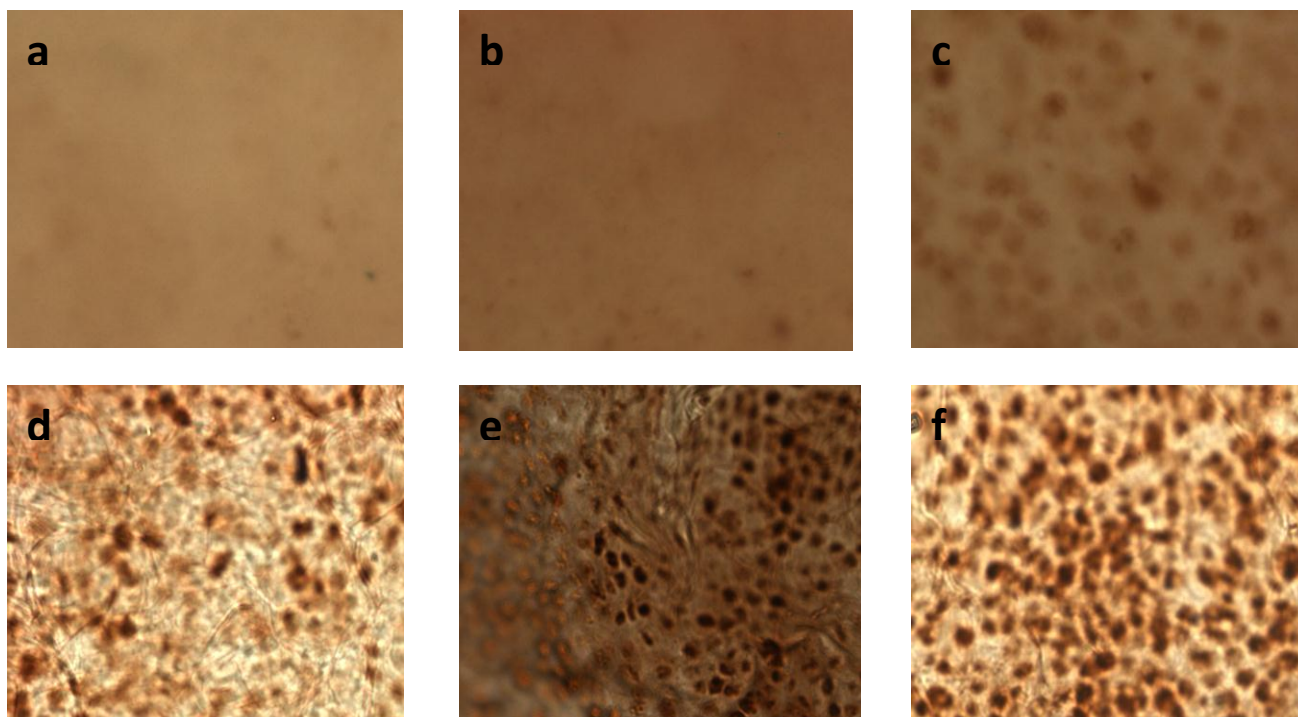


Figure 4-13 **UVR induces the expression of p53 in *ex vivo* skin.** a) Epidermis from fresh skin, b) epidermis from UVR sham skin, incubated overnight at 37°C, c-f) epidermises treated with 379.2, 758.4, 1516.8, 3033.6 mJ/cm<sup>2</sup> of UVR respectively. Photographs were taken of whole-mount immunohistochemically stained epidermis under conventional light microscopy conditions; due to the multiple cell layer nature of the epidermis, some nuclei were in focus whereas other nuclei remained out of focus.

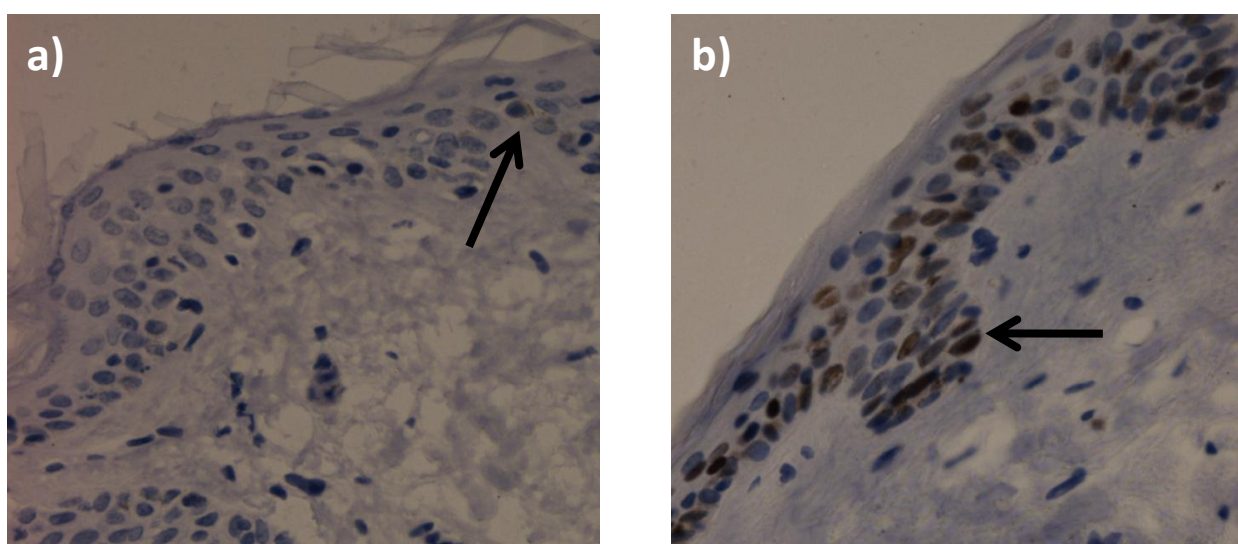


Figure 4-14 **Example of p53 staining in cross-dissected human skin explants, stained with a nuclear counterstain.** a) UVR sham control, with the arrow pointing to a cell staining for p53 in the cytoplasmic region, b) UVR irradiated sample, with the arrow showing at the nuclear loci of p53.

The next step was to verify the dose of UVR that could cause enough damage to the skin in order for more than 50% of the epidermal cells to stain positive for p53, so that the levels of miR-34s can be comparable to sham controls. To achieve that, redundant skin from 10 mastectomy patients was irradiated with 379.2, 758.4, 1516.8, 3033.6, 4550.4mJ/cm<sup>2</sup> of UVR, stained for p53 and then scored depending on the intensity and percentage of staining cells. The skin explants were cultured overnight post irradiation and then the epidermis was split from the dermis before acetone fixation. The skin explants were irradiated under a TL12 UVR emitting lamp.

Photographs shown in Figure 4-13 were taken of whole-mount immunohistochemically stained epidermis under conventional light microscopy conditions; due to the multiple cell layer nature of the epidermis, some nuclei were in focus whereas other nuclei remained out of focus. Stained nuclei are depicted by circular round spots; staining intensity varied demonstrating different amounts of p53 protein.

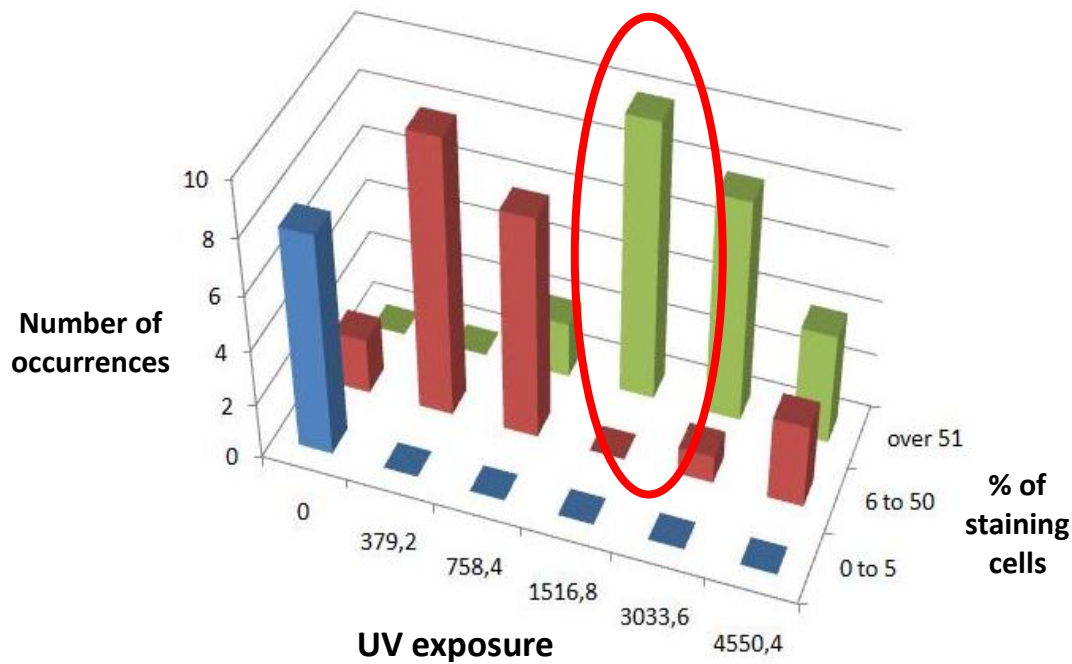


Figure 4-15 **Grading of the p53 response to UVR in human skin explants.** Human skin explants from 10 different patients received a range of single doses of UVR ( $379.2\text{mJ/cm}^2$ ,  $758.4\text{mJ/cm}^2$ ,  $1516.8\text{mJ/cm}^2$ ,  $3033.6\text{mJ/cm}^2$ ,  $4550.4\text{mJ/cm}^2$ ). 24 hours later the epidermises were removed, stained and graded for p53 expression.

For the shammed control (no UVR applied) in 8 of the patients the staining was at physiological levels: 0-5% with two exceptions where the percentage fluctuated between 6 and 10%. Previous work from this group showed evidence that non-irradiated human skin, appears to have these occasional p53 positive cells that are thought to be undergoing a cell cycle differentiation switch (Dazard *et al*, 2000; Murphy *et al*, 2002). The circled column represents the effective dose of UVR needed to produce over 51% of p53 positive epidermal skin cells in 9 out of 10 volunteers' skin assessed.

