Towards topical microRNA-directed therapy for epidermal disorders

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Abbreviations

cSCC	Cutaneous squamous cell carcinoma
DOTAP	1,2-dioleoyloxy-3-trimethylammonium propane
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
hBD-2	Human beta defensin 2
IFNγ	Interferon gamma
IL	Interleukin
Keratin 17	KRT17
LCNPs	Liquid crystalline nanoparticles
miRNA	MicroRNA
MO	Monoolein
siRNA	Short/small interfering RNA
SNA-NC	Spherical nucleic acid-nanoparticle conjugates
STAT3	Signal transduction and activator of transcription 3
TACE	Tumour necrosis factor alpha (TNFα)-converting enzyme
TGFβ	Transforming growth factor beta
TIMP-3	Tissue inhibitor of metalloproteinase 3
TNFα	Tumour necrosis factor alpha

Abstract

There remains an unmet dermatological need for innovative topical agents that achieve better long term outcomes with fewer side effects. Modulation of the expression and activity of microRNA (miRNAs) represents an emerging translational framework for the development of such innovative therapies because changes in the expression of one miRNA can have wide-ranging effects on diverse cellular processes associated with disease. In this short review, the roles of miRNA in epidermal development, psoriasis, cutaneous squamous cell carcinoma and re-epithelisation are highlighted. Consideration is given to the delivery of oligonucleotides that mimic or inhibit miRNA function using vehicles such as cell penetrating peptides, spherical nucleic acids, deformable liposomes and liquid crystalline nanodispersions. Formulation of miRNA-directed oligonucleotides with such skin-penetrating epidermal agents will drive the development of RNA-based cutaneous therapeutics for deployment as primary or adjuvant therapies for epidermal disorders.

Introduction

Few discoveries in recent bioscience history have had as wide-ranging an impact as the observation that small endogenous non-protein coding RNAs regulate the expression of multiple gene targets in diverse species. Following early reports by the Victor Ambros (1) and Gary Ruvkun (2) laboratories, the breakthrough came in 2001 with 3 *Science* papers that showcased the widespread nature of microRNA (miRNA) expression in metazoan organisms (3-5). Since then, the number of microRNA-related entries in PubMed has grown exponentially to over 64,000 (Fig. 1A).

FIGURE 1

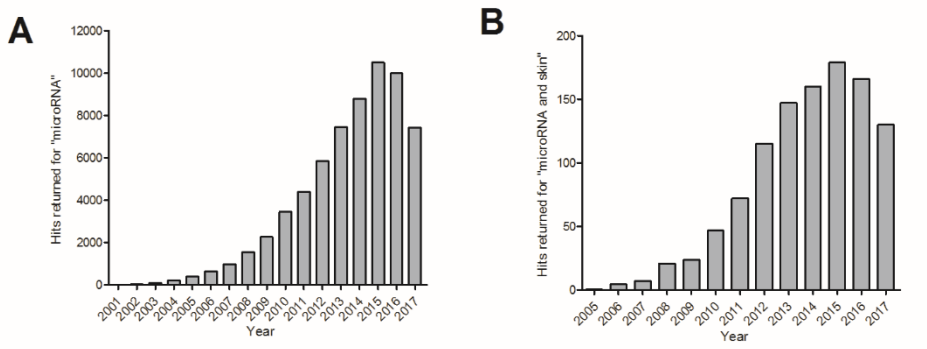

Fig. 1: Growth in the PubMed records returned using the search term (A) "microRNA" (A) or "microRNA and skin (B).

The miRNA revolution stems from the ability of these small non-coding RNA (ncRNA) molecules, typically ~22 nucleotides long, to regulate post-transcriptional expression across most of the genome, thereby fine-tuning numerous pathways that control cell behaviour (6, 7). Small ncRNAs are defined as being less than 200 nt long, and in addition to miRNAs, include small-interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNA (snoRNAs) (8-10).

More recently, long ncRNAs (defined as >200 nt long) have garnered attention as central regulators of physiological and pathological processes, despite being expressed at 15-230 lower levels than protein coding transcripts (8). One such lncRNA, PRINS (Psoriasis susceptibility-related RNA Gene Induced by Stress), has long been associated with psoriasis, a debilitating skin disorder characterised by complex interplay of cytokines from immune and skin cells (11, 12). Recent efforts have defined a plethora of differentially expressed lncRNAs psoriatic skin (13-15) and in cutaneous squamous cell carcinoma (cSCC) (16-18). The roles of lncRNA in epidermal homeostatic and skin diseases have been very recently been reviewed by Botchkareva (19) so will not be considered further here.

As the largest and most accessible organ of the body, the skin represents a major tissue for pharmacologic drugs targeting cutaneous disorders as well as systemic delivery of active agents. The goal of this short review is to highlight the emerging consensus on the global mechanism of miRNA action, the roles of miRNA in development and disease with reference to the epidermis, the outer layer of the skin. The translational prospects for topical delivery of RNA-directed oligonucleotides for skin disorders will focus on cell penetrating peptides (CPPs), spherical nucleic acid (SNA)-gold nanoparticles, deformable liposomes and liquid crystalline nanoparticles (LCNPs). Nanoparticles based on natural or synthetic polymers such as chitosan or poly(lactide-co-glycolic acid) will not be considered herein as they have received little attention for cutaneous oligonucleotide delivery and have been reviewed elsewhere for dermatological disorders (20). For broader consideration of the issues associated with the design and delivery of oligonucleotide-based therapy to diverse tissues, see the recent review by Juliano (21).

MicroRNA Biogenesis

Mature miRNAs arise from hairpin precursors (pre-miRNAs) which are themselves largely derived from intergenic or intronic regions of coding and non-coding transcripts as reviewed elsewhere (22-24). Gene repression by miRNAs is mediated by the RNA-induced silencing complex (RISC), the assembly of which has been reviewed recently (25). In its mature form, the RISC consists of a single-stranded mature miRNA bound to an Argonaute (AGO) protein, of which there are four paralogs in human cells (AGO1–AGO4). By guiding AGO proteins to the 3' untranslated region (3' UTR) of target mRNA, the miRNA facilitates sequence-specific repression of gene output (6, 22-26). Recent structural studies have yielded exquisite insight into the mechanistic and conformational basis for guide miRNA binding to AGO2 and target RNAs (27, 28).

Mechanisms of microRNA action

Mature miRNA duplexes consist of a guide strand which mediates RISC action and a passenger or minor strand (miRNA*) that was considered to be degraded (29). However, recent studies have shown miRNA* also regulate gene expression via RISC-dependent binding to the 3'UTR of mRNA transcripts (30, 31). For this and other reasons (32, 33) mature miRNAs are now designated miR-#-5p or miR-#-3p according to the precursor hairpin arm from which they arise.

