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**Regulation of NF- κ B-mediated inflammatory
responses by miR-378a-3p in 3D epidermal model**

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Regulation of NF- κ B-mediated inflammatory responses by miR-378a-3p in 3D epidermal model

Abstract

miR-378a-3p has previously been shown to be upregulated under the inflammatory stimulus and in psoriatic skin lesions, suggesting a possible role in psoriasis-associated inflammatory signalling. Previous studies have used two-dimensional (2D) keratinocyte culture model and reported that miR-378a-3p activates NF- κ B signalling, resulting in increased *CXCL8* (IL-8) expression. However, the effects of miR-378a-3p in differentiated keratinocyte culture models have not previously been investigated. Therefore, this thesis focuses on analysing the role of miR-378a-3p in three-dimensional (3D) keratinocyte culture models by comparing its effects on inflammatory gene expression in conventional 2D and differentiated 3D keratinocyte cultures.

The study examined the effect of miR-378a-3p transfection on inflammatory gene expression, with particular focus on NF- κ B pathway. In the course of the study, expression levels of *CXCL8* expression and selected NF- κ B-associated genes (*TRAF6*, *NFKB1A*, and *EGFR*) were evaluated under both unstimulated and IL-17A-stimulated conditions. In addition, the effect of miR-378a-3p transfection on keratinocyte differentiation- and proliferation-related genes (*KRT5*, *KRT14*, *KRT10*, and *INV*) were investigated.

The obtained results demonstrated distinct effect of miR-378a-3p between 2D and 3D culture system. In 2D cultures, miR-378a-3p increased *CXCL8* expression, consistent with previously reported pro-inflammatory effects. In contrast, differentiated 3D cultures showed reduced *CXCL8* expression together with downregulation of *TRAF6* and *EGFR* following miR-378a-3p transfection. Overall, the findings suggest that the effect of miR-378a-3p may depend on differentiation state and culture conditions.

Keywords

microRNA-378a-3p, 3D cell culture, keratinocytes, NF- κ B signalling, psoriasis

CERCS: B500 Immunology, serology, transplantation

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NF-κB-vahendatud põletikuliste vastuste reguleerimine miR-378a-3p poolt 3D epidermaalses mudelis

Kokkuvõte

Varasemates uuringutes on näidatud, et miR-378a-3p ekspressioon suureneb põletikulistes tingimustes ja psoriaatilistes löövetes, viidates selle võimalikule rollile psoriaasiga seotud põletikku reguleerivas signaalrajas. Senised uuringud on peamiselt keskendunud kahemõõtmeliste (2D) keratinotsüütide rakukultuuri mudelitele ning on näidatud, et miR-378a-3p aktiveerib NF-κB signaalirada, mille tulemusena suureneb *CXCL8* (*IL-8*) ekspressioon. Samas ei ole miR-378a-3p mõju diferentseerunud keratinotsüütide mudelites varem uuritud. Käesolev bakalaureusetöö keskendub miR-378a-3p rolli analüüsimisele kolmemõõtmelistes (3D) keratinotsüütide rakukultuuri mudelites, võrreldes selle mõju põletikuga seotud geeniekspressioonile tavapärastes 2D ja diferentseerunud 3D keratinotsüütide rakukultuurides.

Töös uuriti miR-378a-3p transfektsiooni mõju põletikuga seotud geenide ekspressioonile, keskendudes eelkõige NF-κB signaalrajale. Uuringu käigus hinnati *CXCL8* ning valitud NF-κB-ga seotud geenide (*TRAF6*, *NFKB1A* ja *EGFR*) ekspressioonitasemeid nii stimuleerimata kui ka IL-17A-ga stimuleeritud tingimustes. Lisaks uuriti miR-378a-3p transfektsiooni mõju keratinotsüütide diferentseerumise ja proliferatsiooniga seotud geenidele (*KRT5*, *KRT14*, *KRT10* ja *INV*).

Tulemustest selgus, et miR-378a-3p toime erineb 2D ja 3D rakukultuuri mudelite vahel. 2D rakukultuurides suurendas miR-378a-3p *CXCL8* ekspressiooni, mis on vastavuses varasemalt kirjeldatud põletikku soodustava toimega. Seevastu diferentseerunud 3D rakukultuurides täheldati pärast miR-378a-3p transfektsiooni *CXCL8* ekspressiooni vähenemist koos *TRAF6* ja *EGFR* ekspressiooni allareguleerimisega. Üldiselt viitavad saadud tulemused sellele, et miR-378a-3p toime võib sõltuda keratinotsüütide diferentseerumisastmest ja rakukultuuri tingimustest.

Võtmesõnad:

mikroRNA-378a-3p, 3D-rakukultuur, keratinotsüüdid, NF-κB signaalimine, psoriaas

CERCS: B500 Immunoloogia, seroloogia, transplantoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

2D – Two-dimensional

3' UTR – 3' Untranslated region

3D – Three-dimensional

AD – Atopic dermatitis

AGO – Argonaute

ALI – Air-liquid interface

CCL20 – C-C motif chemokine ligand 20

CCL27 – C-C motif chemokine ligand 27

cDNA – Complementary DNA

CPP – Cell-penetrating peptide

CXCL8 – C-X-C motif chemokine ligand 8

EEF1A1 – Eukaryotic translation elongation factor 1 alpha 1

EGFR – Epidermal growth factor receptor

ELISA – Enzyme-linked immunosorbent assay

I κ B – Inhibitor of kappa B

I κ B α – Inhibitor of kappa B alpha

IKK – I κ B kinase

IL – Interleukin

IL-8 – Interleukin-8

INV – Involucrin

KGM3 – Keratinocyte Growth Medium 3

KRT5 – Keratin 5

KRT10 – Keratin 10

KRT14 – Keratin 14

miRISC – miRNA-induced silencing complex

miRNA – MicroRNA

mRNA – Messenger RNA

MRE – miRNA response element

NF- κ B – Nuclear factor kappa B

NHEK – Normal human epidermal keratinocyte

PF14 – PepFect14

pre-miRNA – Precursor microRNA

pri-miRNA – primary microRNA

PV – Psoriasis vulgaris

RISC – RNA-induced silencing complex

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

TGF – Transforming growth factor

Th17 – T helper 17 cells

TNF – Tumour necrosis factor

TRAF6 – TNF receptor-associated factor 6

$\Delta\Delta$ Ct Delta-delta cycle threshold

INTRODUCTION

Psoriasis is a common chronic inflammatory skin disease characterised by red, raised plaques with silvery scales, epidermal hyperplasia, and abnormal keratinocyte differentiation (Raharja et al., 2021). Such epidermal dysfunction arises from the pathogenic cyclic state, which propagates the inflammatory response: keratinocyte hyperproliferation in response to cytokines produced by immune cells, leading to the production of even more inflammatory signals, which, in turn, recruit more immune cells and sustain inflammation (Griffiths et al., 2021a). Even though the complete mechanism of psoriasis is not fully understood, several miRNAs have been reported to be dysregulated in psoriatic skin and may contribute to inflammatory responses by modulating gene expression through mRNA degradation or translational repression (Dopytalska et al., 2023).

Recent studies have reported increased microRNA-378a-3p (miR-378a-3p) expression in keratinocytes from psoriatic skin lesions compared with keratinocytes from healthy skin (Xia et al., 2022). Additionally, IL-17A, a key cytokine involved in psoriasis, has been shown to induce miR-378a-3p expression in keratinocytes. In cultured keratinocytes, transfection with miR-378a-3p mimic was found to activate NF- κ B signalling by targeting *NFKB1A*, a negative regulator of the NF- κ B pathway (Xia et al., 2022). Furthermore, miR-378a-3p was reported to reduce keratinocyte proliferation and may regulate the pathogenesis of psoriasis through modulation of TGF- β signalling via *BMP2* (Soonthornchai et al., 2021a). These findings suggest that miR-378a-3p may be a key player in the psoriasis signalling pathway, and investigating its role in the inflammation is crucial for understanding the disease mechanism.

Previous studies on miR-378a-3p have mainly used two-dimensional (2D) keratinocyte culture models, which do not fully reflect the structural complexity of human skin. In contrast, three-dimensional (3D) keratinocyte culture systems allow keratinocytes to differentiate and stratify, thereby more closely mimicking tissue structure (Barua et al., 2022; Habanjar et al., 2021; T. Sun et al., 2006). Considering that skin inflammatory diseases are closely associated with epidermal differentiation and barrier formation, 3D culture systems are especially valuable for studying inflammatory pathways.

This study aims to investigate the role of miR-378a-3p in regulating inflammatory gene expression in keratinocytes, mainly focusing on the NF- κ B pathway, using a 3D keratinocyte culture model. By transfecting miR-378a-3p mimic and analysing inflammatory gene expression changes, this study aims to determine whether miR-378a-3p functions as a pro-inflammatory or anti-inflammatory regulator. In addition, the study compares inflammatory gene expression profiles between 2D and 3D keratinocyte culture systems. Finally, because

psoriasis is associated with abnormal keratinocyte proliferation and differentiation (Griffiths et al., 2021b), this study also examines whether miR-378a-3p affects genes related to keratinocyte proliferation and differentiation.