After  $379.2\text{mJ/cm}^2$  of irradiation only two volunteers showed vigorous instant response, while the rest of them showed around 15% of p53 positive staining cells. After  $758.4\text{mJ/cm}^2$  more than 25% of the total population of cells was positively stained for p53.  $1516.8\text{mJ/cm}^2$  is the first UVR dosage where over 60% of the cells were staining for p53. On one hand, some of the volunteers epidermal sheets' show a smooth p53 dose response to UVR and reach almost 85% of positive p53 nuclei, but on the other hand, most of the patients' epidermal sheets had endured too much damage; the disruption of the dermo-

epidermal junction by EDTA during the splitting procedure, was hindered in these samples and p53 staining was scarce, with gaps of completely unstained regions and areas of accumulated damaged-p53 expressing nuclei. Consequently, these samples were considered to be unsatisfactory for microRNA analysis. 1516.8mJ/cm<sup>2</sup> was found by this work to be the prevalent UVR dose where more than 51% of the irradiated epidermal cells stained for p53.

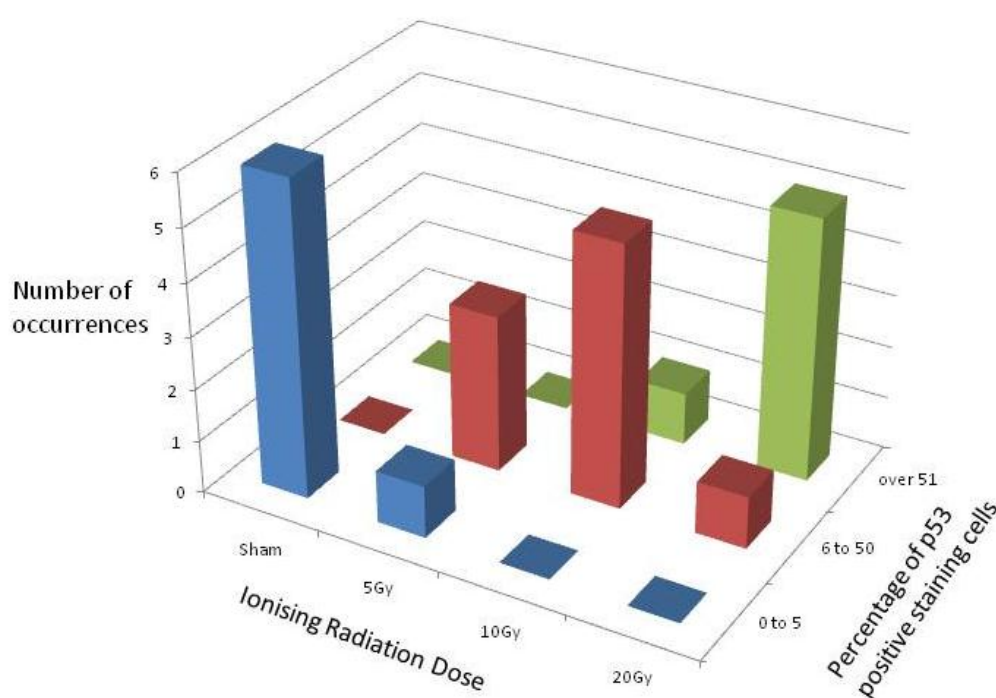


Figure 4-16 **Grading of p53 response to IR in human skin explants.** Human skin explants from 6 different volunteers received 0, 5, 10 or 20Gy of IR. In 5 out of the 6 subjects 20Gy of IR was enough to induce over 51% of the total epidermal population to stain for p53.

As opposed to UVR, IR did not have to be adjusted for the *in vivo* experiments as gamma rays penetrate human tissues without being absorbed but by being attenuated (see section 4.4.3). The depth of the epidermis in the case of gamma rays is negligible; hence the germinal layer of the epidermis encounters the same amount of energy that reaches the cornified outermost epidermal layer.



In this experimental model, the aim is to inflict enough damage to the cells so that the p53 protein is both activated and upregulated in order for p53's downstream effects on microRNA family 34 to be detected and analysed. Hence, three single doses of IR were chosen; 5Gy, 10Gy and 20Gy. As shown in Figure 4-16, a 20Gy dose is enough for over 51% of epidermal cells to stain positive for p53 protein with immunohistochemistry 24 hours post irradiation.

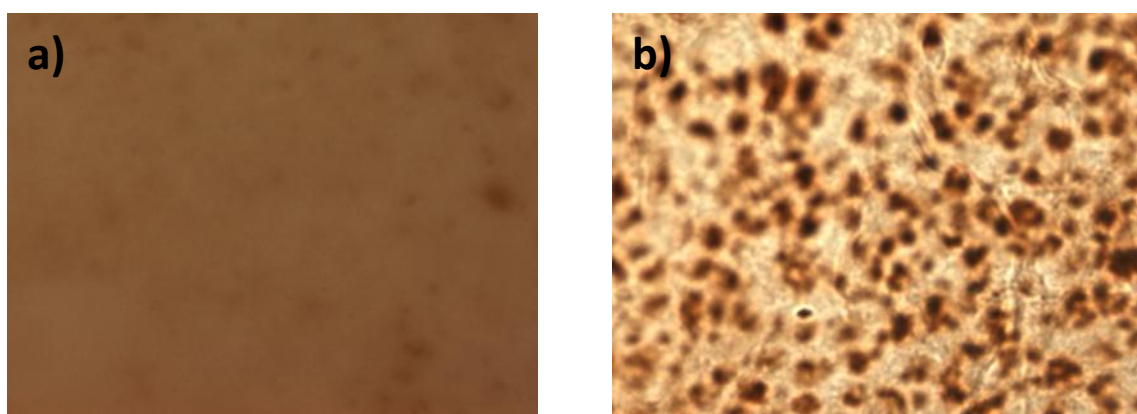


Figure 4-17 **Example of p53 staining in IR exposed human skin explants.** a) IR sham control, b) IR irradiated sample. Human epidermis was separated from the dermis, fixed and stained for p53. Cells positive for p53 are stained with brown colour.

The antibody against p53 used in the above experiments is the DO-7 antibody staining for an epitope of p53 not affected by conformational changes by phosphorylation, acetylation or faulty amino-acids in case of mutated p53. As seen clearly in Figure 4-17, most of the occasional cells staining for p53 in UVR or IR sham control, illustrated cytoplasmic localisation of the protein, while most of the staining cells in treated explants show a massive upregulation of the protein and translocation to the nuclear part of the cell. Once the doses of UVR and IR, which caused over 51% of the total epidermal cell population to stain for p53, were established, these doses were used to irradiate skin explants for the miR-34a detection experiments. The basal keratinocytes do not exist in a strict monolayer (similarly to the *in vitro* models described above) in skin tissue but they are surrounded by other cell types and are protected by the stratified layers of the upper epidermis. In case of damage, the cells receive pro-inflammatory signals through adhesion molecules and cytokines, activating various signalling pathways. In order to confirm that the



*in vitro* observations (Figure 4-9) seen in HPK and HCT116 cells occur in such a different environment such as skin tissue, the tissue was lysed in TRI reagent and miR-34a was quantified by qPCR as seen in Figure 4-18. Adriamycin wasn't used in the ex vivo system, as the effects of the chemical on skin are unknown. Adriamycin is only used intravenously as a chemotherapy drug and its penetration rate in human skin is elusive.

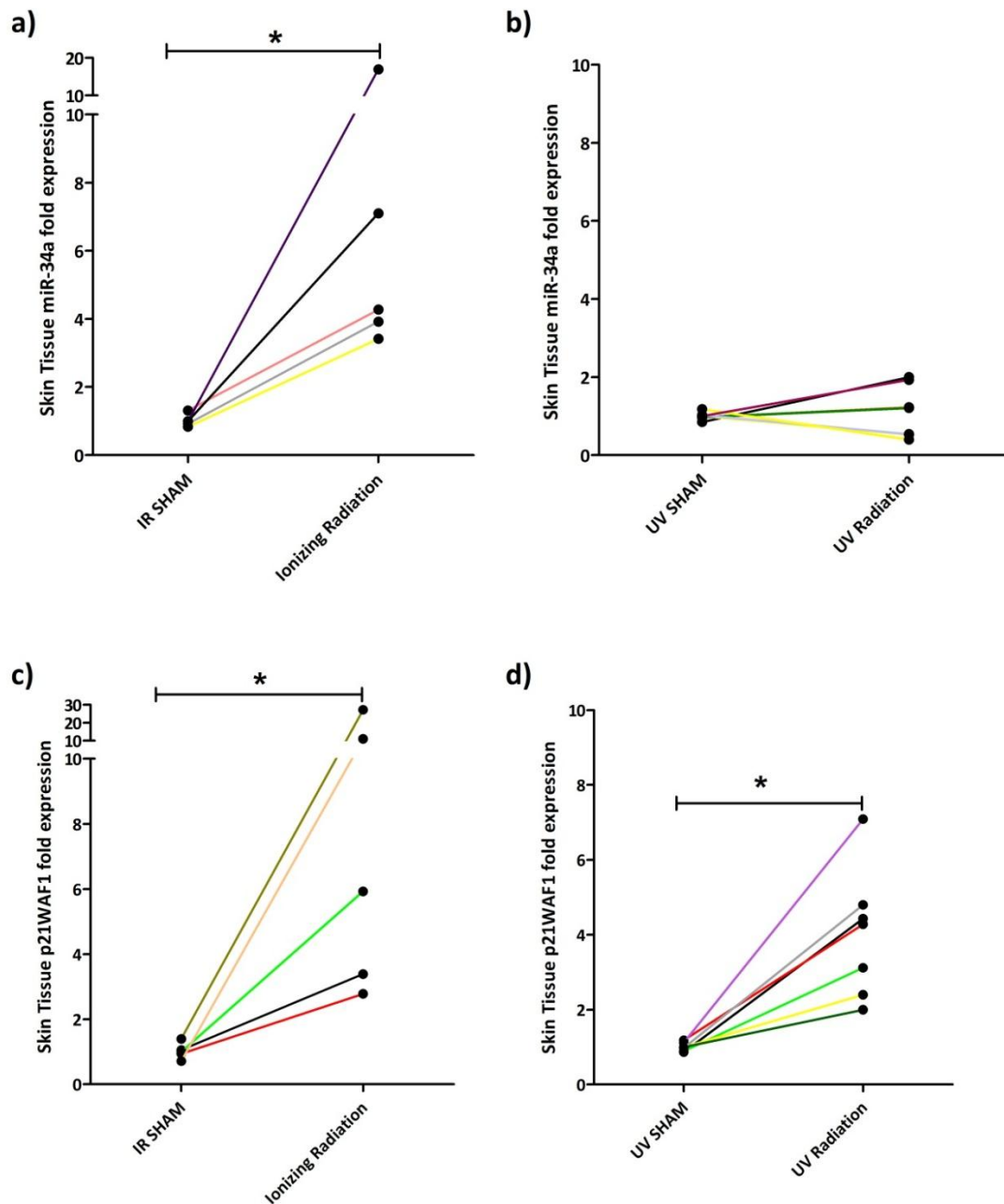


Figure 4-18 **miR-34a and p21WAF1 induction in human skin tissue treated with a), c) IR and b), d) UVR.** The mRNA of p21WAF1 was significantly modulated in both treatments. All samples collected 24 hours post treatment. Expression of microRNAs and p21WAF1 was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=6 independent experiments. Data are means of qPCR triplicates. In all \* $p < 0.05$  compared with its respective untreated group.

miR-34a was significantly modulated by IR in human skin ( $p < 0.05$ ), being expressed at least twice as much in treated samples compared to control ones (FED 3.00-15.64). UVR on the other hand does not present an overall modulation of the microRNA, with the latter

remaining around baseline levels with FED fluctuating between -0.78 to 1.15. IR induced a significant upregulation of miR-34a in skin tissue p53's transcription status was confirmed in both treatments as the p21WAF1 expression results in Figure 4-17 indicate (FED of IR 1.92-11.34, FED of UVR 2.33-4.91).