Early studies indicated that a given miRNA can downregulate the expression of hundreds of target genes at the mRNA and protein levels (34-37). What has been somewhat controversial is whether such miRNA-dependent attenuation of gene output relies primarily on destabilisation and degradation of mRNA or on inhibition of translational (38). Several studies suggested miRNAs function by blocking initiation

or elongation phases of translation (39-43) and that, at least in HeLa cells, translational inhibition was the dominant effect of miRNAs (44, 45). However, the Bartel group observed little change in the translational efficiency of ribosomes on target mRNAs in HeLa cells, accounting for around 16% of the observed miRNA-dependent repression. In contrast 84% of miRNA-mediated gene repression could be attributed to mRNA destabilisation (46). Subsequent investigations on cell lines, primary macrophages, mouse liver and primary B cells revealed that while translational repression dominates miRNA action immediately following robust miRNA induction, this is soon subsumed within a much greater mRNA destabilization effect that maintains steady-state repression (47).

MicroRNAs and epidermal development

The epidermis forms the outer part of the skin and consists predominantly of keratinocytes stratified from a basal layer of viable cells to an outermost layer of terminally differentiated keratinocytes. Studies returned by PubMed in relation to “microRNA and skin” have also grown exponentially over the last decade, showcasing the efforts that have been made to define the contributions of microRNAs to normal and pathological skin biology (Fig. 1B). From a developmental perspective, studies with mouse models have revealed that miR-203, miR-205 and miR-214 function as central controllers of epidermal morphogenesis. Several independent laboratories revealed that by downregulating the stem-cell associated transcription factor p63 along with other targets, miR-203 supports the initial commitment of embryonic stem cells to the keratinocyte lineage and bolsters the transition from proliferation to differentiation during stratification of the epidermis (48-51). Loss of miR-205 led to derepression of phosphatases and other antagonists of

the pro-survival protein Akt, resulting in a dramatic reduction in Akt activation in interfollicular progenitors and HFSCs (52). Hence the main function of miR-205 appears to be maintenance of the proliferative capacity of basal cells in the nascent epidermis, hair follicle stem cells (HFSCs), outer root sheath, oesophagus and tongue (52).

For miR-214, studies on transgenic mice have revealed multiple roles in the regulation of both embryonic hair follicle development and postnatal hair cycling (53). Keratinocyte-specific overexpression of miR-214 reduced proliferation of hair matrix cells and interfollicular keratinocytes, leading to thinner hair shafts and thinner epidermises, respectively (53). For further details on miRNA functions in skin development, see the excellent review by Yi and colleagues (54).

Mouse studies have provided valuable insights concerning miRNA function in the developing epidermis. However, little consideration has been given to concerted efforts to use human cell and tissue models to uncover similar understanding of miRNA function. Ablation or ectopic expression of miRNAs in human induced pluripotent stem cells (hiPSCs) could provide a distinctive framework for characterising miRNA function in epidermal stratification and folliculogenesis, given that both mouse and human pluripotent SCs have been differentiated into multipotent keratinocytes that generate interfollicular keratinocytes, stratified epidermal equivalents and hair follicle cells (55-60). With over 700 hiPSC lines now available from the Human Induced Pluripotent Stem Cells Initiative (61), the path is clear for renewed efforts to decipher miRNA function using these cell lines as an alternative to mouse-based studies. Importantly, miRNA expression can be silenced in pluripotent SCs using transcription activator-like effector nucleases (TALENs) (62, 63) or with Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

and CRISPR-associated protein 9 (Cas9) nuclease gene editing (64, 65). Therefore, CRISPR-Cas9 reagents can be introduced into iPSCs using nucleofection or non-lipid based chemical transfection with GeneJuice® (66, 67). The utility of CPPs and SNAs in delivery of TALEN and CRISPR-Cas9 reagents to iPSCs for subsequent differentiation into epidermal keratinocytes or hair follicle cells for 'omics studies, morphometric assessment, drug screening and exposome analyses (68) warrants detailed investigation.

MicroRNAs and psoriasis

The major inflammatory molecules associated with epidermal inflammation and hyperplasia in psoriasis include interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ) and interleukins (ILs) including IL-1, IL-17 and IL-22). Medium-scale and comprehensive screens revealed multiple miRNAs were dysregulated in psoriasis (69, 70), among which miR-21-5p functions as key mediator of epidermal inflammation (71). As illustrated in Fig. 2, miR-21-5p downregulates tissue inhibitor of metalloproteinase 3 (TIMP-3) in keratinocytes, as reported in several independent studies (71-73). The subsequent elevation of tumour necrosis factor alpha (TNFα)-converting enzyme (TACE; also known as ADAM17) activity results in enhanced release of soluble TNFα from keratinocytes to promote epidermal inflammation (71, 74). Notably, the depletion of TIMP-3 in a psoriasis-like mouse and in patient-derived xenografts on immunodeficient mice was reversed using anti-miR-21 oligonucleotides and this was associated with downregulation of inflammatory cytokines including IL-17, IL-23 and TNFα (71). Importantly, epidermal thickness of the xenografts was reduced by intradermal injection of anti-miR-21 every 48 h for 30 days. However, this decrease

in epidermal thickness was observed in 8 out of 11 psoriasis cases (73%), leaving open the question of what factors precluded anti-miR-21 efficacy in the non-responders.