1 LITERATURE REVIEW

1.1 KERATINOCYTES

Keratinocytes are the most abundant cell type of the epidermis, which serve as the body's outer protective barrier. The primary function of keratinocytes includes restoration of the skin barrier and wound repair by executing migration, proliferation, and differentiation. Additionally, keratinocytes actively participate in immune-sensing functions through pattern-recognition receptors, which constitute the first line of immune response (Al-Dhubaibi et al., 2025). Moreover, keratinocytes can also directly present antigens to T cells to generate a subsequent immune response.

Keratinocytes play a crucial role in driving and amplifying inflammation through the production of inflammatory mediators, such as $\text{TNF}\alpha$, IL-1, IL-6, and IL-36, and chemokines, such as CCL20 and IL-8 (Piipponen et al., 2020). Additionally, keratinocytes participate in immune cell recruitment by secreting factors that activate neutrophils, macrophages, and T cells, thereby recruiting them to the site of inflammation (Piipponen et al., 2020).

Taking the functionality of keratinocytes into account, in chronic inflammatory conditions, including psoriasis, keratinocytes exhibit pathological behaviour. They enter acanthosis, a hyperproliferative, cyclic state, as a response to cytokines produced by immune cells (specifically the IL-23/IL-17 axis). This results in the production of even more inflammatory signals, which, in turn, recruit more immune cells (Griffiths et al., 2021).

1.2 PSORIASIS

Psoriasis is a chronic immune-mediated inflammatory skin disease that affects more than 60 million people worldwide and currently has no cure (Raharja et al., 2021). The main factors in these disorders were identified as genetic susceptibility and environmental triggers. Psoriasis is characterised by thickening of the epidermis and formation of red plaques with silvery scales associated with abnormal keratinocyte proliferation and differentiation. The pathogenesis of psoriasis is driven by both innate and adaptive immune system components. The IL-23/IL-17 cytokine axis is considered one of the central mechanisms driving psoriasis progression. Dendritic cells become activated and release IL-23 signalling molecules, which maintain and expand Th17 and Tc17 cells. T cells release IL-17, which is one of the central cytokines in psoriasis. IL-17 stimulates keratinocytes to hyperproliferation and induces the production of additional proinflammatory mediators, chemokines, and antimicrobial proteins, which attract

more immune cells and generate more inflammatory signals, creating a positive-feedback loop of keratinocyte hyperproliferation (Griffiths et al., 2021a).

1.3 NF- κ B PROTEIN FAMILY

Nuclear factor kappa B (NF- κ B) is a family of transcription factors composed of five structurally related members, which means that they are proteins that enter the cell nucleus, bind to the DNA and regulate the activity of certain genes, more specifically genes responsible for the immune and inflammatory responses (Goldminz et al., 2013a). NF- κ B transcription factors such as RelA (p65), RelB, c-Rel, NF- κ B1 (p50/p105), and NF- κ B2 (p52/p100) are found in many cell types and play an important role in immunity, inflammation, cell survival, and stress response. Under resting conditions, NF- κ B dimers are inactive in the cytoplasm by inhibitor proteins called I κ Bs, which stop the transcription factors from entering the nucleus. Following exposure to inflammatory stimuli, or danger signals, the I κ B kinase (IKK) complex gets activated and phosphorylates I κ B proteins, leading to their ubiquitination and proteasomal degradation. As a result, NF- κ B dimers are released and translocate into the nucleus, where they regulate inflammatory gene transcription (Liu et al., 2017a).

There are two signalling axes that mediate NF- κ B activation: the canonical pathway and the non-canonical pathway. The stimuli and specific protein kinases vary between the two pathways, but both of them are crucial for immune and inflammatory responses (Figure 1) (Goldminz et al., 2013b).

1.3.1. The Canonical Pathway

The canonical NF- κ B pathway is a classical pro-inflammatory signalling pathway, with a rapid and short-lasting nature (Lawrence, 2009). This pathway can be activated by various factors, including pro-inflammatory cytokines (TNF- α and IL-1), microbial products that bind Toll-like receptors (TLRs), and antigens that activate T-cell and B-cell receptors (Liu et al., 2017a) (Figure 1).

Activation of the canonical pathway depends on the IKK complex, which is composed of two catalytic subunits (IKK α and IKK β) and a regulatory subunit NEMO (IKK γ). IKK β phosphorylates the inhibitory protein I κ B α (Liu et al., 2017a). Consequently, after I κ B α is phosphorylated, it undergoes polyubiquitination and proteasomal degradation. After the I κ B α is removed, NF- κ B dimers, predominantly p50/RelA (p65) and p50/c-Rel, translocate into the nucleus to activate the transcription of target genes, such as cytokines, chemokines, and adhesion molecules (Liu et al., 2017a).

1.3.2. The Non-canonical Pathway

The response of the non-canonical signalling axis is slower and is more specialised than the canonical pathway, since it is dependent on protein synthesis (Liu et al., 2017a). The non-canonical pathway is not activated by TNF- α (Lawrence, 2009) and instead triggered by specific subset of the TNF receptor superfamily members, including CD40 ligand, B-cell activating factor (BAFF), lymphotoxin- β (LT β R), and receptor activator of NF- κ B ligand (RANKL) (Goldminz et al., 2013b).

This pathway mainly regulates lymphoid organ development, B-cell activation, and adaptive immune responses (Goldminz et al., 2013b). Notably, unlike the canonical pathway, the non-canonical pathway depends on NF- κ B-inducing kinase (NIK), which activates IKK α instead of IKK β . Instead of degrading I κ B α , the pathway proceeds to the p100 precursor protein, followed by p100 phosphorylation by IKK α . This process triggers ubiquitination of p100 and the degradation of its C-terminal domain, generating the mature p52 subunit. As a result, the p52/RelB dimer is able to translocate into the nucleus (Liu et al., 2017a).

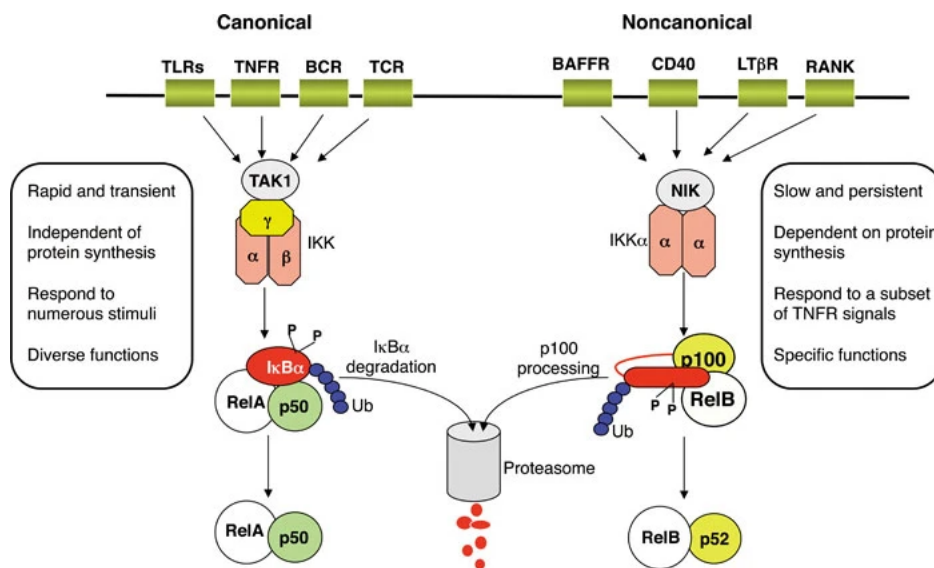


Figure 1. Canonical and non-canonical NF- κ B signalling pathways.

Canonical pathway can be initiated by the signal from both innate and adaptive immune receptors. The trigger response results in the activation of the IKK complex by Tak1. Afterwards, IKK phosphorylates I κ B α , causing its degradation, which leads to the rapid and transient nuclear translocation of the prototypical NF- κ B heterodimer RelA/p50. In contrast, Non-canonical NF- κ B pathway relies on phosphorylation of p100, which is triggered by TNFR members signalling. This pathway is dependent on NIK and IKK α , but not on the trimeric IKK

complex, and mediates the persistent activation of RelB/p52 complex. Source: (S. C. Sun, 2011).

1.3.3. Role in Pro-inflammatory Gene Induction

NF- κ B is a central transcriptional regulator of inflammatory response (Lawrence, 2009). NF- κ B is responsible for regulating a vast array of genes involved in the initiation and amplification of inflammation (Liu et al., 2017a).

NF- κ B induces the expression of major pro-inflammatory cytokines including TNF- α , IL-1 β , IL-6, and IL-12. In addition, it induces several chemokines, such as IL-8, MCP-1, and RANTES, which recruit immune cells to areas of injury or infection (Liu et al., 2017a). In inflammatory skin diseases such as psoriasis, NF- κ B also facilitates the production of CCL20 and CCL27, which recruit dendritic cells and Th17 cells into the skin (Goldminz et al., 2013b).

The NF- κ B pathway also regulates the expression of cell-surface adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, thereby facilitating leukocyte migration from the blood into tissues (Liu et al., 2017a). Furthermore, NF- κ B promotes transcription of NLRP3 and pro-IL-1 β , which serve as precursors for the inflammasome complex and the subsequent secretion of mature IL-1 β (Liu et al., 2017b).