#### 4.4.4. SIRT1, a confirmed target of miR-34a is not downregulated in UVR treated cells

As described in the introduction of this chapter, miR-34a represses the translation of protein SIRT1. SIRT1 is a member of a protein family called sirtuins. Sirtuins are NAD dependent deacetylases with targets in various compartments of the cell (mitochondria, cytoplasm and nucleus) (Vassilopoulos *et al*, 2010). In particular SIRT1 is required for proper DNA repair and genomic stability (Oberdoerffer *et al*, 2008). It also directly implicated with cell senescence and p53 and non-p53 related apoptotic mechanisms (Michan and Sinclair, 2007). SIRT1 deacetylates p53, thus abrogating its transcriptional functions and promoting cell survival; it is in this relationship between apoptotic elements of the cellular network and cell survival that SIRT1 has been baptised both a tumour suppressor and oncogene (Saunders and Verdin, 2007; Deng, 2009). SIRT1 plays a crucial role in cell survival and has been shown to be a direct target of miR-34a, which represses its translation, facilitating the transcriptional activity of p53 (Fujita *et al*, 2008; Luan *et al*, 2010).

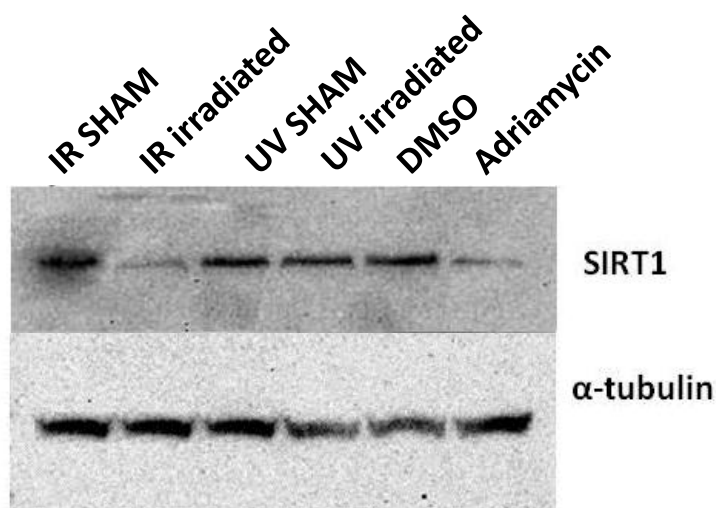


Figure 4-19 **Western blot analysis of SIRT1 protein levels.** HCT116 cells were treated with IR, UVR and adriamycin, lysed 24h later and protein extracts were immunoblotted using the invitrogen NuPAGE technology.

The cells (HCT116) were treated with either adriamycin, IR or UVR and were lysed in RIPA lysis buffer, followed by protein extraction and quantification. The proteins were distributed by molecular weight by electrophoresis on a bis-tris 10 well gel and transferred via semi-dry transfer onto a PVDF membrane. SIRT1 was detected with SIRT1 H-300 antibody and  $\alpha$ -tubulin was used as loading control.

As results show in Figures 4-19 and 4-20, the direct effect of the upregulation of miR-34a in the adriamycin and IR treated samples had a significant impact on the levels of SIRT1 protein ( $p < 0.01$  and  $p < 0.01$  respectively). Intensity ratio difference ranged between -0.26 and -0.81 in adriamycin treated HCT116 cells and -0.42 and -0.86 in IR treated cells. In Figure 4-20c, it is evident that overall, UVR does not modulate the SIRT1 protein content of cells.

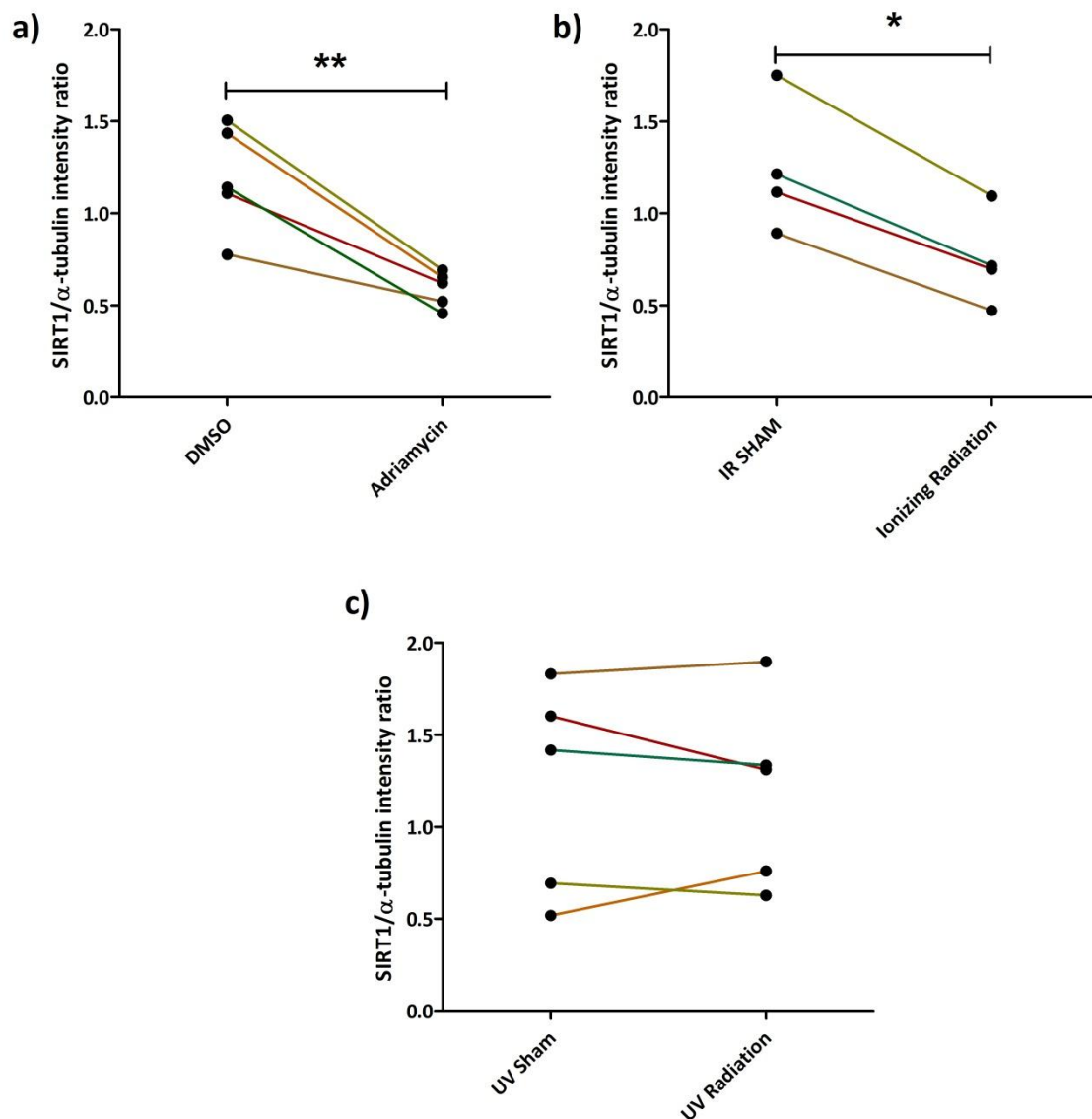


Figure 4-20 **SIRT1 levels in a) adriamycin, b) IR and c) UVR treated HCT116.** Data are results of densitometry analysis of SIRT1 protein levels in PVDF membranes. Pictures were taken and quantified using QuantityOne software. N=5 (a, c) and N=4 (b) independent experiments. In all \*p<0.05, \*\*p<0.01 compared with its respective untreated group.

4.4.5. miR-34b and miR-34c exhibit unique expressions, different to miR-34a following genotoxic damage in cells and skin tissue.

miR-34a belongs to the miR-34 family which has another two members: miR-34b and miR-34c. miR-34a is generated from a transcriptional unit in chromosome 1p36, while miR-34b and miR-34c are born from the processing of a bicistronic transcript of

chromosome 11q23, while 30kb away from the precursor of both sites there is a p53 binding region (Bommer *et al*, 2007; Kumamoto *et al*, 2008). Corney *et al* proved that miR-34b and miR-34c are indeed transcriptional targets of p53 and they are both upregulated following adriamycin and IR treatments (Corney *et al*, 2007). So it seemed interesting to investigate the expression pattern of miR-34b and miR-34c in this system and assess whether the entire miR-34 family expression is altered following UVR.