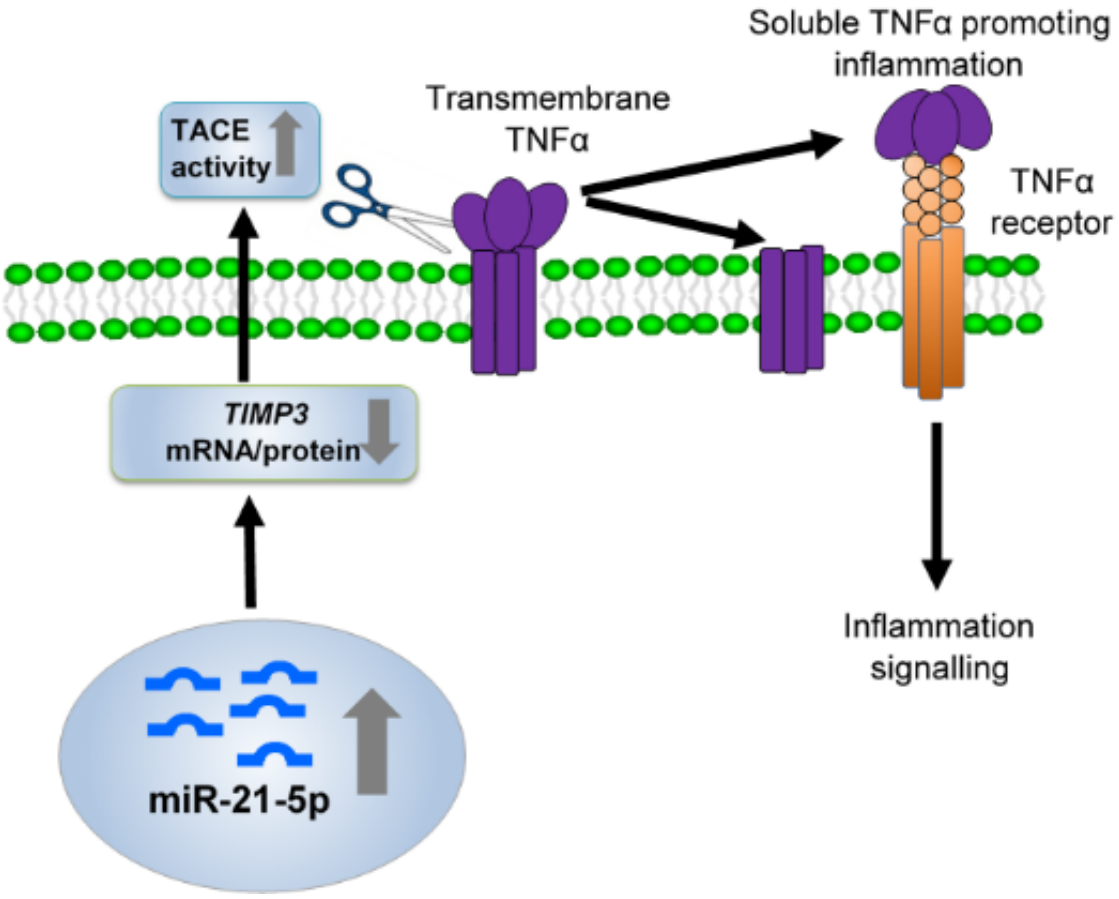

Fig. 2: Schematic depiction of a miRNA-21-dependent inflammation pathway. Elevation of miR-21 through mechanisms that are yet to be fully defined in keratinocytes leads to post-transcriptional repression of TIMP-3. Unfettered from TIMP-3 inhibition, TACE activity is enhanced, boosting the processing of transmembrane TNFα precursor into the soluble form that promotes inflammation through the TNFα receptor.

Interestingly, elevated levels of miR-21-3p have also been linked to skin inflammation (75). As the passenger or minor strand of the pre-miR-21 duplex, miR-21-3p appeared to be around 1,000 times lower than miR-21-5p levels in the mouse epidermis. Nevertheless, pharmacologic or UV-dependent activation of the peroxisome proliferator-activated receptor (PPARβ/δ) nuclear hormone receptor led to a significant increase of miR-21-3p levels in mouse skin and the HaCaT keratinocyte cell line (75). In psoriatic skin, miR-21-3p was raised almost 4-fold compared to healthy skin (75). Importantly, although these observations were made using a rather small cohorts ($n \geq 4$ independent biopsies) earlier work by Bowcock and colleagues had observed an almost 9-fold increase in miR-21-3p levels in lesional psoriatic skin compared to healthy skin, using 24 and 20 independent biopsies, respectively (70). Thus elevation of miR-21-3p appears to be strongly associated with psoriasis. Whether inhibition of miR-21-3p ameliorates psoriasiform inflammation remains to be seen. Nonetheless, topical anti-miR-21 oligonucleotides may need to target both miR-21-5p and miR-21-3p in the psoriatic epidermis for maximal therapeutic efficacy.

The relationships between cytokines and miRNA dysregulation in keratinocytes have not been fully defined. That said, TGFβ1 has been shown to promote miR-21-5p and miR-21-3p expression in HaCaT keratinocytes (72, 73, 75-77). Furthermore, miR-31 was induced by TGFβ1 in primary human keratinocytes, but not significantly changed by TNFα, IL-22, IL-6 or IFNγ (78). This contrasts with recent work by Wang and collaborators, who showed that each of these four cytokines could elevate miR-31 levels in primary keratinocytes by ≥2 fold, depending on concentration (79). In any case, levels of miR-31 were significantly elevated in psoriatic biopsies (78, 79). Importantly, inhibition of miR-31 reduced the basal and

TNFα-dependent expression of inflammatory cytokines and chemokines in human keratinocytes and reduced epidermal thickening and keratinocyte hyperproliferation in the imiquimod mouse model (78, 79). Taken together, these studies also suggest miR-31 functions within a positive feedback loop in keratinocytes: IL-6 activates NF-κB to drive miR-31 expression and miR-31 represses serine/threonine kinase 40 (STK40), a suppressor of NF-κB-dependent transcription to further enhance miR-31 levels. It will be interesting to evaluate this hypothesis and define the implications of such a regulatory loop quantitatively.

The morphological alterations associated with the psoriatic epidermis are strongly linked to IL-22 activity (80). Little is known about the impact of IL-22 on keratinocyte miRNA expression. We have shown that IL-22 induces miR-184 in reconstituted human epidermis and in HaCaT keratinocytes, as does oncostatin M (81). Inhibition of the JAK/STAT pathway abrogated the cytokine-dependent expression of miR-184. Interestingly, we demonstrated the RISC effector AGO2 was downregulated by miR-184, suggesting a miR-184:AGO2 axis of dysregulation in psoriasis AGO2 (81). However, the extent to which miR-184 modulates epidermal homeostasis is unclear though recent studies on HaCaT keratinocytes suggest that miR-184 stimulates keratinocyte proliferation and reduces apoptosis (82). Beyond miR-184, IL-22 has also been shown to promote the expression of miR-197 in keratinocytes, and this was associated with increased binding of STAT3 to the miR-197 promoter (83).

Interestingly, miR-146a was among the first miRNAs found to be elevated in psoriatic skin (69). Evidence from the Sonkoly and Pivarcsi group indicated that Toll-like receptor (TLR) ligands induced a sustained increase in miR-146a in keratinocytes, parsimoniously, to downregulate the expression of inflammatory

chemokines such as IL-8 and CCL-20 (84, 85). The picture that emerges is one in which miR-146a dampens TLR-dependent epidermal inflammation by suppressing TRAF6 (TNF receptor-associated factor 6) and IRAK1 (IL-1 receptor-associated kinase 1) consistent with early work by Baltimore and colleagues (86). Attenuation of TRAF6 and IRAK1, both of which mediate TLR signalling, in turn impaired activation of the nuclear factor kappa B (NF-κB) transcriptional programme and the recruitment of inflammatory cells (84, 85). More recent work showed miR-146a induction in keratinocytes exposed to IL-17, a central driver of psoriatic inflammation in the skin (87). Activation of NF-κB, expression of IL-8 and the ability of keratinocytes to attract neutrophils was abrogated in cells loaded with a synthetic miR-146a mimic, presumably due to downregulation of TRAF6, which is known to mediate IL-17A signalling to NF-κB (88, 89). Importantly, intradermal injection of the miR-146a mimic into mouse blocked the development of psoriasis-like inflammation (87). Obviously, intradermal injection is not appropriate for psoriasis patients, hence, like with anti-miR-21, there is an urgent need for validated topical strategies to deliver miR-146a mimics to psoriatic skin in clinical trials.

Interestingly, a functional polymorphism in the *miR-146a* gene appeared to protect against early onset of psoriasis, apparently due to the anti-inflammatory impact of elevated levels of miR-146a in keratinocytes (87). Patients with the CC rs2910164 genotype were slightly protected against psoriasis when compared to those with the GC or GG genotypes. This raises the intriguing prospect of a prophylactic regimen based on miR-146a delivery to patients at higher risk of developing the disease.

Several miRNAs have also been reported to be downregulated in psoriatic skin, including miR-125b (90), miR-99a (91) and miR-424 (92). The roles of these and other miRNAs in psoriasis have been reviewed in detail elsewhere (93-96).