NF- κ B signalling can also function cooperatively with other inflammatory pathways. For example, the IL-17A/F heterodimer activates genes like DEFB4 and S100A7, which are associated with psoriasis, through a mechanism involving NF- κ B and the transcriptional co-activator I κ B ζ (Bertelsen et al., 2018).

1.3.4. NF- κ B in Chronic Inflammatory Diseases

Since the NF- κ B pathway controls the expression of pro-inflammatory cytokines, chemokines and adhesion molecules that are responsible for continuous tissue damage, the pathway dysregulation is characteristic of numerous chronic inflammatory diseases such as psoriasis, rheumatoid arthritis, and inflammatory bowel disease (Liu et al., 2017b). For example, phosphorylated NF- κ B levels are found to be 60-fold higher in psoriatic skin lesions than in healthy skin (Lizzul et al., 2005). It is assumed that NF- κ B may link keratinocyte hyperproliferation with the overactive states of immune cells in psoriatic conditions (Goldminz et al., 2013a).

One of the main inflammatory cytokines driving the psoriatic pathway is considered to be IL-17A, which is primarily produced by Th17 cells and functions as a signalling molecule that, upon binding to a specific receptor, activates several intracellular signalling pathways, including p38 MAPK, NF- κ B, Act1 and TRAF6. The amplification of the inflammatory

response in psoriasis by IL-17A is mainly mediated through the induction of a transcriptional co-activator I κ B ζ via p38 MAPK and NF- κ B pathways. When activated, I κ B ζ drives the expression of several specific proteins associated with skin inflammation such as CCL20, hBD2, S100A7 and IL-8 (Bertelsen et al., 2018).

1.4. miRNA

microRNAs (miRNAs) are small non-coding RNA molecules approximately 22 nucleotides long that function as post-transcriptional regulators of gene expression. They are involved in crucial cellular processes such as proliferation, differentiation, and apoptosis (O'Brien et al., 2018a).

1.4.1. miRNA Biogenesis

During miRNA biogenesis, long primary RNA transcripts are processed into functional small non-coding RNA molecules. miRNA biogenesis occurs through canonical and non-canonical pathways.

The canonical pathway is the primary mechanism of miRNAs processing, during which miRNAs are transcribed from DNA sequences by RNA polymerase II/III into primary miRNAs (pri-miRNAs) (Figure 2). Afterwards, the RNA-binding protein DGCR8, together with ribonuclease III enzyme Drosha, processes pri-miRNAs into precursor miRNAs (pre-miRNAs) by cleaving the pri-miRNA at the base of its characteristic hairpin structure, generating a 2-nucleotide 3' overhang. The Exportin-5 (XPO5)/RanGTP complex then transports pre-miRNA from the nucleus to the cytoplasm, where the enzyme Dicer (an RNase III endonuclease) removes the terminal loop of the pre-miRNA, resulting in the formation of a mature miRNA duplex (O'Brien et al., 2018b). One strand of the duplex is selected as the guide strand depending on thermodynamic stability and the 5' nucleotide (Winter et al., 2009). The guide strand is subsequently loaded into Argonaute (AGO) protein, forming the miRNA-induced silencing complex (miRISC).

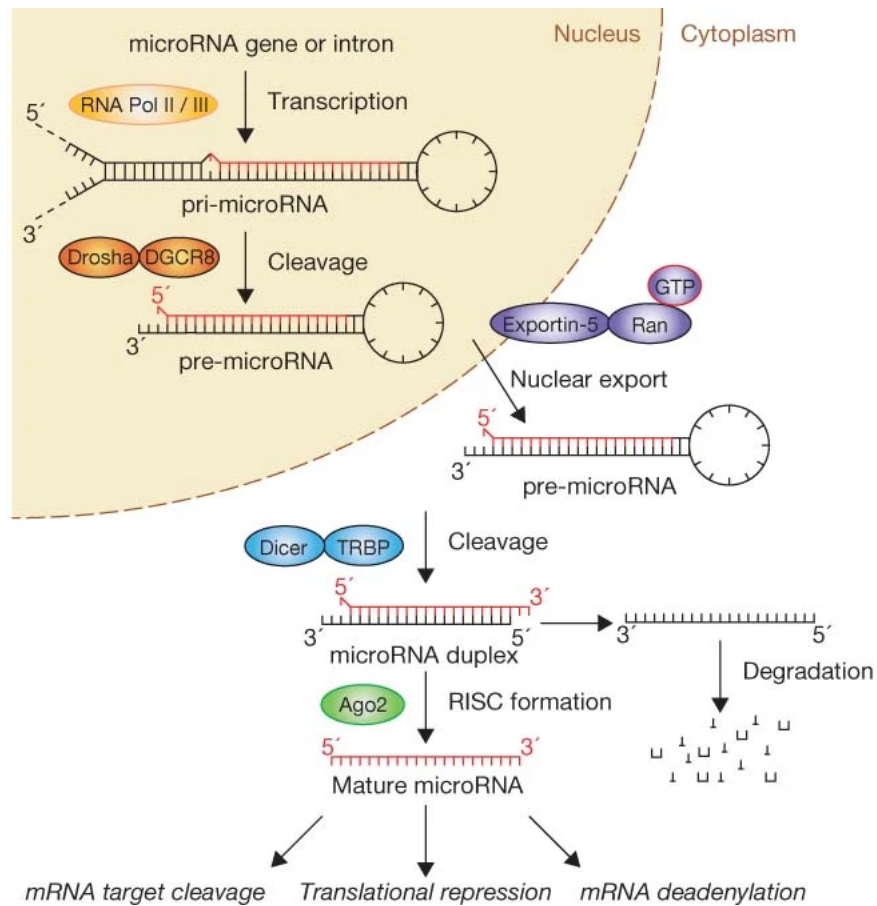


Figure 2. The canonical pathway of microRNA biogenesis and processing. miRNA genes are transcribed by RNA polymerase II and III, generating long primary transcripts (pri-miRNAs). Pri-miRNAs are then cleaved by Drosha-DGCR8 complex into precursor miRNAs (pre-miRNA). Pre-miRNAs are exported into the cytosol by Exportin-5 and its partners. Pre-miRNAs are cleaved by Dicer and TRBP into a miRNA duplex. One of the two strands in the duplex is degraded while the other (mature miRNA) is incorporated into the RNA-induced silencing complex (RISC), which regulates protein expression. Source: (Winter et al., 2009).

There are several types of non-canonical pathways, but the main idea is their ability to bypass certain parts of the canonical machinery, such as Drosha/DGCR8 processing. In such pathway variation, miRNA precursors are produced from mRNA introns during splicing, without the canonical initial Drosha cleavage (Winter et al., 2009). Another non-canonical pathway is Dicer-independent. In this case, miRNAs are processed by Drosha, resulting in too short pre-miRNA fragments that cannot be Dicer substrates. In this pathway, miRNA maturation happens through AGO2 protein within the cytoplasm through slicing and trimming (O'Brien et al., 2018b).

1.4.2. miRNA Activity and Mechanism of Action

miRNAs function as post-transcriptional regulators that control gene expression by interacting with target mRNAs (Carreras-Badosa et al., 2022). Usually, the function of miRNAs is to repress the expression of genes, however the activity localisation and the mRNA binding region are variable (O'Brien et al., 2018b).

The main functional role of miRNAs involves several key steps. Firstly, miRNA and AGO proteins form the miRISC complex, which then proceeds with the target recognition step. In animal cells, miRISC typically identifies its target through the so-called "seed region", which is a 2–8-nucleotide-long region of the miRNA. The region is complementary to miRNA response elements (MREs) and usually located in the 3' untranslated region (3' UTR) of the target mRNA. However, miRNA can also interact and bind to the 5' UTR or coding sequences. If the miRNA and its target have high complementarity, the mRNA can be cleaved directly by AGO2. However, when compatibility is only partially complementary, the translation process is inhibited by interference with translation initiation mechanisms (O'Brien et al., 2018b).

The efficiency of miRNA-mediated regulation is influenced by the cellular localisation of miRISC and its target mRNAs. Additionally, miRISC can interact with polysomes on the rough endoplasmic reticulum, where it suppresses translation before the mRNA is ultimately degraded in endosomes. An individual mRNA may contain multiple MREs, allowing stronger silencing effects. In contrast, competing endogenous RNAs (ceRNAs), including circular RNAs, can act as "sponges" that bind and isolate miRNAs, preventing them from targeting mRNAs and relieving gene repression (O'Brien et al., 2018b).

1.4.3. Role in Inflammation

Since miRNAs function as repressors of mRNA translation, they serve as crucial regulators of the immune response in the skin, influencing the differentiation and activation of immune cells, such as T and B lymphocytes, macrophages, and dendritic cells. In skin inflammatory pathways, miRNAs act as epigenetic factors, modulating gene expression without altering DNA sequences. The role of a miRNA often can be characterised as either pro-inflammatory drivers or negative regulators – anti-inflammatory (Dopytalska et al., 2023).