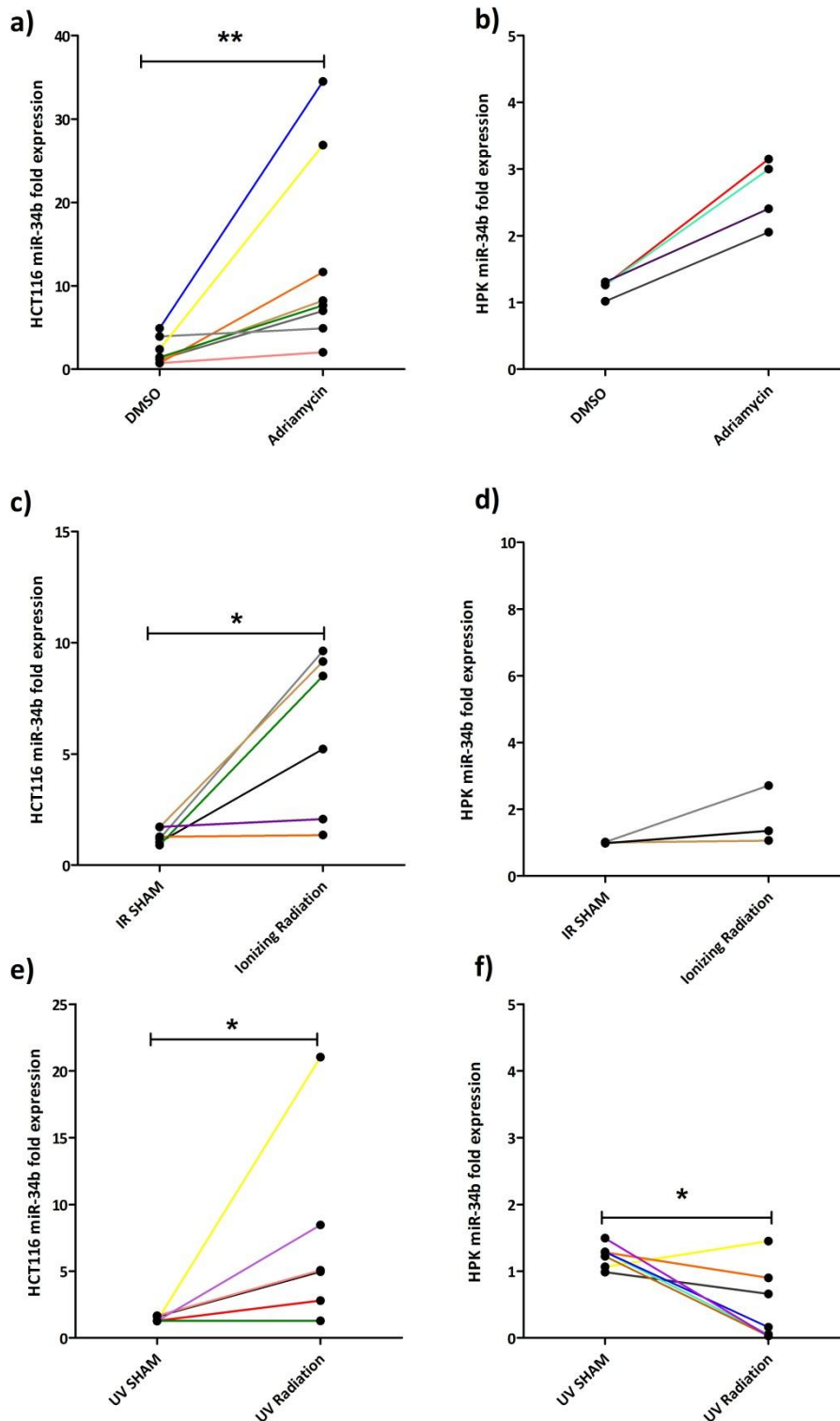


Figure 4-21 **Relative miR-34b expression in a),c),e) HCT116 and b),d),f) HPK cells treated with a),b) adriamycin, c),d) IR and e),f) UVR.** Expression of microRNAs was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=8 (a), N=7 (f), N=6 (c, e), N=4 (b) and N=3 (d) independent experiments. In all \* $p < 0.05$ , \*\* $p < 0.01$  compared with its respective untreated group. Data are means of qPCR triplicates.

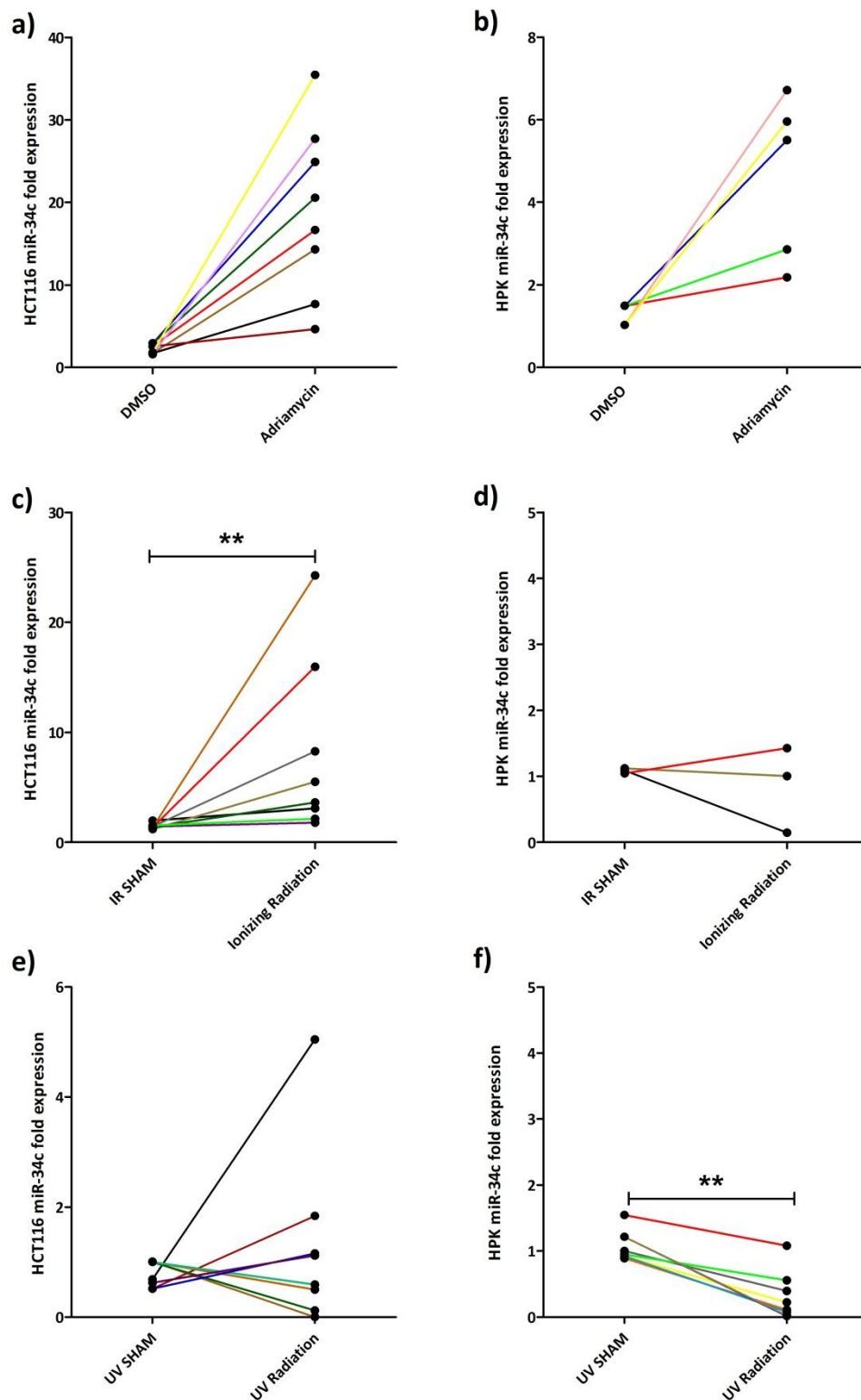


Figure 4-22 Relative miR-34c expression in a),c),e) HCT116 and b),d),f) HPK cells treated with a),b) adriamycin, c),d) IR and e),f) UVR. Expression of microRNAs was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=8 (a, c), N=7 (e,f), N=5 (b) and N=3 (d) independent experiments. In all \*\* $p < 0.01$  compared with its respective untreated group. Data are means of qPCR triplicates.



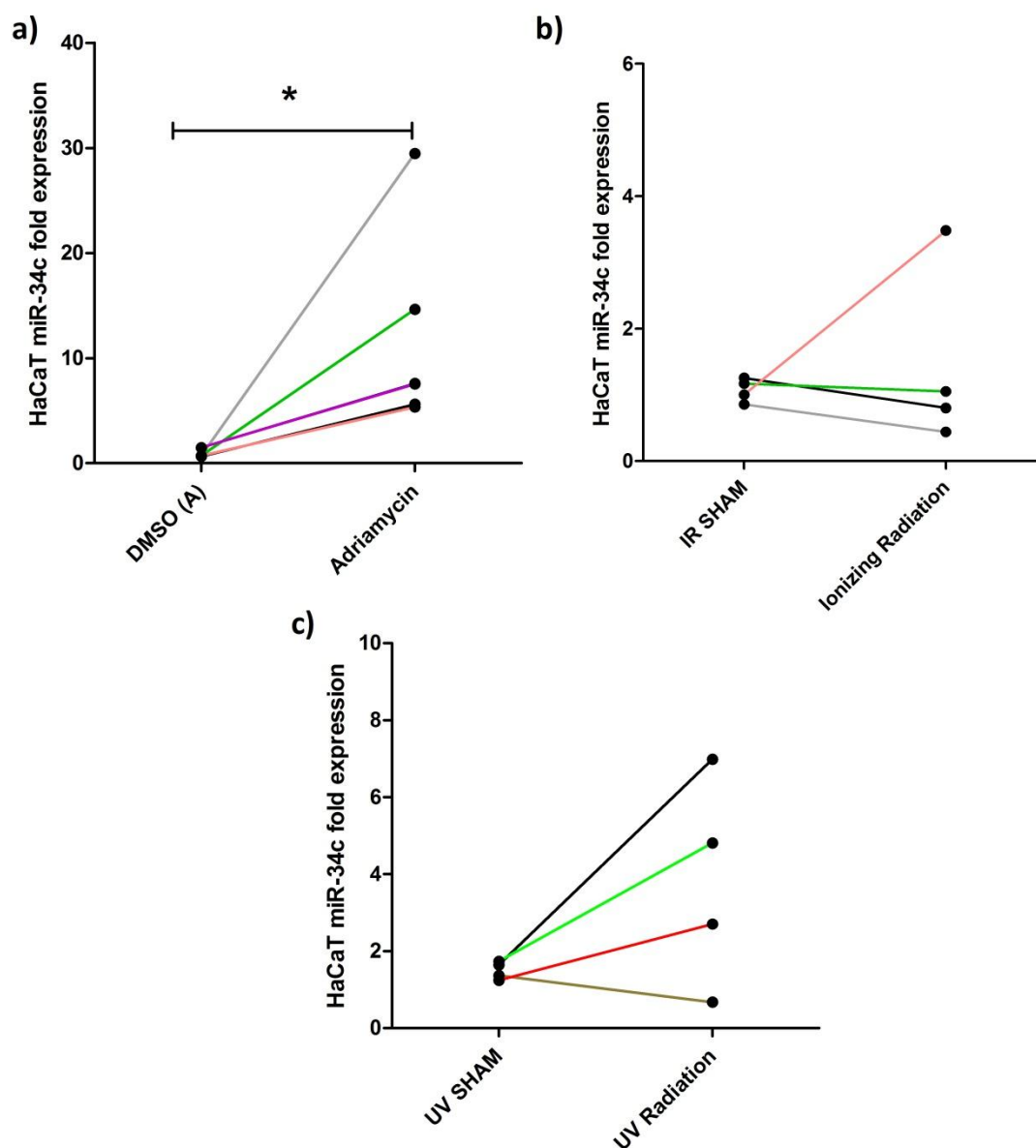


Figure 4-23 **miR-34c modulation by a) adriamycin, b) IR and c) UVR in HaCaT cells.** Expression of miR-34c was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=5 (a, c) and N=4 (b) independent experiments. Data are means of qPCR triplicates.