MicroRNAs and cutaneous squamous cell carcinoma

Non-melanoma skin cancers are the most common malignancies in the world and represent a growing public health challenge due to population ageing, UV exposure, indoor tanning and other environmental factors (97, 98). Although low-risk localised cSCC lesions can be treated by surgical methods, advanced metastatic disease has an estimated mortality rate of >70% despite various chemotherapeutic options (99, 100).

Early work indicated that miR-21-5p and miR-184 were elevated in cSCC compared to normal skin (101). Several independent studies have confirmed miR-21-5p upregulation in cSCC (102-108). Furthermore, miR-21-3p, miR-31 and miR-135b appear to be consistently elevated in cSCC (75, 106, 107, 109). With miR-21 and miR-31 also being strongly linked to psoriasis as discussed above, it would be interesting to unravel the associated genomic or exposomal factors that determine the propensity of keratinocytes with elevated miR-21 or elevated miR-31 to proceed down psoriatic or cSCC paths.

In contrast to the raise levels of a few miRNAs, the majority of differentially expressed miRNAs detected in cSCC were down-regulated (103, 104, 107, 109). This evokes questions about the global mechanisms underpinning miRNA alterations in cSCC and their relevance to cSCC therapy. Conceptually, reversing the overall depletion of miRNAs may offer translational benefits that exceed those of replacing individual miRNAs. However, the key factors leading to miRNA depletion in cSCC

cells have not been elucidated and the mechanistic explanations for downregulation of specific miRNAs, such as miR-124, miR-125b and miR-204 cSCC remain obscure (103, 107, 110, 111). Furthermore, although mutations in NOTCH1 and in TGFβ receptors have been defined as primary drivers of cSCC (112, 113), the relationships between impaired NOTCH1 or TGFβ receptor signalling and miRNA depletion have not been investigated.

Conflicting observations have been made in relation to miR-205 in cSCC, with some studies suggesting no differential expression between normal skin and cSCC (101, 103) and others reporting elevation of miR-205 (105, 108). Notably, in an oral SCC cell line, elevated miR-205 was associated with sustained Akt signalling and cell survival via repression of SH2-containing phosphoinositide 5'-phosphatase (SHIP2) (114). Hence, in cases where miR-205 is raised in cSCC, the mechanisms of carcinogenesis may be related to the roles of miR-205 in epidermal development (52). Other miRNAs implicated in cSCC include miR-34a, miR-199a and miR-361-5p, as reviewed in (115).

MicroRNAs in keratinocyte migration and wound healing

Non-healing chronic wounds are also a burgeoning public health issue associated with the rising incidence of diabetes and the ageing population (116). Co-ordinated re-epithelisation of the wound surface by migrating keratinocytes is a crucial element of wound closure (117). Some miRNAs associated with psoriasis, such as miR-21, miR-203 and miR-31, have also been implicated in keratinocyte migration (72, 118, 119) Raised expression of TGFβ1 in wounded mouse skin correlates with the induction of miR-21-5p in keratinocytes, and upregulation of miR-21 in the migrating cells mediates the early phase of wound contraction (72, 120). However, although

inhibition of miR-21-5p with anti-sense oligonucleotides impaired keratinocyte migration in these studies, the putative ability of a miR-21 mimic to promote wound healing was not examined. Furthermore, as highlighted above, elevated miR-21-5p has been associated with cSCC. Therefore, the translational potential of a miR-21-5p mimic in relation to wound healing remains unclear.

Other miRNAs regulating keratinocyte migration include miR-483-3p, miR-24, miR-205, and miR-132 (121-125). Upregulated expression of miR-483-3p also occurs scratch-injured cultures of human keratinocytes and wounded skin in mice to sustain keratinocyte proliferation, peaking at the final stage of the wound closure process (121). Elevation of miR-24 during keratinocyte differentiation controls cell mobility and promotes differentiation by regulating the expression of several proteins associated with remodelling of the actin cytoskeleton (122). Likewise, miR-205 activity has been shown to stimulate keratinocyte migration, and least partly by regulate filamentous actin polymerization and loosening cell attachment to the extracellular matrix (123). However, more recent studies from Su and colleagues suggest that raised miR-205 in the migratory front of chronic non-healing venous ulcers impairs wound healing (124). Inhibition of miR-205 derepressed integrin subunit alpha 5 (ITGA5), a component of the classical fibronectin receptor, enhancing wound closure in monolayer scratch assays and wounded mouse skin (124)., Interestingly, elevated expression of miR-210 has also been observed in keratinocytes at the edge of ischemic wound tissue (126). Consequently, a clinical trial has been launched to evaluate the relationship between miR-210 and clinical outcomes in patients with chronic venous leg ulcers (NCT02024243). Thus both miR-205 and miR-210 have potential as targets for wound therapy, and vehicles for

effective delivery of anti-miR-205 and anti-miR-210 oligonucleotides to keratinocytes will likely be required for translational purposes.

Topical targeting of microRNAs in cutaneous disease

Validated agents for cutaneous delivery of miRNA-directed oligonucleotides (miRNA mimics or miRNA inhibitors) for translational and clinical benefit have not yet been realised. The epidermis presents a physical and immunological barrier against the external environment and associated pathogens (127, 128). The stratum corneum (SC) provides most of the epidermal barrier function, composed as it is of non-viable keratinocyte squames embedded in a lipid-rich matrix, making it largely impermeable to water and to hydrophilic and lipophilic substances greater than 500 Da (129-131). Hence drug delivery to the viable epidermis and beyond requires optimisation of multiple parameters to secure efficient delivery without evoking an irritation response (132). We now consider emerging approaches for conveying RNA-directed oligonucleotides into the epidermis, focusing on CPPs, SNA-gold nanoparticles, deformable liposomes and LCNPs. The transmission of miRNA-directed oligonucleotides to the epidermis is an emerging field that can take advantage of the technologies already being explore for cutaneous delivery of siRNA and other oligonucleotides. Physical approaches (microneedles, microporation) and active methods (electroporation, iontophoresis, sonophoresis) for oligonucleotide delivery into skin have been review previously by Mitragotri and colleagues (133).

Cell penetrating peptides

Extensive studies on the potential of cell penetrating peptides CPPs for drug delivery have been performed since the HIV TAT peptide and the *Drosophila* peptide

penetratin were first defined as CPPs (134, 135). Sequences presented in Table 1 illustrate the diversity of selected CPPs known to traverse the SC into the viable epidermis. Broadly, CPPs are thought to enter cells through endocytosis-driven pathways or via direct translocation across the lipid bilayer, and the reader is referred to the excellent review by Bechara and Sagan for mechanistic details (136).

Early work by Khavari and collaborators indicated that conjugation of the immunosuppressant cyclosporine A (CsA) to the CPP poly-arginine (R7) enabled CsA to cross the SC of mouse and human skin (137). More recently, a SPACE (skin penetrating and cell entering) peptide was isolated by iterative selection from an in vitro phage display library and shown to facilitate delivery of CsA into porcine epidermis (138, 139). In a comparison of CPP-mediated entry of CsA into porcine skin, Mitragotri and colleagues observed little difference in performance of the SPACE peptide and R7 (140). A third peptide, TD-1, also delivered CsA into the skin, albeit with slightly lower efficiency than SPACE peptide and R7. Although all three skin-penetrating peptides (SPPs) showed minimal irritation, the toxicity profile of the SPACE peptide was lower than that of R7 and TD-1.