Several miRNAs are dysregulated in inflammatory skin diseases, suggesting their involvement in common inflammatory pathways. For example, miR-146a is often upregulated in the inflammatory skin diseases such as psoriasis, atopic dermatitis (AD), and hidradenitis Suppurativa (HS). miR-146a is considered to function as an anti-inflammatory regulator by suppressing innate immune responses in keratinocytes and inhibiting the production of pro-

inflammatory cytokine TNF- α (Dopytalska et al., 2023). In addition, it was confirmed that miR-146a deficiency results in much stronger and earlier inflammation, leading to cellular infiltration in the dermis and elevated levels of IFN- γ , IL-1 β , and CCL (Rebane et al., 2014).

1.4.4. miR-378a-3p

MicroRNA-378a-3p (miR-378a-3p), also referred to as miR-378a, has been reported to be overexpressed in the epidermis of active psoriatic skin lesions compared to healthy skin (Soonthornchai et al., 2021a). The suffix "3p" indicates that the mature miRNA sequence originates from the 3' arm of the precursor miRNA hairpin structure (O'Brien et al., 2018b).

It was found that IL-17A, a key cytokine involved in psoriasis, induces the expression of miR-378a in keratinocytes through the activation of the NF- κ B pathway (Xia et al., 2022). The exact role of miR-378a in psoriasis is still unknown, but the miRNA is alleged to be a pro-inflammatory amplifier in the psoriatic feedback loop.

In two-dimensional keratinocyte culture models, transfection with miR-378a was found to increase expression of inflammatory mediators, including IL-8 and CCL20, a potent chemoattractant for cells and dendritic cells, which helps sustain the local inflammatory environment (Xia et al., 2022).

1.5. CELL PENETRATING PEPTIDES

Cell-penetrating peptides (CPPs) are a group of short peptides, typically fewer than 30 amino acids long, that are able to pass through cellular membranes. CPPs are widely utilised as delivery vehicles for various cargo molecules, including plasmids and different types of nucleic acids, such as DNA, RNA, siRNA, miRNA, and mRNA (Periyasamy et al., 2023). One of the main advantages of CPPs is their lower cytotoxicity and minimal immunogenicity compared to traditional lipid-based delivery systems (Carreras-Badosa et al., 2020).

1.5.1. Mechanisms of Nucleic Acid Delivery

Because nucleic acids are negatively charged, their cellular delivery is quite challenging. CPPs facilitate this process through electrostatic interaction between positively charged peptides and negatively charged nucleic acids, resulting in the formation of nanoparticle complexes. This interaction neutralises the negative charge present on the nucleic acid molecules and allows nucleic acid molecules to pack together more tightly. The complexes are primarily internalised through endocytosis. The mechanism of internalisation depends on the specific CPP, cargo

type, and the target cell, and may include macropinocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis (Carreras-Badosa et al., 2020).

One of the major challenges for efficient delivery is endosomal entrapment and degradation of cargo following cellular uptake. To overcome this limitation, some pH-responsive CPPs are designed to disrupt the endosomal membranes upon the drop in pH, releasing the nucleic acid into the cytosol (Lundberg et al., 2007).

1.5.2. Types and Modifications of CPPs

CPPs can be derived naturally or synthetically designed, and chemical modifications are often introduced to improve their delivery efficiency. For example, stearylation, the addition of stearic acid to the N-terminus of peptides, enhances transfection efficiency and promotes endosomal escape (Periyasamy et al., 2023). In addition, incorporation of histidine residues changes the net charge of the peptide under acidic endosomal conditions, facilitating disruption of endosome membranes and cargo release (Carreras-Badosa et al., 2020).

1.5.3. PepFect14

PepFect14 (PF14) is an amphipathic cell-penetrating peptide belonging to the PepFect family. PepFect family peptides are chemically modified derivatives of the CPP transportan 10 (TP10) and are widely used for the delivery of nucleic acids both *in vitro* and *in vivo* (Ezzat et al., 2011).

PF14 has several crucial modifications that elevate its performance and stability. First of all, like all PepFect family members, it is modified with a stearic acid moiety at its N-terminus. Such modification increases its transfection efficiency. Another feature that differentiates PF14 from its parent peptide, TP10, is the incorporation of the non-proteinogenic amino acid ornithine, which makes the peptide less susceptible to degradation by serum proteases (Ezzat et al., 2011).

PF14 possesses a stable net positive charge (+5) under physiologically relevant pH conditions, which makes it possible for the peptide to function as a delivery vehicle for a wide range of nucleic acids into cells by electrostatically interacting with negatively charged nucleic acids. (Carreras-Badosa et al., 2020). The interaction results in the formation of nanoparticles, which typically range in size from 100 to 300 nm in diameter (Periyasamy et al., 2023).

2 THE AIMS OF THE THESIS

The overall aim of this thesis is to investigate the role of miR-378a-3p in inflammatory responses in 2D and 3D keratinocyte culture models relevant to psoriasis.

Specific objectives are as follows:

1. To optimise miR-378a-3p transfection in 3D keratinocyte cultures

This aim is to develop an efficient and reproducible protocol for miR-378a-3p transfection in a 3D keratinocyte culture model.

2. To evaluate the inflammatory effects of miR-378a-3p in 2D and 3D keratinocyte cultures

The second aim investigates the effects of miR-378a-3p transfection on the expression of inflammation-associated genes and predicted miR-378a-3p target genes in conventional 2D monolayer cultures and differentiated 3D keratinocyte cultures.

3. To evaluate the effects of miR-378a-3p on 3D keratinocyte cell culture stimulated with IL-17A

The aim is to investigate whether miR-378a-3p transfection influences the inflammatory response of 3D keratinocyte cultures following IL-17A stimulation. This seeks to determine whether miR-378a-3p acts as a pro- or anti-inflammatory regulator during the IL-17A signalling in a 3D keratinocyte culture model.

4. To investigate how miR-378a-3p transfection in 3D cell culture affects proliferative and differentiative gene expression

The last aim focuses on the impact of miR-378a-3p transfection on genes involved in keratinocyte proliferation and differentiation in 3D culture model. This study intends to investigate whether miR-378a-3p influences genes related to differentiation and cornified envelope formation.

3 EXPERIMENTAL PART

3.1. MATERIALS AND METHODS

3.1.1. Cell Cultures

Normal human epidermal keratinocytes (NHEK) were obtained from PromoCell (Germany) and stored in liquid nitrogen until use. Cells were thawed and cultured in T75 flasks containing 30 mL of Keratinocyte Growth Medium 3 (KGM3; Promocell, Germany), supplemented according to the manufacturer's instructions. For two-dimensional (2D) cell cultures, KGM3 was further supplemented with 5 U/mL of penicillin-streptomycin (Thermo Fisher Scientific, USA), whereas three-dimensional (3D) cultures were grown without antibiotics. After thawing, cells were incubated at 37 °C in 5% CO₂ for 16 hours. The media was then replaced with 13 mL of fresh KGM3. Cells were grown until approximately 70% confluence and subsequently passaged into two or three flasks, depending on experimental requirements. For 2D conditions, NHEKs were seeded at a density of 7×10^4 cells per well on 12-well plates using 1 mL of KGM3. For 3D cultures, the same number of cells (7×10^4) were seeded onto ThinCert cell culture inserts (0.4 µm pore; Greiner Bio-One, Austria) using a 1:1 mixture of KGM3 (PromoCell, Germany) and Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, USA). After 48 hours, the apical medium was removed to establish an air-liquid interface (ALI), as described previously (Vaher et al., 2023). For 3D cultures, transfection was performed at the time of seeding, allowing transfection complexes to penetrate more effectively through the developing cell layers. For 2D monolayer cultures, transfection was performed on the 1st day after seeding.

3.1.2. Transfection and Stimulation

All transfections were performed using cell-penetrating peptide PepFect14 (PF14; Biosynth, Switzerland). miRNA mimics were complexed with PF14 in a 1:17 molar ratio, using 50 nM and 850 nM of mimics and PF14, respectively. Complexes were formed in 1/10 of the final transfection volume using Milli-Q water and incubated at room temperature for 1 hour before addition to the cells. In all experiments, alongside miR-378a-3p mimic (Horizon Discovery, UK) transfection, untreated cells and cells transfected with a negative control mimic (Horizon Discovery, UK) were included as controls.

For 2D cultures, keratinocytes at approximately 70% confluency were transfected with either a negative control miRNA mimic or a miR-378a-3p mimic or a miR-146a mimic as a positive control. Transfection complexes were added directly to the culture medium 24 hours after seeding, and cells were incubated under standard culture conditions for an additional 24 hours.

Where applicable, cells were then stimulated with 10 ng/mL of IL-17A cytokine (PeproTech, USA) to induce an inflammatory response. Following cytokine stimulation, cells were incubated for another 24 hours and then harvested for downstream analyses.

For 3D cultures, transfection was performed at the same time as seeding. Cells were then cultured for a total of five days. During the first 48 hours, culture medium was added to both the apical and basal compartments. Thereafter, the medium was removed from the apical compartment to promote epidermal differentiation, while the medium in the basal compartment was replaced daily. Where applicable, cytokine stimulation was performed 24 hours before harvesting. After completion of the five-day culture period, cells were harvested for downstream analyses.