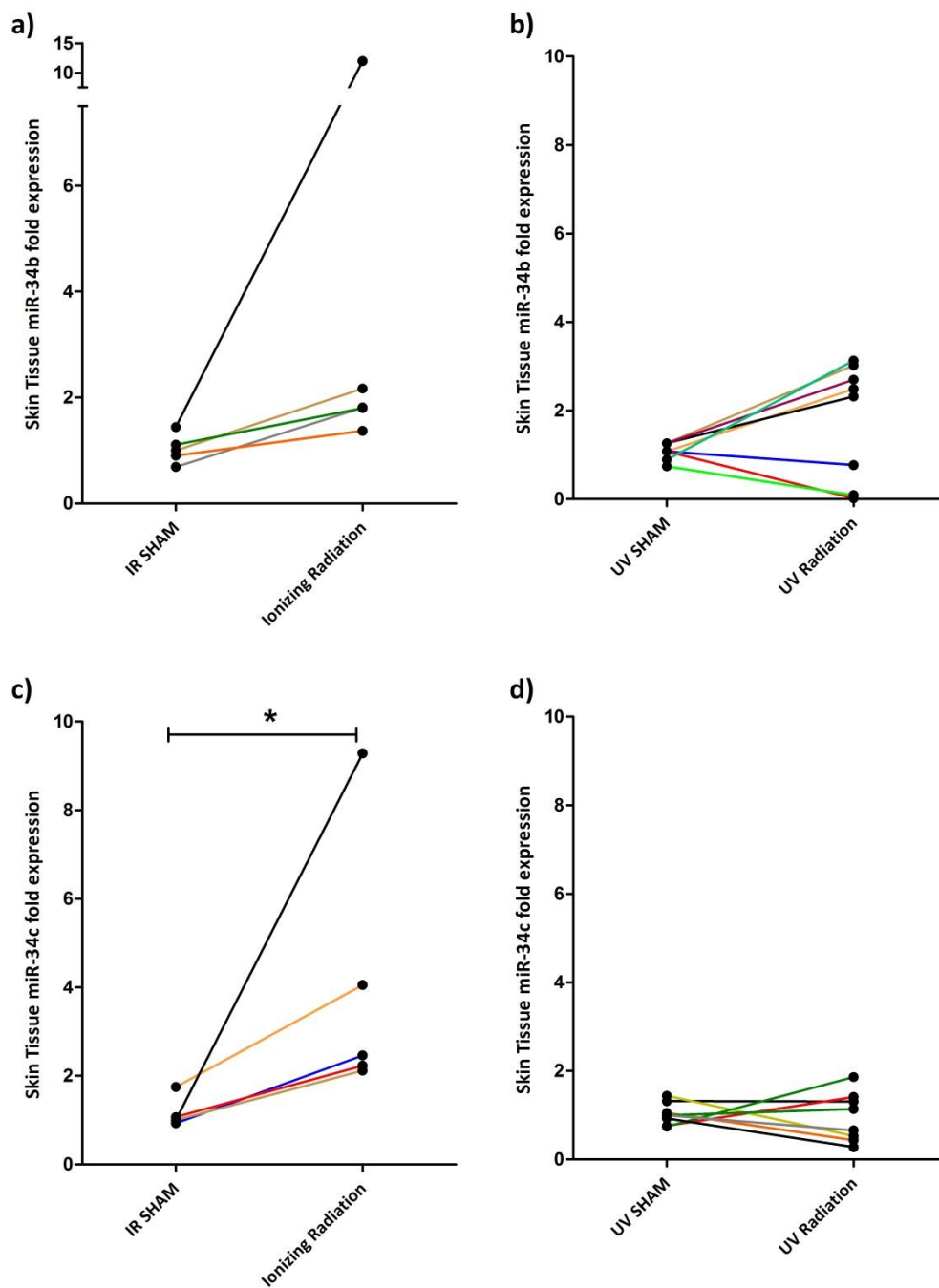


Figure 4-24 **miR-34b and miR-34c modulation by IR a), c) and UVR b),d) in human skin tissue.** Expression of microRNAs was detected by qPCR.  $C_T$  values were determined by SDS 2.0 software. N=8 (b, d) and N=5 (a, c) independent experiments. In all \*\* $p < 0.01$  compared with its respective untreated group. Data are means of qPCR triplicates.

The main focus of the experiments demonstrated in the Figures 4-21 to 4-24 was to establish whether miR-34b and miR34c are modulated similarly to miR-34a in response to UVR in HPK, HCT116, HaCaT cells and skin tissue. Adriamycin and IR were used as positive controls. miR-34b was not detectable by qPCR in HaCaT cells. It is not clear whether this is a result of a mutation or an inhibitory phenomenon.

Adriamycin significantly induced miR-34b in HCT116 cells (FED from 0.98 to 29.58,  $p < 0.01$ ) and in HPK cells (FED from 1.04 to 1.89, ns). IR increased the levels of miR-34b in all samples tested: HCT116 FED from 0.35 to 8.44 ( $p < 0.05$ ), HPK FED from 0.06 to 1.69 and human skin tissue FED from 0.47 to 10.55. UVR significantly induced miR-34b in HCT116 cells (FED from 0.01 to 19.75,  $p < 0.05$ ) whereas it significantly suppressed it in HPK cells (FED from -1.73 to 0.38,  $p < 0.05$ ). Overall, miR-34b expression was not modulated in UVR irradiated skin tissue (FED from -1.06 to 2.24).

Adriamycin induced the expression of miR-34c in both HCT116 (FED from 2.08 to 33.64) and HPK cells (FED from 0.69 to 5.69). Surprisingly miR-34c upregulation in HaCaT cells was significant ( $p < 0.05$ , FED from 4.63 to 28.77). IR did not modulate the expression of miR-34c in HPK (FED from -0.94 to 0.38) and HaCaT cells (FED from -0.46 to 2.48). On the other hand, miR-34c was significantly in HCT116 cells (FED from 0.36 to 22.89,  $p < 0.01$ ) and skin tissue (FED from 1.12 to 7.35,  $p < 0.05$ ). UVR significantly suppressed miR-34c expression in HPK cells (FED from -0.39 to -1.20,  $p < 0.01$ ). miR-34c failed to show a trend of expression in response to UVR in HCT116 (FED from -4.54 to 1.60), HaCaT (FED -0.69 to 5.34) and skin tissue (FED from -0.91 to 1.12).

#### 4.5. DISCUSSION

In 2007 a new element of the p53 network was discovered; miR-34 family which caused a surge in p53-related microRNA research. In particular miR-34a, miR-34b and miR-34c, belong to two different genes miR34A and miR34BC respectively and they are directly transcribed by activated p53 upon DNA damage caused by etoposide, 5-fluorouracil, nutlin-3, adriamycin and ionising radiation (IR) (Bommer *et al*, 2007; Corney *et al*, 2007; Raver-Shapira *et al*, 2007; Tarasov *et al*, 2007). Out of all the miR-34s, miR-34a is the most investigated member of the family. There was no controversy as far as the upregulation of this microRNA is concerned post p53 activation by adriamycin or IR across the literature and the data presented in this project (Figures 4-9 and 4-18); miR-34a is upregulated as suggested by literature in all HPK, HCT116 and skin tissue samples treated with positive controls (adriamycin and IR). miR-34a, in its mature form, it acts as a cell cycle arrest and pro-apoptotic agent by lowering the levels of a) proteins that allow the cell to pass the G1/S and S/G2 transitions of the cell cycle pertinent checkpoints: CDK6, cyclin-E2, CDK4, CCND1, CCNE2 and pRB (Bommer *et al*, 2007; He *et al*, 2007, Sun *et al*, 2008; Navarro *et al*, 2009) b) proteins that enhance cell survival and proliferation without being members of the checkpoint mechanism: BCL-2, C-MET and SIRT1 (Bommer *et al*, 2007; He *et al*, 2007; Yamakuchi *et al*, 2008; Ji *et al*, 2009; Sotillo *et al*, 2011) and last c) the mitogenic protein c-MYC (Wei *et al*, 2008). Kato *et al* reported that miR-34a mutant *C.elegans* worms were susceptible to non-apoptotic death post irradiation from an IR source (Kato *et al*, 2009), which is confirmed in mammalian cells (CCL primary cells) from Zenz *et al* (Zenz *et al*, 2009). In addition Zenz *et al* showed that leukaemia cells with mutated miR-34a exhibit an impaired induction of p53 downstream genes. Corney *et al* first reported that miR-34b and miR-34c inhibit cell proliferation together synergistically, supported by a later paper from Ji *et al* that illustrate all members of the miR34 family to effectively activate caspase-3 and target Notch1 and Notch2 proteins (Corney *et al*, 2007; Ji *et al*, 2009). Mutated p53 gene or absence of p53 protein has been proved to alter miR34s expression profile to minimum detection by qPCR in most of these studies, proving the direct relationship of p53 and miR-34 family. However, in Figure 4.23a it is demonstrated that in HaCaT cells that possess non-functional p53 protein miR-34c is significantly upregulated by adriamycin, thus suggesting

that there can be a compensating mechanism present. In summary, miR-34a transcription and concomitant maturation accelerate the apoptotic function of p53 protein upon DNA damage insults, either by enhancing p53's downstream genes' expression or by silencing cell propagating proteins.