Table 1: Sequences of selected skin penetrating peptides

CPP	Primary Sequence	Refs.
IMT-P8	RRWRRWNRFNRRRCR	(141)
PepFect6	Stearyl-AGYLLGK(K(K2(tfq4)))INLKALAALAKKIL-NH2L*	(142)
Poly-Arginine (R7)	RRRRRRRR	(140)
SPACE peptide	AC-TGSTQHQ-CG	(138)
TD-1	ACSSSPSKHCG	(143)

*PepFect6 is stearylated analogue of transportan-10, with four trifluoroquinoline moieties attached via a lysine triplex

The SPACE peptide has been shown to deliver covalently conjugated siRNA through the SC to silence target gene expression in mouse skin (138, 144, 145). In the rat footpad model, TD-1 transmitted siRNA throughout the epidermis and reduced expression of a target gene (143). Hence, although the use of SPACE peptide, R7 or TD-1 to deliver miRNA-directed oligonucleotides has not been evaluated to my knowledge use of these CPPs should be generalizable to miRNA mimics or inhibitors.

An *in silico* screening approach recently yielded a 15-amino acid arginine-rich CPP, IMT-P8, penetrated cells 10 times more efficiently than TAT or a related IMT-P4 peptide (141). The efficiency of IMT-P8 appears to stem not just from the helical structure it adopts but also the specific clustering of arginine residues on one phase of the helix and two critical tryptophan residues on the opposite phase (Fig. 3).

The main mechanism of cell entry by IMT-P8 appears to be macropinocytosis mediated by cell-surface proteoglycans (141). Interestingly, the N-terminal portion of the IMT-P8 primary sequence (RRWRRWNRFNRRRCR) is similar to the R_6W_3 peptide (RRWWRRWRR) reported by Sagan and colleagues (146). A comparison of the skin-penetrating capacities of these two peptides should be revealing in terms of the extent to which residues 10-15 of IMT-P8 are required for activity.

In tests on shaved mouse skin, FITC-labelled IMT-P8, with or without a peptide cargo, appeared to partition predominantly to the SC (147). Relatively little distribution was evident in the bulk of the epidermis. Similarly, a large (green fluorescent protein) cargo attached to IMT-P8 appeared to be targeted mainly to hair follicles (147). Hence, utility of IMT-P8 for therapeutic miRNA transport into the epidermis will depend on the emergence of further evidence that IMT-P8 cargoes localise and function within interfollicular keratinocytes. Alternatively, IMT-P8 may

prove useful for miRNA delivery specifically to the hair follicle, for instance as an intervention for alopecia or cylindroma (148-150).

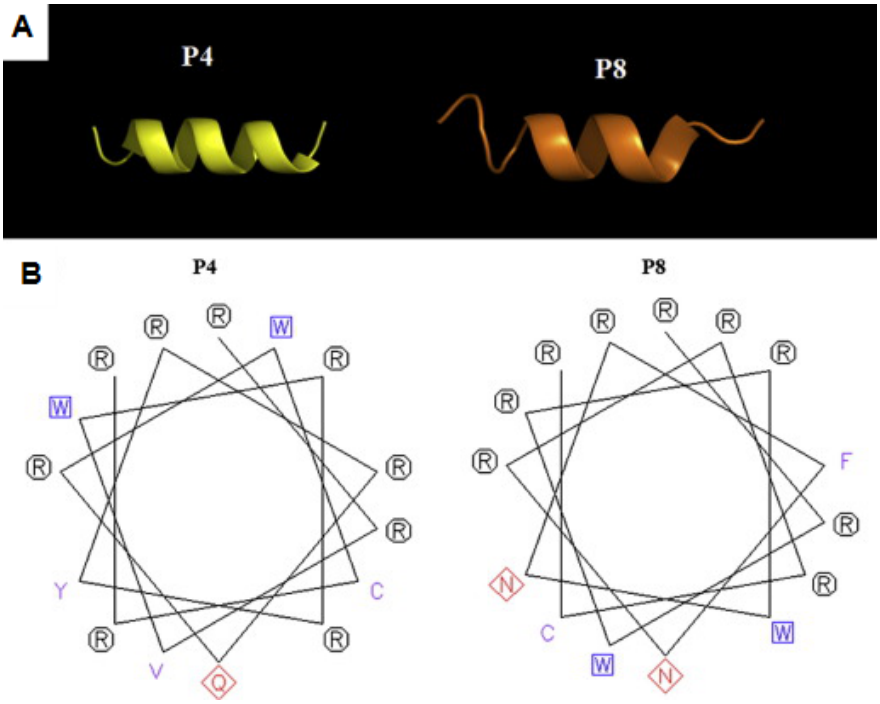

Fig. 3: Representative CPPs of the IMT family. (A) Predicted helical structures of IMT-P4 and IMT-P8. (B) Helical wheel projections of IMT-P4 and IMT-P8 showing the tryptophan (W) residues positioned opposite arginines (R) in IMT-P8 but interspersed between arginines in IMT-P4. Adapted from Gautam et al., ref. (141) with permission from Elsevier.

One limitation of the CPPs covered above is the requirement for covalent conjugation to their cargoes. In contrast, PepFect6 (PF6), developed by the Langel group, formed stable complexes with siRNA simply upon mixing the two molecules (142, 151). PepFect6 is an analogue of transportan 10 designed with an N-terminal stearyl moiety that enhances membrane insertion and four trifluoromethylquinoline derivatives to promote endosomal escape (142, 152). Recently, subcutaneous injection of PF6 CPP complexed with a miR-146a mimic was shown to suppress inflammatory gene expression in a mouse model of irritant contact dermatitis (153). There is arguably a case, therefore, for merging PF6 with SPACE peptide, R7 or TD-1 in order to combine the oligonucleotide-binding capacity of PF6 with the ability of the SPPs to cross the SC into the viable epidermis.

Spherical nucleic acids and gold nanoparticles

Various gold nanoparticles (AuNP) are also under investigation for oligonucleotide delivery, reviewed in (154). Spherical nucleic acid nanoparticle conjugates (SNA-NC) are particularly intriguing AuNPs that have been shown to rapidly enter over 50 cell types (155). The original SNA-NC (depicted in Figure 4) consisted of a 3-dimensional (3D) gold core decorated with a densely packed shell of covalently immobilized, highly oriented oligonucleotides (156). Uptake of SNA-NC appears to occur via lipid-raft–dependent, caveolae-mediated endocytosis upon binding to class A scavenger receptors (157).