3.1.3. RNA Purification, cDNA Synthesis and Quantitative RT-PCR

For both 2D and 3D keratinocyte cultures, the cells were lysed using Qiazol reagent (Qiagen, Germany), and RNA was isolated using miRNeasy Mini Kit (Qiagen, Germany). RNA concentration and purity were measured with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

For quantification of mRNA expression levels, cDNA was synthesised from 300–500 ng of total RNA using oligo(dT), dNTP Mix, RevertAid Reverse Transcriptase, and RiboLock RNase Inhibitor (Thermo Scientific, USA). Then the qPCR was performed using 5× HOT FIREPol EvaGreen qPCR mix (Solis BioDyne, Estonia) on QuantStudio 12K Flex system (Thermo Fisher Scientific, USA). Relative expression levels were calculated using the comparative Ct ($\Delta\Delta C_t$) method with *EEF1A1* as the reference gene. The list of primers used was included in the table. (Table 1)

For quantification of miRNA expression level, TaqMan MicroRNA Reverse Transcription Kit, microRNA Assays (Thermo Fisher Scientific, USA) and 5× HOT FIREPol Probe qPCR Mix (ROX; Solis BioDyne, Estonia) were used according to the manufacturer's instructions. Relative expression levels were calculated using the comparative Ct ($\Delta\Delta C_t$) method, and let-7a was used for normalisation.

Table 1. Primer sequences used in the study.

Primer		Sequence
<i>EEF1A</i>	F	CCACCTTTGGGTCGCTTTGCTGT
	R	TGCCAGCTCCAGCAGCCTTCTT
<i>CXCL8</i>	F	GCAGCTCTGTGTGAAGGTGCAGTT
	R	TTCTGTGTTGGCGCAGTGTGGTC
<i>TRAF6</i>	F	CCGCGCACTAGAACGAGCAAGT
	R	CCATGGCCACACAGCAGTCACT

<i>NFKBIA</i>	F	GATGTCAATGCTCAGGAGCCC
	R	CCCCACACTTCAACAGGAGT
<i>EGFR</i>	F	CAGCTATGAGATGGAGGAAGACG
	R	CTATTCCGTTACACACTTTGCGG
<i>KRT10</i>	F	TCCCAACTGGCCTTGAAACAATCCC
	R	AGAGCTGCACACAGTAGCGACCT
<i>INV</i>	F	GCCTTACTGTGAGTCTGGTTGACA
	R	GGAGGAACAGTCTTGAGGAGCT
<i>KRT14</i>	F	GCAGTCATCCAGAGATGTGACC
	R	AGCCTCAGTTCTTGGTGCG
<i>KRT5</i>	F	AGGAATGCAGACTCAGTGGAG
	R	AGGAATGCAGACTCAGTGGAG

3.1.4. Protein Expression Analysis

The amount of secreted IL-8 levels in cell culture supernatants was quantified using an ELISA kit (BioLegend, USA) according to the manufacturer's instructions. Prior to analysis, all samples were diluted 1:3, with the exception of the miR-146a samples, which were analysed undiluted. Absorbance was measured using an ELISA plate reader at 450 nm with a reference wavelength of 540 nm.

3.1.5. Data Analysis

Experiments were independently repeated at least twice, and similar trends were observed between experiments. Representative experiments are shown in the figures with at least three technical replicates per condition. Data is represented as mean $\pm$ standard error (SE). Statistical analyses were carried out using GraphPad Prism software (version 11, GraphPad Software, USA). Differences between experimental groups were analysed using Student's t-test or one-way ANOVA followed by Tukey's, Dunnett's, or Šidák's multiple comparisons test, as indicated in the figure legends. A p-value < 0.05 was considered statistically significant.

3.2. RESULTS

3.2.1. Optimisation of miRNA transfection in 3D keratinocyte cell culture

A schematic of the experimental workflow is shown in Figure 3A. In 2D cultures, normal human epidermal keratinocytes (NHEKs) were seeded on day 1 and transfected with miR-378a-3p mimic or negative control mimic on day 2. On day 3, the cells were lysed, and RNA was isolated. In the case of 3D cultures, seeding and transfection were performed on day 1, followed by apical media removal on day 3 to establish an air-liquid interface (ALI). On day 6, cells were harvested for RNA isolation.

To assess the formation of a multilayered, differentiated 3D culture, the expression levels of proliferation (*KRT14*, *KRT5*) and differentiation-associated (*KRT10*, *INV*) genes were measured in both culture models (Figure 3B). In 3D cultures, strong upregulation of differentiation-associated genes, such as *KRT10* and *INV*, was observed compared to 2D culture (Figure 3B). As shown in Figure 3B, proliferation markers did not strongly differ between the culture systems, with increased levels of *KRT14* found in 3D. These results indicate that, in contrast to the 2D model, the 3D culture model effectively supports keratinocyte differentiation, confirming the efficiency of this 3D culture system.

Transfection efficiency was assessed by measuring miR-378a-3p levels using RT-qPCR (Figure 3C). In both 2D and 3D cultures, miR-378a-3p levels were significantly higher in miR-378a-3p-transfected samples than in negative control, which confirms successful and efficient miR-378a-3p transfection in both culture models.

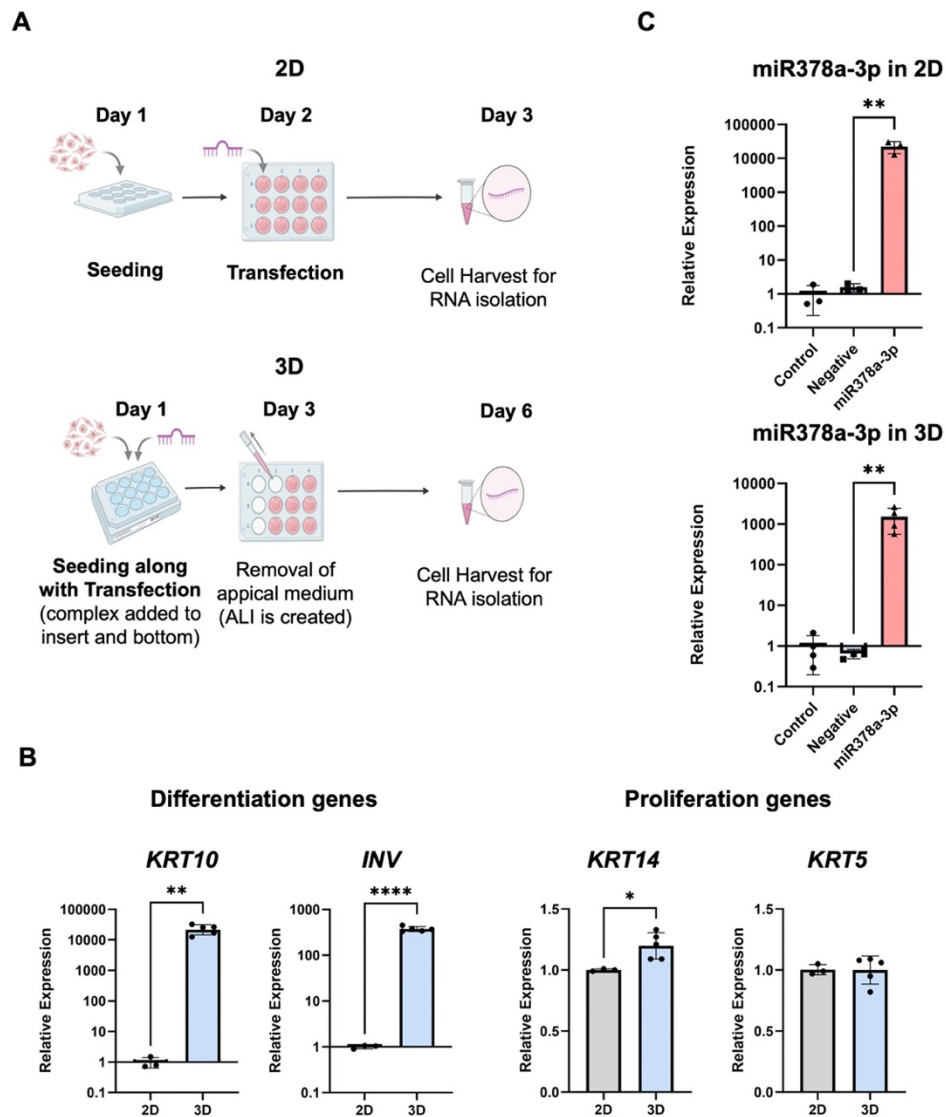


Figure 3. Validation of miR-378a-3p transfection and its effects on proliferation and differentiation-associated gene expression in 2D and 3D keratinocyte cultures. (A) Schematic of the experimental workflow. In 2D cultures, NHEKs were seeded on day 1 and transfected with miR-378a-3p or a negative control on day 2, followed by cell lysis for RNA isolation on day 3. In 3D cultures, NHEKs were seeded and transfected on day 1, followed by apical media removal on day 3 to create an air-liquid interface (ALI). On day 6, the cells were harvested and lysed to proceed with RNA isolation. Created with BioRender.com (Toronto, Canada). (B) Relative mRNA expression of indicated proliferation and differentiation-associated genes in 2D and 3D, measured by RT-qPCR. (C) Relative miR378a-3p expression level measured by RT-qPCR in 2D and 3D cell cultures. Control designates untransfected and untreated cells. Statistical analysis was performed using Unpaired two-tailed Student's t-test (B), One-way ANOVA (Dunnett's) (C). ns, not significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. For 2D cultures, $n = 3$ technical replicates per condition. For 3D cultures, $n = 5$ technical replicates per condition.