As mentioned in the previous sections of this chapter, given the literature just reviewed, it was hypothesised that since UVR is a major DNA damage factor and it has proved to elevate p53 protein in human skin, then miR-34a would be transcribed by the UVR induced p53 and its levels would be increased. However, strikingly miR-34a levels decreased in UVR irradiated HPK and HCT116 cells while they remained on baseline expression in UVR irradiated skin tissue. p53 protein activation was established by p21WAF1 expression which was increased in all the treatments used for this project and all tissues and cell lines apart from HaCaT cells where p21WAF1 remained the same between control samples and treated. Therefore, this is the first time that miR-34a expression post DNA damage caused by UVR is shown to be possibly caused by an alternative activation pattern of p53.

Firstly, DNA damage is detected preferentially by ATR and PI-3 kinases in UVR irradiated cells but in the case of IR it is mostly by ATM in a distinctly different pattern than in UVR induced DNA damage which affects the concomitant cell signalling pathways (Uncal-Kacmaz *et al*, 2002; Garcia-Muse and Boulton, 2005; Yajima *et al*, 2006). Secondly, p53 has been reported to be phosphorylated differently upon different DNA damage factors; upon UVR insults p53 is phosphorylated on Ser-46, Ser-15 and Ser-392 (Lakin *et al*, 1999; Espinosa, 2008). Particularly the latter phosphorylation (Ser-392) does not occur following IR resulting in sequence specific DNA binding of p53 and impaired growth arrest (Lakin *et al*, 1999). Moreover, there have been several reports on the diversity of the downstream genes of p53 following IR and UVR (Zhao *et al*, 2000; Rieger and Chu, 2004; Latonen and Laiho, 2005). This diversity can be supported by the fact that DNA photoproducts inhibit RNA Polymerase II elongation action and RNA Polymerase II is not affected by p53, so anything that has an impact on RNA Polymerase II in different genotoxic damage treatments will change p53's downstream genes' expression (Latonen and Laiho, 2005; Espinosa 2008; Paris *et al*, 2008). In particular, Paris *et al* exhibit another control

mechanism of the p53 response which accounts for differences in p53 responses of both different cell types and treatments; p21WAF1 mRNA has individual half lives in various cell types after the same treatment and within the same cell type with various treatments, a procedure that is not dependent on p53 (Paris *et al*, 2008). The latter observed phenomenon must be affecting more mRNAs than just p21WAF1, which allows the p53 network to build an immense diversity in its intertwined cell signalling pathways and cell survival outcomes. Notwithstanding, it can be assumed that p53 levels is not the factor that causes the downregulation of miR-34a, but the type of DNA damage insult; as seen in Figures 4-5 and 4-7, miR-34a expression is not proportional to p53 quantity, for example IR induces a higher p53 expression than adriamycin in HPK but a lower miR-34a transcription levels. What's more, the amount of p53 has also been linked to the cell fate post DNA damage; Santoro and Blandino present a scheme where low p53 results in cell proliferation, high p53 results in growth arrest and moderate amounts of p53 lead to cell senescence, all this through the impact of p53 on the balance between mTOR and p21 pathways (Santoro and Blandino, 2010). This observation could be accounted partially to SIRT1 that has been proved to negate the mTOR protein downregulation by stress signals through TC2 (Ghosh *et al*, 2010). Zeng *et al* reported in 2000 that UVR irradiated cells exhibited extensive apoptosis post irradiation in comparison with gamma irradiated cells. In addition, they observed that UVR induced inhibition of MDM-2 protein, a protein that binds p53 and facilitates its degradation. In accordance with this data, the results shown on Figures 4-2 and 4-8, indicate a resistance to gamma radiation from all the cell types examined; p53 was robustly upregulated in UVR irradiated HPK while it was moderately increased in HCT116.

Despite the differences in p53 levels, in Figures 4-2, 4-5 and 4-8, it can be observed that miR-34a exhibits a comparable modulation in HCT116 and HPK cells in all examined treatments. Moreover, in all treatments miR-34a tends to be driven back to its baseline expression levels at 48h post treatment/application of the DNA damaging stimuli (Figures 4-4 and 4-6). Taking all this into account, it can be assumed that miR-34a's role is very specific and it is mostly p53's action that determines cell fate.

In studies assessing the interchanging p53 responses between UVR and IR, a) there was a general consensus that p53 levels rise in response to increasing doses of both UVR

and IR with UVR induced p53 usually peaking around 12-30 hours post irradiation and IR induced p53 varying between 3-48 hours depending on the cell type, b) p53 is stabilised faster in IR treated cells by phosphorylation and acetylation reactions but its turnover is also faster than in UVR treated cells (Lu and Lane, 1993; Liu *et al*, 1994; Ponten *et al*, 2001; Latonen and Laiho, 2005). Furthermore, treatment aside, there is a heterogeneity in the p53 response that is closely related to aging. The amount of p53 produced also determines the downstream genes that will be transcribed depending on the p53's affinity to their promoters; low p53 production favours only the promoters with high affinities while with high p53 production the specificity of the genes transcribed is lower (Espinosa, 2008).

The family of p53 proteins has another two members: p63 and p73. Opinions on their functions vary a lot, with p63 compensating for p53 in p53 null cells under genotoxic stress (Yao *et al*, 2010) or that p63/p73 are required for p53 dependent apoptosis (Flores *et al*, 2002) and finally that p63/p73 are not dispensable for apoptosis (Senoo *et al*, 2004). Protein p63 comes in two splice variants, TAp63 (transcriptionally active) and  $\Delta$ Np63 (transcriptionally inactive).  $\Delta$ Np63 is highly expressed in keratinocyte stem cell, playing a role in stemness and differentiation of the keratinocytes (Koster *et al*, 2004). It was implicated with microRNA research when Antonini *et al* reported that on the presence of p63, miR-34 family is downregulated in mice (Antonini *et al*, 2010). However, they did not investigate how SIRT1 is expressed in this system. They also report that miR-34b and miR-34c were found to be expressed intrinsically low in skin tissue, a finding that is in accordance with my data. On the other hand, in another mouse model,  $\Delta$ Np63 $\alpha$  overexpression gives an accelerated aging phenotype and SIRT1 protein levels are remarkably decreased (Sommer *et al*, 2006). In this case, the scientific group did not investigate miR-34 family expression. No DNA damage agent was used in either of these studies. There is an elusive link between p63, miR-34s and SIRT1 that needs to be clarified.

p63 protein has been associated with UVR induced DNA damage because it has been stated to be degraded upon UVB radiation insult, by p38 MAPK and MDM2 and that this degradation is required for apoptosis to take place (Liefer *et al*, 2000; Galli *et al*, 2010). In an extensive study on the role of p63 in UVB induced DNA damage, Papoutsaki *et al*, unravel that  $\Delta$ Np63 is bound to p53 downstream genes' promoters and upon UVB damage

in human keratinocytes, it is phosphorylated to be degraded and detaches from specific promoters to allow p53 to initiate the transcription of these genes (Papoutsaki *et al*, 2005). They also mention that  $\Delta Np63$  binds to these promoters to promote proliferation in stem keratinocytes. The most striking finding was that the promoter detachment of  $\Delta Np63$  is a p53 independent phenomenon, happening in p53 mutated or p53 null cell lines (Galli *et al*, 2010).

In this project SIRT1, miR-34a's confirmed target does not exhibit a particular modulation post UVR treatment, remaining in the same levels as in the control samples, Wang and Chen show the same result in U2OS and HCT116 cells that were UVR irradiated and they claim that SIRT1 protects the cells from further reactive oxygen species damage (Wang and Chen, 2010). SIRT1 seems to be important in UVR insulted cells according to some recent findings; SIRT1 targets MMP-9 in UVR irradiated skin (Lee *et al*, 2010) and it also regulates UVR induced DNA damage repair through deacetylation of XPA (Fan and Luo, 2010). MMP-9 is a protein that degrades collagen in skin, thus conferring to the loss of thickness of the extracellular matrix in the dermis and XPA is a protein involved in the nucleotide excision repair DNA repair mechanism that is responsible for removing the UVR generated nucleotide photoproducts. As seen on the time course illustrated in Figure 4-6, the expression profile of UVR modulated miR-34a correlates with reported data on UVR irradiated skin CPD formation; CPDs form immediately following UVR exposure and are still detectable up to 24-30hours later but have faded away 2 days post irradiation (Burren *et al*, 1998; Ling *et al*, 2001; Qin *et al*, 2002). In a similar pattern, miR-34a is downregulated in HPK cells (Figure 4-4) 24hours post UVR irradiation and comes back to baseline levels at 48hours. Therefore it can be assumed that UVR induced miR-34a suppression occurs in order to allow SIRT1 to enhance DNA repair mechanisms and promote the division of a mutations-free cell.

SIRT1 modulation post DNA damage occurs through another mechanism shown in human chondrocytes post IR treatment, where SIRT1 was found to be downregulated by p38, which forms a protein complex with it (Hong *et al*, 2009) a phenomenon which could explain why in p53 mutated HaCaT cells protein SIRT1 is downregulated post UVB treatment (Cao *et al*, 2008).



miR-34a seems to be absent from the UVR induced DNA damage response probably to enhance DNA repair and promote cell proliferation via SIRT1. From this literature review it is evident that the cell chooses very carefully how to react to different DNA damaging assaults. The finding of the present project adds up to the building picture of UVR responsive pathways. Nevertheless, the way with which the cells decide to recuperate as much of the DNA lesions as possible, could account for the rising numbers of skin cancers lest mutations are propagated by this perpetuation of UVR damaged cells. Of course, all this data has been derived from lab models that use single doses of UVR deriving from UV lamps instead of solar radiation. Repetitive solar irradiation might have a different impact on the aforementioned factors.

## 5. General Discussion and Future Work

### 5.1. THE HYPOTHESES

#### 5.1.1. microRNAs in ACD

ACD is a very common skin disease affecting 20% of the general population who is allergic to at least one allergen (Lushniak *et al*, 2004; Diepgen and Weisshaar, 2007). These allergens can be found anywhere, from the workplace to the household environment: metals such as nickel, additives such as parabens and solvents such as DNCB and fragrances such as musk are a few examples. ACD can be confirmed with patch testing and the only treatment available is the avoidance of the chemical which can lead to a severe impact in the quality of life of ACD sufferers because it may necessitate having to change jobs (Kadyk *et al*, 2003).