Work Paller and colleagues showed that such SNA-NC constructed with siRNA duplexes against the epidermal growth factor receptor (EGFR) distributed rapidly and extensively throughout the epidermis of hairless mouse skin and human skin equivalents upon topical application (158). More importantly, the expression of

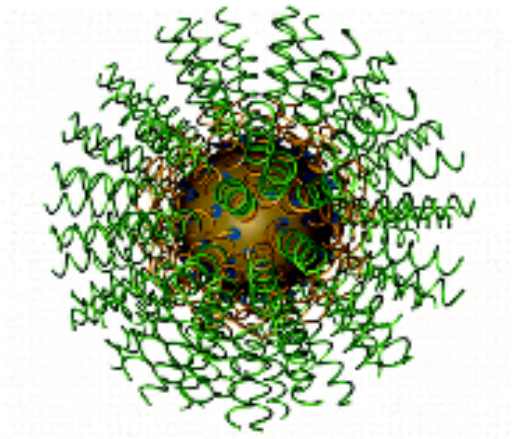

Fig 4: Depiction of a spherical nucleic acid illustrating a gold core surrounded by a densely packed layer of covalently attached oligonucleotides. The core can be based on other metals or on polymers, and appears dispensable for SNA function. Reprinted with permission from Cutler et al., ref. (155). Copyright 2012, American Chemical Society.

EGFR at the mRNA and protein levels was strongly suppressed in both skin models, with no apparent toxicity (158). Similar siRNA-based SNA-NCs were found to promote wound healing by downregulating ganglioside-monosialic acid 3 (GM3) synthase (159). Notably, the gold core of SNA-NC can be replaced with a biocompatible porous silica (SiO_2) shell without loss of SNA functionality (160). This enhances the translational potential of SNAs given that silica can be degraded under physiological conditions (161). Furthermore, exchanging the gold core for a hollow silica shell showed that the emergent ability of SNAs to function as delivery agents is due to the dense layer of oriented oligonucleotides as opposed to the inorganic core (160).

The SNA-NC approach has been explored for targeting miRNA. Mirkin and co-workers produced SNA-NC with miR-205 mimics to facilitate miRNA replacement in prostate cancer cells (162). Although SNA-NC:miR-205 lowered the expression of a target gene by 52%, compared to a non-targeting control SNA-NC, the effects of SNA-NC:miR-205 on the transcriptome as a whole have not been established. Such insights would reveal the putative differential impact that SNA-NC:miR-205 has on miR-205-regulated transcripts. In any case, as miR-205 has been reported to promote epidermal and corneal keratinocyte migration (123) the wound healing potential of SNA-NC:miR-205 deserves exploration.

Whereas the SNA-NC:miR-205 study aimed to raise effective miR-205 levels, a recent study constructed SNA-NC with antisense oligonucleotides to inhibit miRNA function. Using SNA-NC:anti-miR-99b, inflammation-related depletion of a miR-99b target gene was reversed in a mouse model of sepsis (163).

These miRNA-directed SNA-NC proof-of-concept studies combined with the established abilities of topically applied SNA-NC to permeate the epidermis support the development of SNA-NC as modulators of miRNA activity in the skin. Initial efforts focussed on SNA-NC:miR-146a and SNA-NC:anti-miR-21 will be highly relevant for psoriasis.

Non-covalent AuNP conjugates have also been evaluated for oligonucleotide delivery (154). Recently, a layer-by-layer approach was used to generate chitosan-coated AuNPs/siRNA/chitosan formulations that penetrated porcine ear skin under iontophoresis (164). However, although AuNPs coated with chitosan or other polymer is of broad biomedical interest, there is a paucity of data regarding their utility for oligonucleotide delivery to the skin. The reader is referred to an excellent review on polymer-coated AuNPs for further insight (165).

Deformable liposomes

Various “elastic” “flexible” or “ultradeformable” liposomes have recently emerged for topical delivery of therapeutic agents (166-169). Elasticity in this context relates to the presence of both stabilizing and destabilizing molecules within a given lipid bilayer and the ability to redistribute within that bilayer (166). The exploitation of liposomes for siRNA delivery has been broadly reviewed recently by Chourasia and colleagues (170). Here, Transfersomes®, ethosomes®, transethosomes and SECosomes have been selected to illustrate the potential of deformable liposomes for topical cutaneous drug delivery.

Transfersomes: The first generation of elastic vesicles developed by Cevc and colleagues were Transfersomes® consisting of phosphatidylcholine and a single chain surfactant such as sodium cholate as an edge activator (171-174). Other edge activators include Span 60/65/80, and Tween 20/60/80 (175, 176). Transfersomes® have been shown to mediate transcutaneous delivery of large macromolecules including protein immunogens (171), DNA vaccines (177, 178), insulin (173), interleukin-2 (179), hydrophobic macromolecules such as lycopene (180) and tretinoin (181). In addition, both glucocorticosteroids (182) and the nonsteroidal anti-inflammatory drugs diclofenac (183, 184) and meloxicam (184-186) have been formulated in Transfersomes®. Efforts to deploy Transfersomes® specifically for inflammatory skin disease include formulation of tacrolimus for atopic dermatitis (187) but use of Transfersomes® in miRNA-directed applications has not been reported to our knowledge. The microscopic structures of Transfersomes®, ethosomes® and transethosomes are depicted in Fig. 5.

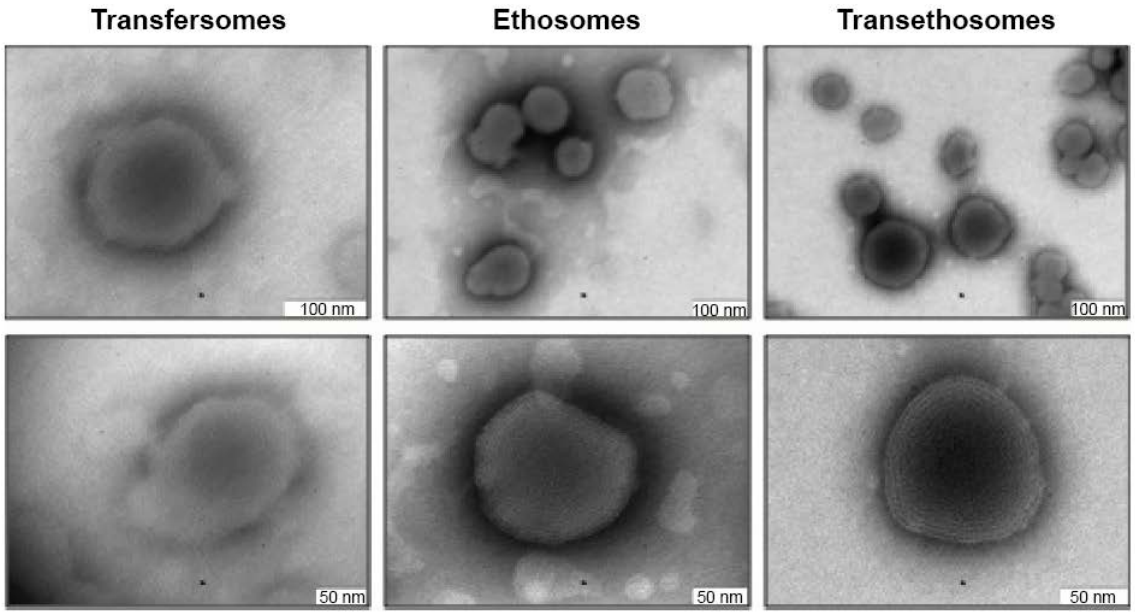

Fig 5: Transmission electron micrographs of ultradeformable liposomes that been compared directly for topical epidermal drug delivery. Reprinted from Ascenso et al., ref. (176) under the Creative Commons Attribution Non-Commercial (unported, v3.0) License. Dove Medical Press Limited.