3.2.2. Regulation of NF- κ B-associated genes and predicted targets of miRNA-378-3p

In order to investigate the inflammatory effect of miR-378a-3p, the expression of selected inflammation-associated genes and predicted target genes was assessed following miRNA transfection in 2D and 3D keratinocyte cultures (Figure 4A, B).

IL-8, encoded by *CXCL8*, is a pro-inflammatory chemokine that promotes the recruitment of immune cells to sites of inflammation, and its expression is regulated through the NF- κ B signalling pathway (Hou & Huttenlocher, 2024). Because miR-378a-3p has previously been linked to NF- κ B signalling, *CXCL8* expression levels were evaluated in 2D and 3D cell culture models. Consistent with previous studies, transfection with miR-378a-3p mimic in 2D cultures significantly increased *CXCL8* expression compared to the negative control (Figure 4A) (Xia et al., 2022). Interestingly, in 3D cultures, an opposite effect was observed: miR-378a-3p transfection led to a significant decrease in *CXCL8* expression (Figure 4A). Transfection with miR-146a, used as a positive anti-inflammatory control, significantly reduced *CXCL8* expression in both culture models, consistent with its previously established role as a negative regulator of NF- κ B signalling (Figure 4A) (Saba et al., 2014).

To further investigate the effects of miR-378a-3p, the expression of *TRAF6*, *NFKBIA* and *EGFR* was measured in both 2D and 3D cultures. These genes were chosen because they are predicted targets of miR-378a-3p according to TargetScan (McGeary et al., 2019) and are associated with inflammatory and NF- κ B-related signalling pathways (Figure 4B). *TRAF6* is an adaptor protein involved in NF- κ B activation (Walsh et al., 2015), whereas *NFKBIA* encodes I κ B α , an inhibitor of NF- κ B signalling (Zhang et al., 2021). *EGFR* can indirectly influence NF- κ B activity through PI3K/AKT-mediated activation of IKK or via MAPK-dependent transcriptional regulation of NF- κ B target genes (Shostak & Chariot, 2015).

In 2D cultures, miR-378a-3p transfection did not significantly alter the expression of *TRAF6*, *NFKBIA* or *EGFR* compared to the negative control (Figure 4B). As expected, transfection with miR-146a significantly decreased the expression of its known target genes, including *TRAF6* and *EGFR* (Park et al., 2015; Xu et al., 2012). In addition, it was also found that *NFKBIA* was downregulated by miR-146a. Notably, in 3D cultures, miR-378a-3p transfection significantly reduced the expression of all three genes (Figure 4B). Together, these results suggest that miR-378a-3p may act as an anti-inflammatory regulator in the 3D culture model.

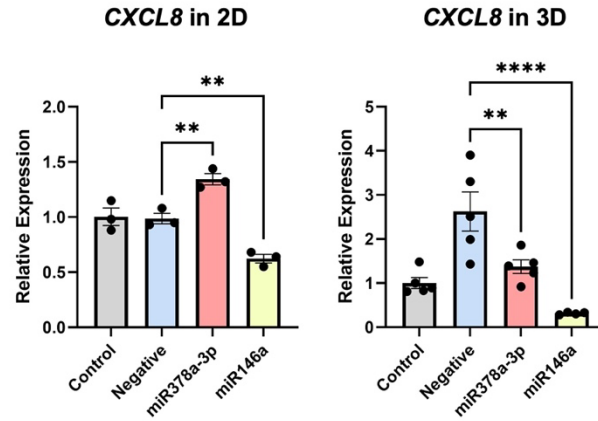
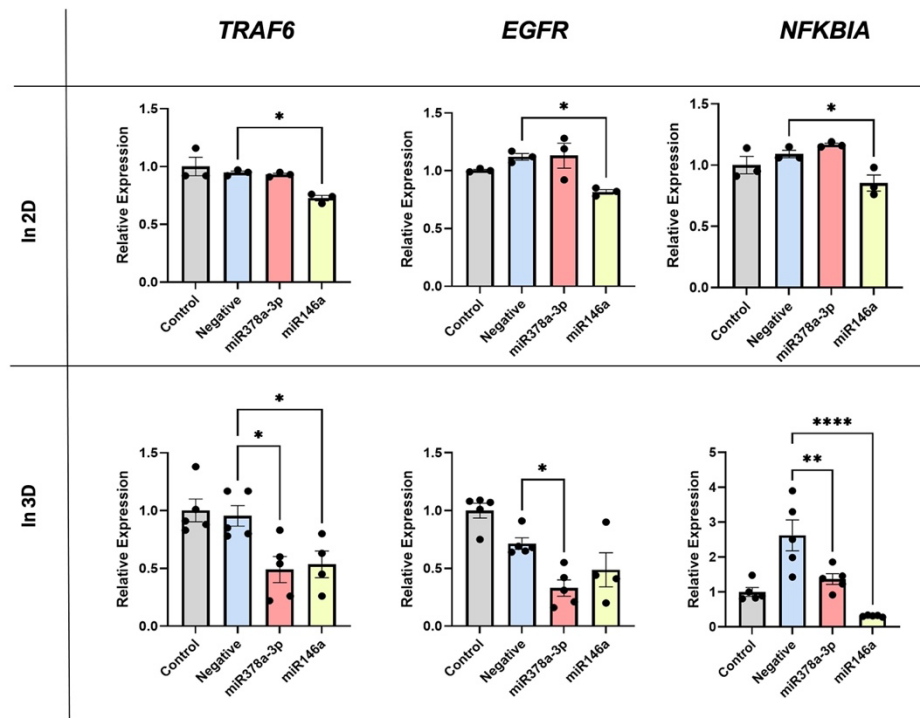
A**B**

Figure 4. Inflammatory gene expression in 2D and 3D keratinocyte cultures following miR-378a-3p transfection. (A) Relative mRNA expression of *CXCL8* in 2D and 3D keratinocyte cultures transfected with miR-378a-3p mimic, negative control or miR146a mimic, measured by RT-qPCR. (B) Relative mRNA expression of miR378a-3p predicted direct targets (*TRAF6*, *EGFR*, *NFKBIA*) in 2D and 3D cultures under the same conditions. Control designates untransfected and untreated cells. One-way ANOVA (Dunnett's). ns, not significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. For 2D cultures, $n = 3$ technical replicates per condition. For 3D cultures, $n = 5$ technical replicates per condition, except for miR-146a-transfected samples, where $n = 4$ technical replicates.

3.2.3. Effect of miR-378a-3p transfection on IL-17A-stimulated cells in 3D culture

Next, we focused on the effects of miR-378a-3p under IL-17A-stimulated conditions using a 3D culture model. IL-17A is a key inflammatory cytokine involved in psoriasis and has also been shown to regulate miR-378a-3p expression in keratinocytes (Xia et al., 2022).

A schematic of the experimental workflow is shown in Figure 5A. 3D keratinocyte cultures were transfected with miR-378a-3p mimic or negative control mimic and stimulated with 10 ng/mL of IL-17A for 24 hours prior to cell harvesting. First, miR-378a-3p expression was measured to evaluate transfection efficiency under IL-17A-stimulated conditions. Transfection with a miR-378a-3p mimic resulted in significantly elevated miR-378a-3p levels compared to the negative control (Figure 5B).

Next, we measured *CXCL8* expression, as shown in Figure 5C. miR378a-3p transfection significantly downregulated *CXCL8* expression compared to the negative control, indicating a possible anti-inflammatory effect of miR378a-3p in the presence of an IL-17A stimulation. Finally, the expression of the predicted direct targets of miR-378a-3p, directly involved in the inflammatory pathway (*TRAF6*, *NFKB1A*), was assessed under IL-17A-stimulated conditions. Both genes showed a trend towards downregulation following miR-378a-3p transfection compared to the negative control (Figure 5D).