The chemicals initially make contact with the epidermis and due to the small molecular weight of most allergenic compounds, they penetrate the skin barrier and enter into the epidermis. There they form complexes with local proteins that are antigenic and as such are called haptens. The local skin microenvironment is perturbed; keratinocytes secrete pro-inflammatory cytokines and chemokines and local DCs (LCs and dDCs) take up the haptenated proteins (if the proteins are extracellular), process them and migrate from the skin to present them to T-cells in lymph nodes. Memory T-cells are generated and home to the skin. This is the sensitisation phase of ACD. Upon re-exposure to the allergen the T-cells are activated and proliferate (Friedmann *et al*, 2003; Cumberbatch *et al*, 2000; Kripke *et al*, 1990). This phenomenon combined with all the subsequent chemokine and cytokine release from the activated keratinocytes initiates an inflammatory response. Monocytes, macrophages and cytotoxic T-cells are recruited to the site of exposure inflicting tissue injury that is experienced by the patient as a rash with vesicle formation and extensive pruritus (Karlberg *et al*, 2007).

microRNAs are small non-coding RNA transcripts that control gene expression in a post transcriptional manner (Bartel 2004). They have been found to be very important in DC

maturation and antigen presentation processes. miR-155, miR-125b and miR-146a are the most extensively studied microRNAs in innate epithelial and DC immunobiology; miR-146a and miR-125b act as negative feedback mediators for inflammatory signals and miR-155 is a molecular switch for DC maturation and a determinant factor for T<sub>H</sub>1/T<sub>H</sub>2 responses (Taganov *et al* 2006; Rodriguez *et al*, 2007; Thai *et al*, 2007).

Keratinocytes actively participate in the initiation of ACD by secreting TNF- $\alpha$  and IL-1 $\beta$ , two cytokines that facilitate the maturation and migration of skin DCs from the skin to the lymph nodes (Cumberbatch, 2000). The mere maturation and migration of DCs from the skin is a key event of the sensitisation phase of ACD. microRNAs are indispensable in the bridging of innate and adaptive immunity by affecting the maturation of DCs post LPS, TNF- $\alpha$ , IL-1 $\beta$  challenges and since potent allergens have distinct effects on skin DCs, it was therefore hypothesized that DNCB will modulate microRNA expression in MoDCs as well.

#### 5.1.2. miR-34a in UVR skin responses

Exposure of cells to genotoxic agents, including IR, cytotoxic agents and UVR leads to DNA damage which is detected by ATR/ATM proteins which trigger the activation and upregulation of the p53 protein (Kaufmann and Paules, 1996). It is recognised that p53 protein activation in this situation plays a key role in determining cell fate. p53 protein is a tumour suppressor protein; it functions as a transcription factor that inhibits cell proliferation in the presence of DNA damage to maintain the integrity of the genome that will be passed to the daughter cells during cell division (Yoshida and Miki, 2010). p53 protein commences a DNA damage response by inducing cell cycle arrest through the actions of its transactivated genes. Following cell cycle arrest, DNA repair mechanisms take place and depending on the cell type, the dose of the genotoxic stimulus and differentiation status the cell can enter a programmed cell death called apoptosis or survive and resume the normal cell cycle (Lockshin and Zakeri, 2004; Okada *et al*, 2004; Sumpter and Levine, 2010).

In the case of IR and chemical genotoxic agents, it has been reported that p53 protein triggers transcriptional activation of miR-34A and miR-34BC genes which regulate

cell cycle and DNA damage response genes (Tarasov *et al*, 2007; Zenz *et al*, 2009). miR-34a is an essential part of the cellular apoptotic machinery by targeting cyclins and other proliferation promoting proteins (Bommer *et al*, 2007; He *et al*, 2007; Sun *et al*, 2008; Navarro *et al*, 2009). Following miR-34a deletion the cell becomes cancerous; in fact, miR-34a has been found to be epigenetically inactivated in lung, breast, colon, bladder and melanoma cancers (Tarasov *et al*, 2007). miR-34a represses the translation of silent mating type information regulation 2 homolog 1 (SIRT1) (a NAD dependent histone deacetylase) leading to downregulation of its protein expression (Yamakuchi *et al*, 2008; Luan *et al*, 2010). Apart from histones, SIRT1 activates or inhibits the function of an array of proteins involved in cell cycle regulation, cell senescence, DNA repair and telomere maintenance via deacetylation (Deng, 2009; Palacios *et al*, 2010). The effect of SIRT1 is cell longevity and DNA repair enhancement upon DNA damage (Kwon and Ott, 2008).

However the effect of UVR on miR-34a (or miR-34b,-34c) has not been investigated as yet in published work. Biological responses of human skin to UVR are of vital importance for the human body's homeostasis. However, UVR can be equally harmful since it causes DNA damage by directly interacting with DNA bases forming CPDs and 6-4 photoproducts which are subsequently repaired by NER (Trautinger *et al*, 2001; Jenkins 2002; Ichihashi *et al*, 2003; Tsourelis-Nikita *et al*, 2005). p53 protein is activated and upregulated post skin exposure to UVR (Burren *et al*, 1998; de Winter *et al*, 2001; Murphy *et al*, 2002). p53 protein is epigenetically inactivated, mutated or deleted through loss of heterozygosity in many cancer types (Bates *et al*, 1999; Gruijl *et al*, 2001).

IR and adriamycin induce the transcription of miR-34a, and decrease the protein levels of SIRT1 through a p53 dependent pathway. This project's aim is to elucidate whether miR-34a and SIRT1 are parts of the p53 dependent UV induced DNA damage response.

## 5.2. THE FINDINGS

### 5.2.1. microRNA signature in ACD

The potent sensitizer DNCB was employed in order to investigate miR-155, miR-125b and miR-146a in skin sensitisation *ex vivo* and *in vitro*. Redundant skin from mastectomy operations was mounted on Franz diffusion chambers that mimic physiological skin conditions. DNCB was applied onto the epidermal side of the skin biopsies and the epidermises were then fixed and stained with anti-DC1a/FITC antibodies (CD1a is a LC marker). LC migration was measured in two different Franz diffusion apparatuses with different sensitisation areas; 3mm and 7mm respectively. Consistent and reproducible LC migration post DNCB application was noted on skin explants that had been mounted on the 7mm diameter sensitisation area diffusion chambers (Figure 3-3). The *ex vivo* skin explant model in this project is a suitable model of skin sensitisation since the control *ex vivo* skin samples exhibit a similar LC morphology to *in vivo* skin and LC numbers were consistently decreased in the DNCB treated *ex vivo* samples which is similar to what is seen in the *in vivo* situation following DNCB application. This is very important as animal testing will be banned soon and there is a need for other lab techniques that can predict contact allergens (Aeby *et al*, 2010).

Expression of miR-155, miR-125b and miR-146a was quantified in DNCB treated skin explants and HPK (human primary keratinocytes). miR-125b has been reported to be significantly downregulated in psoriatic keratinocytes with a concomitant increase of its target FGFR2, thus promoting cell proliferation (Sonkoly *et al*, 2007; Xu *et al*, 2011). In addition, miR-155 has only recently been implicated in skin biology; Wald *et al* report that miR-155 is down-regulated in HPV positive SCCs (Wang *et al*, 2011). Therefore there were no predictions concerning any alteration of the expression profile of these microRNAs caused by DNCB treatment. In fact, DNCB failed to cause a significant modulation on the overall expression of these microRNAs in skin tissue and keratinocytes.

MoDCs were treated with DNCB using two different protocols: a) direct application of DNCB or b) MoDCs were suspended in DNCB treated HPK supernatants (i.e. DNCB\*). DCs

are affected by the tissue environment they reside in and specifically LCs' function is enhanced in the presence of keratinocytes (Asahina and Tamaki, 2006; Sugita *et al* 2006; Chorro *et al*, 2009). Therefore it was considered important that miR-155, miR-125b and miR-146a expression was assessed in MoDC samples treated with both DNCB and DNCB\* in order to elucidate the impact of keratinocyte derived mediators or keratinocyte processed DNCB on the candidate microRNAs expression profile.

Direct DNCB application in MoDC cultures failed to show a distinct modulation of miR-155 and miR-125b (Figure 3-11a,-b). However, DNCB treatment inflicted a significant downregulation of miR-146a (Figure 3-11c) while this was not seen with DNCB\* treatment. miR-146a is a transcriptional target of transcription factor NF- $\kappa$ B and is induced by cytokines TNF- $\alpha$  and IL-1 $\beta$  (Taganov *et al*, 2006). miR-146a is a negative regulator of IL-1 $\beta$  and IFN pathways (Perry *et al*, 2008; Tang *et al*, 2009). Interestingly, this microRNA promotes gut epithelial immune tolerance (Chassin *et al*, 2010). IL-1 $\beta$  is a key cytokine in LC migration and keratinocyte-LC communication, its upregulation is crucial in inflammatory responses as it induces inflammasome activation and chemokine release in keratinocytes (Griffiths *et al*, 2005; Watanabe *et al*, 2007). As previously stated in section 3.5, miR-146a suppression implies a communication step between DCs and keratinocytes, during which keratinocytes may modify or limit the pro-inflammatory response of the DCs to DNCB. This is also supported by the fact that DNCB\* treatment significantly induces the upregulation of miR-125b that represses the translation of pro-inflammatory cytokine TNF- $\alpha$  and promotes the downstream effects of NF- $\kappa$ B (Tili *et al*, 2007; Murphy *et al*, 2010). NF- $\kappa$ B is a transcription factor that coordinates effective antigen presentation in DCs which suggests that the effects on DNCB via miR-125b might modify antigen presentation by the LCs or dermal DCs (Yoshimura *et al*, 2001).

MoDC maturation markers expression was assessed by flow cytometry in order to compare DNCB and DNCB\* treatments (Figure 3-14). Overall, CD86 ( $p < 0.05$ ) and HLA-DR (ns) levels showed a trend towards downregulation in MoDCs treated with HPK supernatant controls (supernatants from HPK treated with DMSO) (Figure 3-14b). Downregulation of HLA-DR and CD86 has been reported to be an effect of calcitonin gene related peptide on mature DCs and part of endotoxin tolerance in macrophages (Carucci *et al*, 2000;

Muthukuru and Cutler, 2008). A tendency towards CD86 and HLA-DR upregulation was observed in DNCB treated MoDCs whereas CD1a showed a tendency towards downregulation in DNCB\* treated MoDCs. The observed changes were subtle but still, the absence of significant changes in MoDC maturation markers post HPK supernatants treatments supports more the existence of an HPK derived immunosuppressive agent in Control\* and DNCB\* samples.