Ethosomes: Work by Touitou and collaborators led to the development of transfersome–like liposomes in which high dosing with ethanol was deployed to enhance skin permeation (188). Ethosomal lipids assembled into phospholipid bilayers in dispersions of up to 45% ethanol, and any solubilisation of the phospholipid appeared to be limited (188). A diverse range of drugs have been loaded into ethosomes® (168, 189). For psoriasis, these include methotrexate (190), tretinoin (191) and 5-aminolevulinic acid, a second-generation photosensitizer for a photodynamic therapy (192). Ethosomal entrapment has also been reported to enhance the permeation of paclitaxel across the SC and improve anticancer activity of the drug in a cSCC cell line (193). As with Transfersomes® though, the potential

of ethosomes® for oligonucleotide delivery into the epidermis remains an underexplored area of research.

Transethosomes: By combining the edge activator approach of Transfersomes® with the high ethanol dose of ethosomes®, Song and colleagues designed transethosomes (175). Initial studies suggested enhance penetration of a lipophilic drug compared to Transfersomes® or ethosomes® (175). In more recent tests using porcine ear skin, Simões and colleagues found that transethosomes enabled deeper penetration of vitamin E into the viable epidermis compared to both Transfersomes® and ethosomes® (176). A similar comparison of Transfersomes®, ethosomes® and transethosomes for delivery of fluorescently-labelled miRNA inhibitors or mimics would provide a strong framework for pushing the use of these deformable vesicles for miRNA-directed therapy.

Specific comparison of Transfersomes®, ethosomes® or transethosomes for oligonucleotide transport into the epidermis does not appear to have been reported. However, cationic elastic liposomes based on 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) and sodium cholate have been shown to convey oligonucleotides into mouse epidermis (194, 195) and cadaveric human skin (196). Functional efficacy was demonstrated using antisense oligonucleotides targeting IL-13 for atopic dermatitis (194) and siRNA targeting BRAF in melanoma cells (196). Similar liposomes comprising 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and ceramide in addition to DOTAP and sodium cholate have also been tested for siRNA delivery in A431 cSCC cells. Liposomes loaded with siRNA against signal transducer and activator of transcription 3 (STAT3) attenuated target gene expression in A431

cells (197). However, no permeation of porcine ear skin was observed beyond the SC unless the specimens were also subjected to iontophoresis (197).

Silencing of keratin 17 (KRT17) has also been explored for anti-psoriatic potential through topical application of siRNA in a liposome-emulsion formulation though the details of the liposome were not reported (198). Importantly, however, Wang and colleagues showed siKRT17 silenced expression of KRT17 human psoriatic skin xenografts on mouse models, and this was associated with histological and clinical improvement including a reduction in epidermal thickness and substantial clearance of erythema and scales (198).

SECosomes: Building on initial work showing siRNA-delivery to melanocytes using DOTAP/sodium cholate elastic liposomes (199), Lambert and co-workers combined DOTAP and sodium cholate with cholesterol as a stabilizer and 30% ethanol to enhance penetration, creating surfactant-ethanol-cholesterol-osomes (SECosomes). These SECosomes were shown to transmit siRNA to a skin-humanized mouse model of psoriasis to silence expression of human beta-defensin 2 (hBD-2, encoded by *DEF4*), an anti-microbial peptide that is highly over-expressed in psoriatic skin (200, 201). More recently, by altering the cholesterol composition and replacing sodium cholate with DOPE, the group produced a modified SECosome (DDC642) with increased ability to deliver siRNA into the viable epidermis of normal and psoriatic skin explants (202). The penetration of DDC642:siRNA lipoplexes into *ex vivo* psoriatic was associated with strong suppression of the target hBD-2 within 48 h. In addition, DDC642 mediated pre-miR-145 and anti-miR-203 oligonucleotide in melanocytes and keratinocytes, respectively, to modulate target mRNA levels (202). However, anti-miR-203 had little effect on target protein levels raising questions

about the optimisation required to achieve biologically relevant miRNA-dependent outcomes with DDC642. Very recently, the group also demonstrated the ability of DDC642 complexed with siRNAs to repress targets in the reconstructed 3D psoriasis skin model available from MatTek Corporation (203). Combining siRNAs against hBD-2, thymic stromal lymphopoietin (TSLP) and KRT17 into a single DDC642 formulation silenced the first two of these genes by 38% and 45%, respectively (203). However, individual siRNA formulations, including siKRT17 were more effective at reducing the levels of distinct psoriasis markers indicating the synergistic potential of a multi-targeted approach requires further evaluation.

LeciPlex

Liposomal vesicles in the form of self-assembled nanocarriers composed of lecithin phospholipids and cationic lipids such as didodecyldimethylammonium bromide (DDAB) or cetyltrimethylammonium bromide (CTAB) have also been developed recently for drug delivery (204, 205). These LeciPlex nanocarriers transported hydrophobic drug molecules to the SC, viable epidermis and dermis. Some evidence of *in vivo* drug efficacy on a rat model of acne was observed when LeciPlex vesicles were loaded with the antibacterial agent azelaic acid (204). However, the utility of LeciPlex nanocarriers for transcutaneous delivery of oligonucleotides remains to be determined. Although the abilities of DDAB and CTMA to trigger irritation raises concerns over their suitability for skin therapy (204), the low cost of DDAB is an important consideration for development of cost-effective liposomal nanocarriers, given that DDAB h

as been estimated to cost 1/800 the price of DOTAP (206). Hence, the abilities of recently reported DDAB-poly(ethylene glycol) nanoassemblies to deliver miRNA-

directed oligonucleotides across the SC into the viable epidermis warrants investigation (206).

Liquid crystalline nanoparticles

Liquid crystalline phase aggregates are lipid-based alternatives to liposomes that have received attention for topical siRNA delivery. When amphiphilic lipids are placed in an aqueous environment, they can self-organise into diverse liquid crystalline structures including the lamellar phase, cubic phase and reverse hexagonal phase (207-209), depicted in Fig. 6.

Monoolein (MO; glycerol monooleate) is widely used for the generation of such liquid crystalline nanoparticles (LCNPs) and the geometries of cubic phase (cubosomes) and reverse hexagonal phase LCNPs make them particularly attractive vehicles for drug delivery and controlled release (210-213).