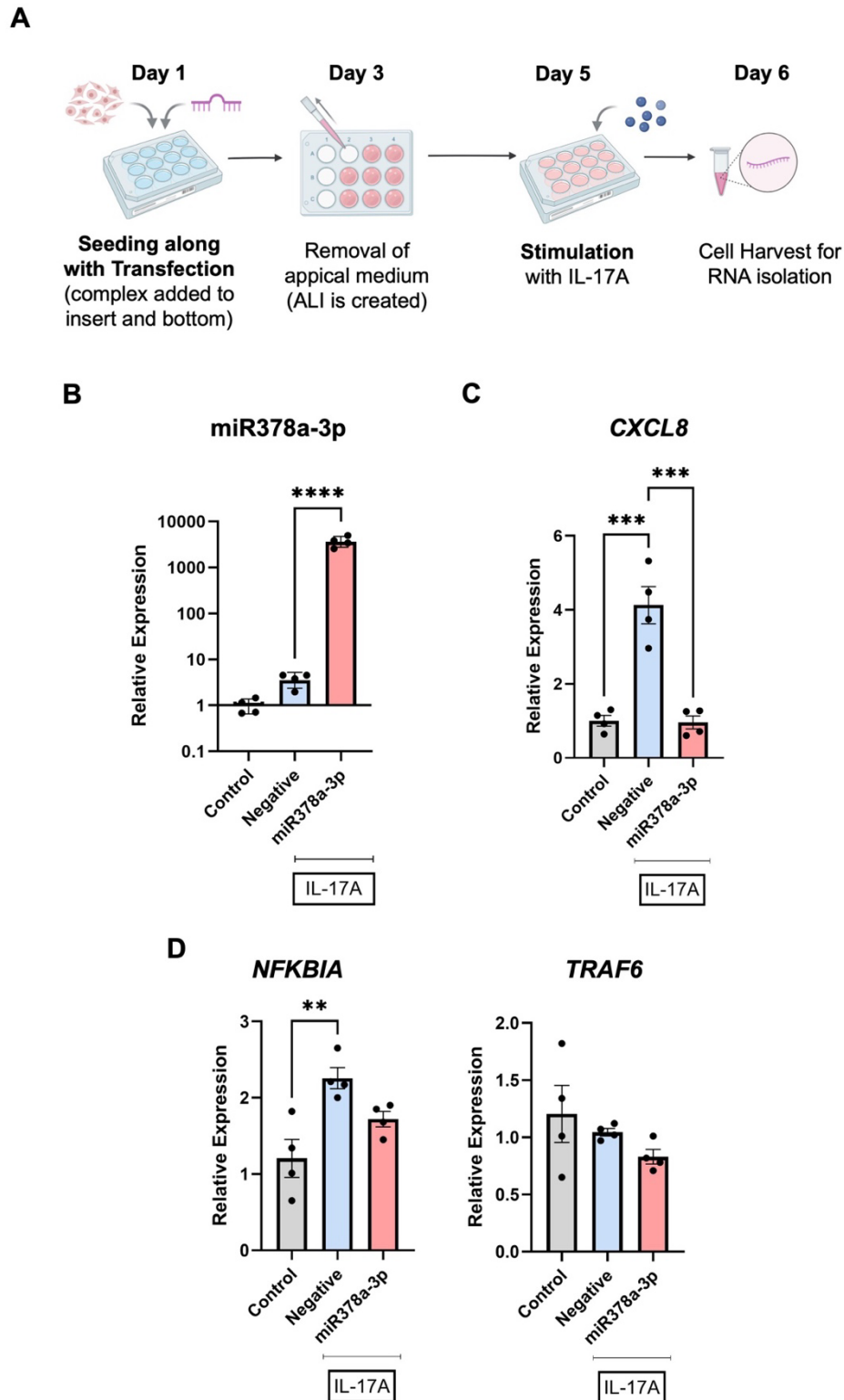


Figure 5. Effect of IL-17A stimulation on miR-378a-3p transfection and inflammatory target gene expression in 3D keratinocyte cultures. (A) Schematic representation of the experimental workflow. 3D keratinocyte cultures were transfected with miR-378a-3p mimic or negative control, followed by stimulation with 10 ng/mL of IL-17A for 24 hours prior to cell harvesting. Created with BioRender.com (Toronto, Canada). (B) Relative expression of miR-378a-3p in 3D cultures under IL-17A-stimulated conditions measured by RT-qPCR. (C) Relative mRNA

expression of *CXCL8* (IL-8) under the same conditions, determined by RT-qPCR. (D) Relative mRNA expression of predicted miR-378a-3p target genes under IL-17A-stimulated conditions. Control designates untransfected and untreated cells. One-way ANOVA (Šídák's). ns, not significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. For all experiments, $n = 4$ biological replicates per condition.

After verifying that the reduction of *CXCL8* mRNA expression following miR-378a-3p transfection was also present under IL-17A-stimulated conditions, an ELISA assay was conducted to determine whether this effect was reflected at the protein level. Figure 6 shows IL-8 secretion levels in 3D keratinocyte cultures following miR-378a-3p transfection and IL-17A stimulation as described in Figure 5A. miR-146a-transfected samples were used as a positive control.

Consistent with the *CXCL8* mRNA expression data (Figure 5C), miR-378a-3p transfection significantly decreased IL-8 protein levels compared to the negative control under IL-17A stimulation (Figure 6). In contrast, under unstimulated conditions, miR-378a-3p transfection did not significantly alter IL-8 secretion, whereas miR-146a transfection resulted in a stronger reduction in IL-8 levels (Figure 6). These results suggest that miR-378a-3p reduces IL-8 level at both mRNA and secreted protein levels, especially under IL-17A-stimulated conditions.

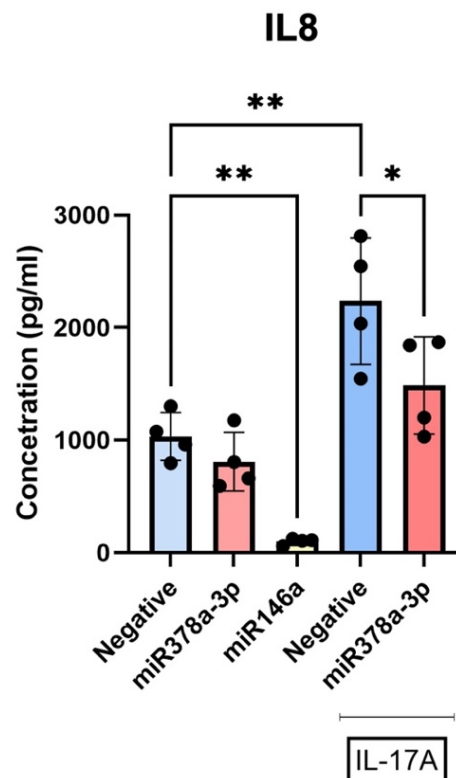


Figure 6. IL-8 protein levels in 3D keratinocyte cultures following miR-378a-3p transfection and IL-17A stimulation. 3D keratinocyte cultures were transfected with miR-378a-3p mimic, negative control or miR146a followed by IL-17A stimulation as described in Figure 5. Supernatants were collected, and IL-8 protein levels were quantified by ELISA. One-way ANOVA (Šídák's). ns, not significant, * $p < 0.05$, ** $p < 0.01$. $n = 4$ technical replicates per condition.

3.2.4. Effect of miRNA-378a-3p on proliferation and differentiation of keratinocytes

Since psoriasis is associated with abnormal keratinocyte proliferation and differentiation, and given that the previous experiment revealed different effects of miR378a transfections between 2D and 3D cultures, we next examined whether miR-378a-3p differentially influences the expression of genes involved in proliferation and differentiation between the two culture models. For that, the expression of differentiation markers (*KRT10*, *INV*) and proliferation markers (*KRT14*, *KRT5*) was assessed in both culture models following transfection with miR-378a-3p mimic, negative control, or miR-146a mimic (Figure 7).

In 2D cultures, neither miR-378a-3p nor miR-146a transfection significantly altered the expression of proliferation or differentiation-associated genes compared to the negative control (Figure 7). In 3D cultures, *KRT10* expression was strongly reduced in negative control mimic-transfected samples compared with untreated controls. However, miR-378a-3p transfection significantly increased *KRT10* expression compared with the negative control. Transfection with miR146a showed an even greater increase in *KRT10* expression. In contrast, the expression of another differentiation marker *INV*, showed no significant changes following transfection with miR-378a-3p or miR-146a (Figure 7). Among the proliferation-associated genes, *KRT14* expression was reduced following miR-378a-3p transfection in 3D cultures, whereas *KRT5* expression remained unchanged (Figure 7).