In the search of other microRNAs involved in ACD sensitisation phase two sets of Taqman qPCR arrays were performed. MoDCs were treated with DNCB for the first set and DNCB\* for the second. The qPCR array cards contained primers for ~380 well characterised microRNAs. DNCB\* treatment failed to yield any new significantly modulated microRNAs. DNCB treatment on the other hand resulted in an upregulation of miR-138 in MoDCs. miR-138 has been reported as an apoptosis promoting microRNA triggered by DNA damage (Wang et al, 2011), however, SOCS5, SOCS6, PPAR-D and GTP binding protein are some of miR-138 targets as predicted by TargetScan, implying an immunomodulatory role for this microRNA.

The third approach utilised for the investigation of microRNAs in the sensitisation phase of ACD was the silencing of microRNA biogenesis component Dicer. The rationale of these experiments was to block the microRNA machinery and observe the impact of the absence of microRNAs in MoDC maturation. However Dicer knockdown with a specific siRNA did not alter microRNA levels (Figures 3-18, 3-19) therefore this approach was abandoned.

#### 5.2.2. miR-34a in response to UVR

p53 protein expression in response to different doses of UVR was quantified in HPK. 151mJ/cm<sup>2</sup> of UVR induced a significant increase in p53 protein levels (Figure 4-2). This dose was then utilised in a time course to investigate miR-34a expression, but miR-34a did not increase following UVR and in fact decreased at 24 hours (Figure 4-4).

UVR treatment was compared with IR and adriamycin treatments in cells and with IR in tissue. IR and adriamycin induced upregulation of p53 protein levels in HPK and HCT116 cells (Figure 4-5 and 4-8) followed by the induction of miR-34a transcription (Figure 4-6 and 4-9). Different doses and application periods for UVR, adriamycin and IR treatments were employed in miR-34a detection experiments of HCT116 and HPK cells in order to see whether the downregulation of miR-34a after 151mJ/cm<sup>2</sup> of UVR at 24h post irradiation is an isolated phenomenon or whether this suppression is also seen at other doses of, or time points following UVR (Figure 4-7). Downregulation of miR-34a was consistent at all UVR doses. Similarly upregulation of miR-34a was seen at all IR and adriamycin doses. miR-34a transcription levels reduce towards baseline levels in IR and adriamycin treated samples collected at 48h. Transcriptional activation of p53 protein was established by p21WAF1 mRNA levels which were induced in HCT116 and HPK cells by all treatments but not in HaCaT cells (Figure 4-11). Levels of miR-34b and -34c mainly followed a similar pattern to miR-34a post UVR in HPK; they were both significantly suppressed (Figures 4-21 and 4-22).

p53 protein levels were increased in skin explants by UVR and IR treatments as established by immunohistochemistry of epidermal sheets (Figures 4-13, 4-14 and 4-17). miR-34a was significantly induced in *ex vivo* IR treated skin tissue whereas UVR did not modulate miR-34a expression (Figure 4-18a). p21WAF1 was significantly induced post both treatments.

miR-34a's confirmed target SIRT1 was detected by western blotting. Adriamycin and IR were expected to induce a suppression of the protein's levels as a consequence of miR-34a upregulation. Indeed, SIRT1 was found to be significantly suppressed in IR and adriamycin treated samples whereas it remained unchanged in UVR irradiated samples (Figure 4-20).

p53 protein is differentially phosphorylated following different DNA damaging agents (Lakin et al, 1999; Espinosa, 2008) which affects the subsequent activation of downstream genes (Zhao et al, 2000; Rieger and Chu, 2004; Latonen and Laiho, 2005) this differential phosphorylation might explain the increase in miR-34a after IR and the absence of miR-34a induction in UVR treated cells and skin tissue. In addition, p53 protein nuclear



concentration is a determinant of the repertoire of downstream p53 protein genes; in low p53 protein concentrations, p53 protein induces the transcription of genes that harbour promoters with high affinity for p53 while low affinity genes are transcribed in high p53 concentrations (Resnick-Silverman et al, 1998; Zhao et al, 2000; Szak et al, 2001). However, the mean fluorescence intensity of p53 did not seem to differ between the three stimuli (IR, adriamycin and UVR) so an alternative possible explanation for the difference between UVR and the other stimuli might be that the cell signalling pathways after UVR, but not after IR and adriamycin, results in the production of an inhibitor of miR-34a transcription. It is also possible that p63, a homologue of p53, is involved. In an extensive study on the role of p63 in UVB induced DNA damage, Papoutsaki et al, unravel that  $\Delta$ Np63 is bound to p53 protein downstream genes' promoters and upon UVB damage in human keratinocytes, it is phosphorylated to be degraded and detaches from specific promoters to allow p53 protein to initiate the transcription of these genes (Papoutsaki *et al*, 2005). They also mention that  $\Delta$ Np63 binds to these promoters to promote proliferation in stem keratinocytes. The most striking finding was that the promoter detachment of  $\Delta$ Np63 is a p53 protein independent phenomenon, happening in p53 protein mutated or p53 protein null cell lines (Galli *et al*, 2010) which adds a new light in the modulation of miR-34a in UVR biology since it has also been proved that mice overexpressing p63 present low miR-34a,-34b,-34c levels (Antonini *et al*, 2010).

SIRT1 has been recognised recently as an important protein in skin biology. SIRT1 inactivates MMP-9 in UV irradiated skin (Lee *et al*, 2010) and it also enhances UV induced DNA damage repair through deacetylation of XPA (Fan and Luo, 2010). As UVR is known to induce differentiation in keratinocytes via the Vitamin D and CaR pathways, the maintenance of SIRT1 levels post UVR exposure seems necessary since SIRT1 induces differentiation in keratinocytes that is very important in UVR exposed skin (Bikle, 2004; Blander *et al*, 2009). SIRT1 inhibits apoptosis and promotes DNA repair instead by binding E2F1 apoptotic transcription factor which could facilitate the survival of cells containing DNA mutations and concomitant carcinogenesis; SIRT1 has been found to be overexpressed in several cancers (Wang *et al*, 2006; Saunders and Verdin, 2007).

Biological responses to UVR and contact allergens are interconnected in skin tissue. As mentioned in Introduction, UVR induces immunosuppression and tolerance to contact allergens. SIRT1 can be implicated in the UV induced immunosuppression pathways since it repressed NF- $\kappa$ B transactivation potential in pancreatic  $\beta$ -cells and protects them from cytotoxicity caused by IL-1 $\beta$  and IFN- $\gamma$  (Lee *et al*, 2009). In addition SIRT1 is involved in epigenetic reprogramming that results in endotoxin tolerance in a THP1 model, by repressing TLR4 induced TNF- $\alpha$  transcription (Liu *et al*, 2010).

Both ACD flares and UV induced apoptosis are aggravated when FAS receptor is absent in keratinocytes (Hedrych-Ozimina *et al*, 2011). Interestingly, FAS is predicted by TargetScan version 5.2 to be targeted by miR-138 and miR-146a.

### 5.3. CONTRIBUTION OF THESE FINDINGS TO SKIN BIOLOGY AND FUTURE WORK

microRNAs have not been linked directly with ACD sensitisation as yet. miR-146a, miR-125b and miR-138 have been depicted as possible key players of the DC response to the potent contact allergen DNCB. Of course, more work is required in order to elucidate the exact role of each of the afore-mentioned microRNAs in ACD initiation.

It would be interesting to assess the expression of maturation markers, cytokine release and T-cell proliferation by DNCB treated MoDCs with overexpressed or silenced miR-146a, miR-125b and miR-138. In addition, HPK-MoDC or HPK-*in vitro* generated LC co-cultures could be used instead of MoDCs in order to investigate the direct impact of keratinocyte-DC communication in microRNA expression and sensitisation progression.

In the case of miR-146a and miR-125b that are implicated in the IL-1 $\beta$  pathway, transfected MoDC or *in vitro* generated LC migration models could elucidate the orchestration of DC motility and maturation upon DNCB exposure.

miR-138 is a rising star in the biology of ACD sensitisation, since according to TargetScan version 5.2 (July 2011), NLRP1 is a predicted target of miR-138. NLRP1 is a structural and functional component of one of the two inflammasome conformations implicated in ACD. As miR-155 promotes DC maturation by limiting the ability of the DC to

phagocytose more antigens, miR-138 could also be controlling inflammasome activation in order for no more DNCB to be uptaken by the DC and to polarise the cell towards presentation.

An array of other potent sensitizers and weaker sensitizers could be assessed in the MoDC model presented in this thesis and the expression of miR-146a, miR-125b and miR-138 could be investigated. If the expression profiles are similar to the ones portrayed here, then these microRNAs can be used as prediction markers for antigenicity of potential contact allergens. In addition, it is proved that DNCB has an impact on microRNA expression but further investigation is needed in order to verify whether any artificial interference on the expression of these microRNAs (miR-146a, miR-125b and miR-138) can act as a halt to the progression of DNCB induced ACD reactions.

UVR has been proven not to induce miR-34a in HPK, HCT116 cells and skin tissue through the work done for this project. This is a novel discovery in UVR induced DNA damage responses and combined with the facts that a) miR-34a has been found inactivated in many cancers and b) SIRT1 (miR-34a silencing target) inhibits apoptosis favouring differentiation and DNA repair, it leads to the question of whether the lack of an increase in miR-34a following UVR could account for the high occurrence of non-melanoma skin cancers due to UVR.

It is of great importance to investigate the survival or apoptotic status of UVR exposed normal HPK cells and cancerous skin cells that overexpress miR-34a. This experiment will provide more information concerning the exact role of miR-34a in skin tissue. In addition, investigations on p63 could also identify whether this transcription factor prevents p53 from inducing miR-34a in UVR exposed cells and tissue. The results of chapter 4 indicate that the p53-miR-34a-SIRT1 pathway is indeed differently modulated in UVR exposed cells and tissue. As discussed in section 4.5, this gives UVR exposed cells a cell cycle 'window' where DNA mutations could potentially be propagated. Further work is needed in order to confirm whether this novel behaviour of miR-34a in response to DNA damage is responsible for the high occurrence of sun induced non-melanoma skin cancers.

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