Early work by the Bentley group found that both cubic and reverse hexagonal phases of aqueous MO LCNPs enhanced the accumulation of CsA in the epidermis and dermis of porcine skin and hairless mice (214). Addition of oleic acid (OA) enabled formation of the reverse hexagonal phase at room temperature (214). More recently, MO:OA nanodispersions incorporating cationic polymer polyethylenimine (PEI) or cationic lipid oleylamine (OAM) were shown to transmit siRNA across the SC into the viable epidermis of hairless mice and silence expression of the GAPDH target (215). Importantly, using optimised MO:OA:PEI:aqueous phase dispersions, siRNA was targeted to IL-6 in a reconstituted human psoriasis skin model, leading to a 3-fold reduction in secreted IL-6 levels (216). Interestingly, functionalisation of

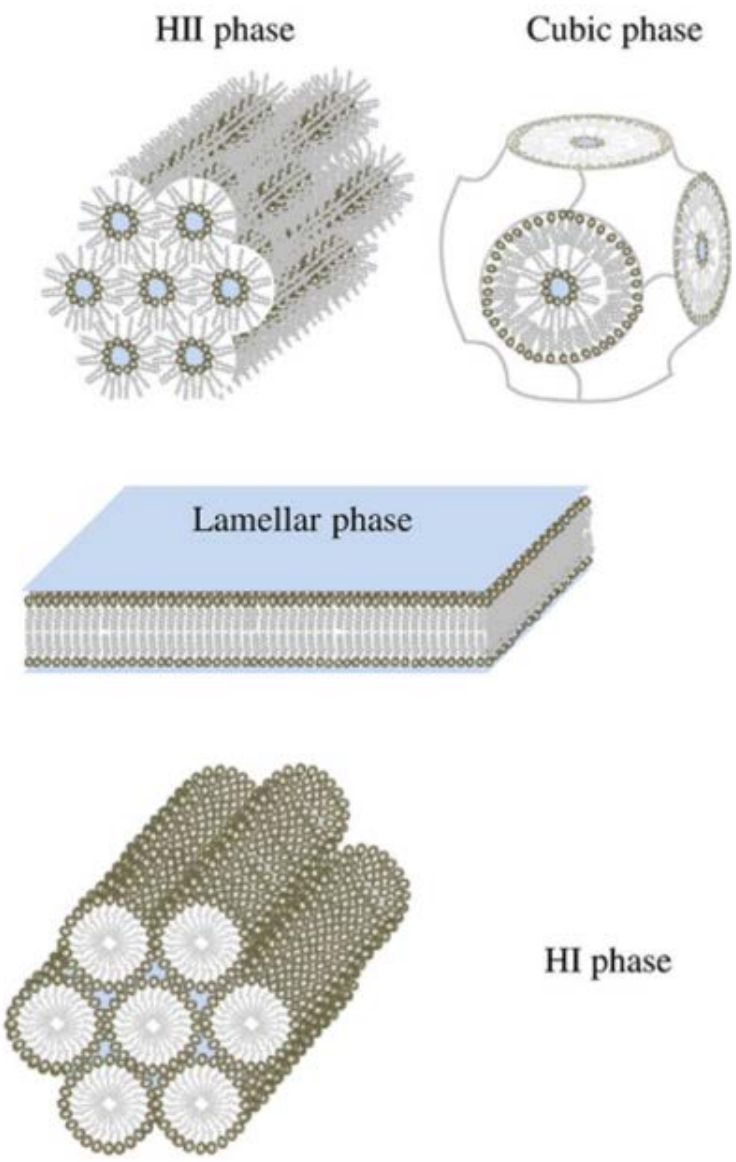

Fig. 6: Liquid crystalline phase structures. Representation of the reverse hexagonal (HII), cubic, lamellar and tubular (HI) phases that can emerge depending on the relative cross-sectional dimensions of polar head groups and hydrophobic regions. Adapted from Jouhet, ref. (209) under the Creative Commons Attribution License. Frontiers Media.

MO:OA:PEI:aqueous phase nanodispersions enhanced siRNA-mediated suppression of TNFα in a phorbol ester-induced model of inflammation on hairless mouse skin (217). Together, these developments suggest Bentley's LCNPs should prove useful in studies aimed at delivering miRNA-directed oligonucleotides, especially anti-miR-21 and miR-146a mimics, to reconstituted in vitro and mouse models of psoriatic skin. In addition, functionalisation with SPACE peptide, polyarginine or IMT-P8 may enhance the penetration of Bentley's LCNPs into the epidermis even more than with the TAT peptide.

Targeting the epidermal keratinocyte

Diverse receptors including scavenger receptors, receptor tyrosine kinases, G protein-coupled receptors, integrins and TLRs can be selectively targeted for oligonucleotide delivery (21). In addition, glycosaminoglycan (GAGs) have also been targeted to promote uptake of a range of cargoes in hard-to-transduce cells (218). For specific targeting of keratinocytes, a particularly novel approach has been developed using an anti-desmoglein (Dsg) monoclonal antibodies (219). Desmoglein is a non-classical cadherin found in the desmosomes of the epidermis (220). Stanley and collaborators cloned a single-chain variable fragment (scFv) of a patient-derived anti-Dsg antibody to yield Px44. As Px44 does not include the antibody effector region, antibody-induced inflammation is avoided (219). In their studies, specific localization of a Px44-conjugated protein cargo to epidermal keratinocytes was observed after intradermal injection of the complexes into human skin xenografts on mice (219). The obvious corollary would be to determine whether Px44 can enhance the overall efficacy of epidermal oligonucleotide delivery with the vehicles surveyed above.

Conclusions

In summary, we are beginning to witness the deployment of the diverse delivery vehicles surveyed above for topical delivery of siRNA, miRNA mimics and miRNA inhibitors to the epidermis (Table 2). Translating the promising initial findings to successful epidermal miRNA-dependent therapy will need to be supported with system-wide proteogenomic analyses of reconstituted human psoriatic epidermis models (221-223) exposed to the various topical vectors surveyed herein. In line with the need for affordable psoriasis treatments highlighted by the World Health Organisation (224), it will also be important to maintain cost-effectiveness for any therapies that emerge. In the meantime, clinical trials of nanoscale approaches for topical miRNA-directed therapy are likely to emerge in the near future for psoriasis, cSCC and wound healing.

Table 2: Topical RNA interference for epidermal disease

Target	Vehicle	Model	Refs.
miR-146a	PepFect6/miR-146a mimic[1]	Mouse ear irritant contact dermatitis	(153)
EGFR	SNA-NC/siRNA	Hairless mouse skin; reconstituted skin equivalents	(158)
GM3S	SNA-NC/siRNA	Diabetic mouse wounds	(159)
STAT3	chitosan-coated AuNPs/siRNA/ chitosan[2]	Porcine ear skin	(164)
STAT3	Cationic liposomes/siRNA[2]	Porcine ear skin	(197)
DEFB4	DDC642 SECosome/siRNA	Reconstituted psoriatic skin	(202)
SOCS3	DDC642 SECosome/anti-miR-203	Keratinocytes	(202)
FSCN1	DDC642 SECosome/pre-miR-145	Melanocytes	(202)
IL-6	Liquid crystalline nanodispersions/siRNA	Reconstituted psoriatic skin	(216)
TNFα	Liquid crystalline nanodispersions with TAT peptide	Hairless mouse skin with chemically-induced inflammation	(217)

[1] PepFect6/miR-146a mimic delivered locally by subcutaneous injection

[2] Iontophoresis was required for penetration beyond the stratum corneum

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