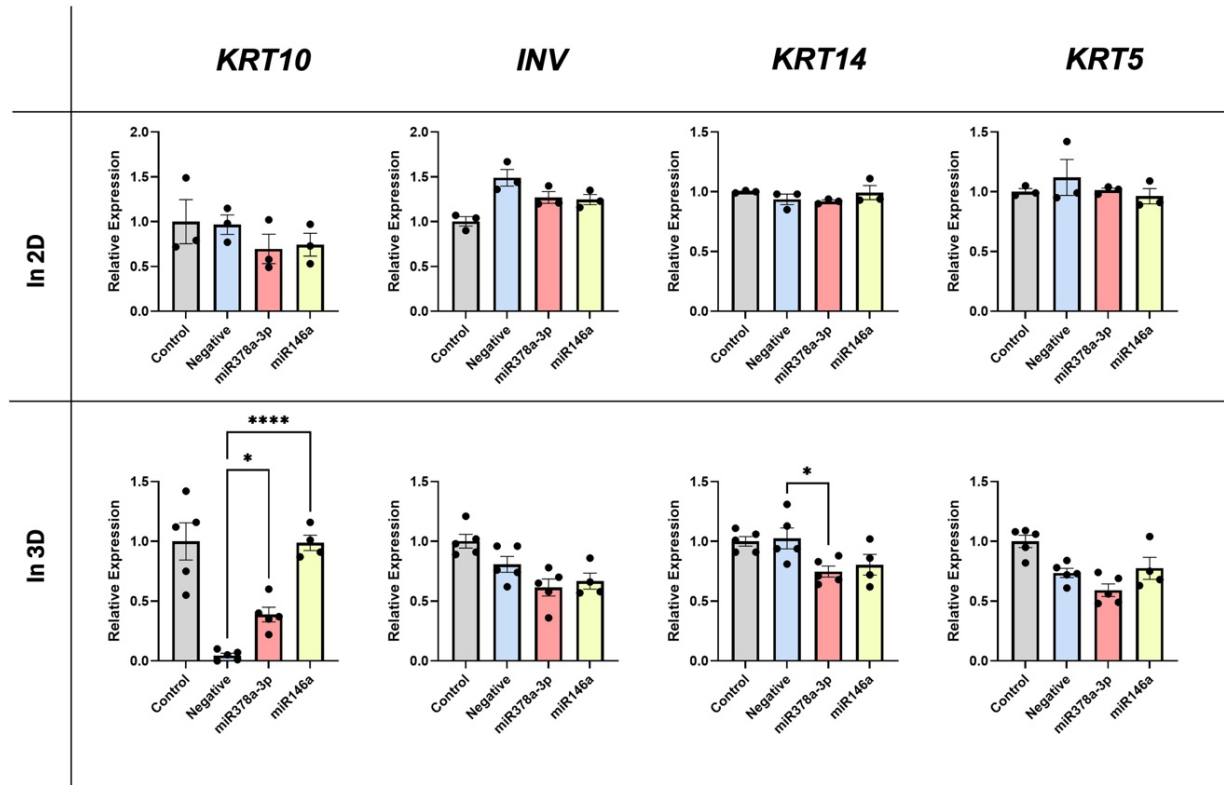


Figure 7. Effect of miR-378a-3p transfection on proliferation and differentiation marker gene expression in 2D and 3D keratinocyte cultures. Relative mRNA expression of differentiation (*KRT10*, *INV*) and proliferation (*KRT14*, *KRT5*) associated genes in 2D and 3D keratinocyte cultures transfected with miR-378a-3p mimic, negative control or miR-146a mimic, measured by RT-qPCR. Control designates untransfected and untreated cells. One-way ANOVA (Dunnett's). ns, not significant, * $p < 0.05$, **** $p < 0.0001$. For 2D cultures, $n = 3$ technical replicates per condition. For 3D cultures, $n = 5$ technical replicates per condition, except for miR-146a-transfected samples, where $n = 4$ technical replicates.

3.3. DISCUSSION

This study investigated the effects of miR-378a-3p on inflammatory gene expression in conventional 2D and differentiated 3D keratinocyte culture models. The findings of this study suggest the distinct inflammation-related functionality of miR-378a-3p in 2D and 3D cell culture models.

miR-378a-3p levels were approximately 10 times higher in 2D transfected samples compared to 3D transfected samples. The reason for the difference may be due to differences in culture and transfection procedures between the two models. In 3D cultures, transfection was performed at the time of seeding to allow the transfection complexes to penetrate through developing cell layers before stable cell-cell contacts and multilayer structures were formed. If the transfection was performed later, penetration of transfection complexes through differentiated cell layers would likely be reduced, resulting in lower transfection efficiency. In contrast, 2D cultures do not form multilayered structures, allowing transfection to be performed one day after seeding when cells are already attached and actively proliferating. In addition, 3D cultures were maintained for a longer period, during which continuous proliferation and differentiation may have contributed to lower detectable levels of transfected miR-378a-3p.

The notable finding of this study was the opposite effect of miR-378a-3p transfection on *CXCL8* expression in 2D and 3D cultures. Previous studies conducted in 2D keratinocyte cultures reported that miR-378a-3p transfection increased *CXCL8* expression, suggesting a pro-inflammatory effect (Xia et al., 2022). Consistent with their findings, miR-378a-3p transfection increased *CXCL8* expression in 2D keratinocyte cultures in this study. But in contrast, in differentiated 3D keratinocyte cultures, miR-378a-3p transfection significantly reduced *CXCL8* expression. It is also important to note that negative transfection increased *CXCL8* level, and miR-378a-3p reduced this transfection effect. Further, reduction of secreted IL-8 level was also found particularly under IL-17A stimulated conditions, confirming the effect also at the protein level. These results suggest that miR-378a-3p may exert different effects depending on the differentiation state and culture conditions of keratinocytes.

To explore the potential mechanism for the opposite effects on *CXCL8*, the expression of predicted miR-378a-3p target genes associated with NF- κ B signalling was analysed. In 2D cultures, even though miR-378a-3p transfection increased *CXCL8*, it did not significantly alter the expression of *NFKBIA*, contradicting a previous study that showed a reduction in *NFKBIA* (Xia et al., 2022). However, there are differences in cell culture conditions, media composition, and experimental setup between studies that may contribute to these differences (Xia et al., 2022).

In 3D cultures, miR-378a-3p transfection significantly downregulated *TRAF6*, *NFKBIA*, and *EGFR*. *TRAF6* functions as an important upstream adaptor protein involved in NF- κ B activation, and therefore its reduced expression may contribute to the observed decrease in *CXCL8* expression (Saba et al., 2014). Although *EGFR* is not directly associated with the NF- κ B pathway, it has been shown to induce inflammatory cytokines (Mascia et al., 2009). Interestingly, *EGFR* is also a known target of the anti-inflammatory miR-146a, which downregulates *EGFR*, so given that miR-378a-3p similarly reduced *EGFR* expression in the 3D cultures, this raises the possibility of overlapping anti-inflammatory effects between these miRNAs. Notably, none of these genes was significantly altered in 2D cultures, further supporting the possibility that inflammatory signalling pathways are differently regulated between cultures.

A paradoxical observation was the downregulation of *NFKBIA* following miR-378a-3p transfection in 3D cultures, as *NFKBIA* encodes I κ B α , a negative regulator of NF- κ B signalling. Reduced *NFKBIA* expression potentially decreases I κ B α availability and thereby promotes NF- κ B activation. However, because we only analysed mRNA expression in this study, no direct conclusions can be made regarding I κ B α protein levels or overall NF- κ B activation. This is particularly important because NF- κ B signalling is strongly regulated at the protein level through phosphorylation, ubiquitination, and nuclear translocation events.

miR-146a's effect on inflammatory gene expression was consistent between the two culture models. As reported in previous studies, miR-146a acts as an anti-inflammatory regulator by inhibiting target genes *TRAF6* and *IRAK1/2*, which later prevents *NFKBIA* activation (Saba et al., 2014). Such a functional role of miR-146a should result in decreased expression of inflammation-related genes (*CXCL8*, *TRAF6*), as observed in both 2D and 3D models. The observed consistency of the miR-146a transfection effect between 2D and 3D cell cultures supports its suitability as a positive anti-inflammatory control in this study.

Because psoriasis is associated with abnormal keratinocyte proliferation and differentiation, this study also investigated whether miR-378a-3p affects the expression of proliferation and differentiation-associated genes. The 2D model showed no significant change in expression of proliferation and differentiation markers following miR-378a-3p transfection. In contrast to 2D, the negative control transfection significantly suppresses *KRT10* expression in the 3D culture model, suggesting that the transfection procedure itself induced cellular stress or inflammatory signalling. Both miR-378a-3p and miR-146a restored *KRT10* expression, with miR-146a showing a stronger effect. Previous findings reported that inflammatory cytokines suppress *KRT10* expression (Omori-Miyake et al., 2014). Therefore, restoration of *KRT10* expression by

miR-378a-3p and miR-146a may further support reduced inflammatory signalling in differentiated 3D cultures. Neither *KRT14* nor *KRT5* expression was altered by miR-378a-3p in 2D cultures. In 3D cultures, only *KRT14* showed a modest decrease, while *KRT5* remained unchanged. These results suggest that miR-378a-3p is not primarily responsible for the regulation of basal keratinocyte proliferation.

SUMMARY

This study explored the functional effects of miR-378a-3p in three-dimensional (3D) keratinocyte culture models, comparing them with more conventional two-dimensional (2D) culture. The results showed that miR-378a-3p affected inflammatory gene expression differently in the two culture systems. In 2D cultures, miR-378a-3p transfection increased *CXCL8* expression, whereas in 3D cultures, miR-378a-3p transfection reduced *CXCL8* expression, suggesting that the differentiation state of keratinocytes and culture conditions may largely influence the inflammatory effects of miR-378a-3p.

In addition, miR-378a-3p transfection in a 3D culture model decreased the expression of *TRAF6* and *EGFR*, genes associated with inflammatory and NF- κ B signalling pathways. Under IL-17A stimulation, which is a key driver of psoriatic inflammation, miR-378a-3p maintained its anti-inflammatory effect, significantly reducing both *CXCL8* mRNA and IL-8 protein levels.

Importantly, this study mainly analysed inflammatory responses at the mRNA level, whereas NF- κ B signalling is extensively regulated at the protein level. Therefore, additional experiments involving protein level analyses would be needed to confirm the proposed mechanisms. Also, it is important to note that observed differences may partially be due to differences in culture conditions and transfection procedures rather than the differentiation state alone.

Overall, the study's findings demonstrated the importance of physiologically relevant culture models for studying miRNA function in inflammatory skin diseases such as psoriasis.

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