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Nucleic acid delivery into skin for the treatment of skin disease: proofs-of-concept, potential impact, and remaining challenges

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Abstract

Nucleic acids (NAs) hold significant potential for the treatment of several diseases. Topical delivery of NAs for the treatment of skin diseases is especially advantageous since it bypasses the challenges associated with systemic administration which suffers from enzymatic degradation, systemic toxicity and lack of targeting to skin. However, the skin's protective barrier function limits the delivery of NAs into skin after topical application. Here, we highlight strategies for enhancing delivery of NAs into skin, and provide evidence that translation of topical NA therapies could have a transformative impact on the treatment of skin diseases.

Graphical abstract

Keywords

Nucleic acids; dermal drug delivery; skin disease

Introduction

Nucleic acids (NAs) hold great potential for the treatment of various diseases, and there has been a significant amount of both academic as well as commercial interest in a variety of NA-based therapeutics including genes, antisense oligodeoxynucleotides (ODNs), siRNA, aptamers, and CpG oligonucleotides [1–4]. However, translation of these platforms to the clinic has been significantly limited by challenges associated with delivering NAs to the diseased site. Enzymatic degradation in the blood, rapid clearance from systemic circulation,

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poor bioavailability at the target site, and immunological response have yet to be adequately addressed for the implementation of systemically administered NA therapies [1, 4].

Delivery of NAs through the skin offers a potential solution to these issues, especially for the treatment of dermatological diseases (Table 1). The skin is the largest organ of the body, and can provide a pain free and compliant interface for drug delivery [5, 6]. Topical delivery of NAs offers several advantages over alternative delivery routes including avoidance of major degradative pathways in the GI tract, avoidance of enzymatic degradation and clearance from the bloodstream, sustained and controlled delivery, reduction in systemic toxicity, the ability to easily observe and treat sites of adverse reactions, and improved patient compliance [6]. Concurrently, it provides a means to directly target the diseased sites for the treatment of dermatological diseases e.g. skin cancer, psoriasis, and atopic dermatitis.

Topical delivery of NAs, however, is quite challenging. The challenge originates from the outermost layer of the skin – the stratum corneum (SC) – which serves as a formidable barrier to the entry of topically applied drugs. Nevertheless, significant efforts have been expended over the years to overcome the skin barrier. This review highlights strategies to effectively deliver NAs into the skin. Focus is placed on their applications for the treatment of dermatological diseases. Further, we provide evidence of the transformative impact of topical NA therapies on the treatment of skin diseases. The efforts here highlight significant advances in topical delivery of NAs over the last decade, and aim to guide future technologies and their translation into the clinic.

The Skin Barrier

The outermost layer of skin, the SC, is primarily responsible for its barrier function. The SC is a thin layer only 10–20 μm thick that is made up of corneocytes. Corneocytes are anucleate cells heavily enriched with intracellular keratin filaments. Corneocytes are held together in a “brick and mortar” structure by a lipid matrix composed of ceramides, free fatty acids, and cholesterol. Materials traversing the skin barrier must, therefore, diffuse through the tortuous lipid channels, and/or traverse transcellularly through corneocytes, or enter the skin through hair follicles or sweat ducts (Fig. 1). Transport within the lipid bilayers, however, is the most common mode of passage through the skin. This results in the exclusion of most foreign materials, and more specifically, renders passage of large, hydrophilic molecules (>500 Da and $\text{Log } P_{\text{o/w}} < 1.5$) such as NAs (typically $\gg 10,000$ Da, $\text{Log } P_{\text{o/w}} < 0$) to virtually negligible levels without some form of enhancement strategy.

The layer underlying the SC, the epidermis, can also serve as another transport barrier [7]. The epidermis is the first viable tissue layer of the skin where the pathology of several dermatological disorders resides. The epidermis is 50–100 μm thick and is composed primarily of keratinocytes. As keratinocytes migrate upward from deeper portions of the epidermis to the SC they gradually begin to keratinize and secrete lipids that eventually form the SC bilayers. This process continues as keratinocytes terminally differentiate into corneocytes and serves to rejuvenate the SC from underneath while the outermost layer of the SC sloughs away. Within the epidermis, keratinocytes are held together by cell-cell tight junctions. In the epidermis, claudin-1, claudin-4, occludin, and zonula occludens-1 are

responsible for inhibiting paracellular transport [8]. This makes transport of NAs, other large macromolecules, and drug carriers such as nanoparticles and NA-lipid complexes difficult both vertically, deeper into the skin, as well as laterally from the site of administration to peripheral areas of the skin. For effective treatment, both the SC as well as epidermal transport barriers must be overcome to deliver NAs to all areas of the disease.

Methods of Transport Enhancement

Over the years, a large number of strategies have been devised for perturbing the SC to enhance the delivery of drugs into and through the skin. These strategies can be generally categorized into three main groups: physical, active, and passive methods. The advantages and disadvantages of each class of perturbation methods are summarized in Table 2. Their use for the delivery of NAs is described below.

NA delivery using physical methods

Microneedles—Intradermal injections are the simplest and most direct method for delivering NAs into the skin. Here, the barrier properties of the SC are overcome completely by injecting NAs directly into the viable tissue layers of the skin. Intradermal injections are typically used for evaluating efficacy of NAs or other cutaneous therapeutics, or as the positive control for evaluating dermal delivery technologies, however, the downsides of intradermal injection for treating skin disease are overwhelming. Needle-phobia is a serious concern for a large number of children as well as adults leading to significant patient non-compliance [9]. Moreover, intradermal injections are limited only to the site of application, and injection into multiple sites during a single administration is challenging. To avoid many of these drawbacks, microneedle arrays have been developed. Microneedle arrays comprise needles that are only 100–700 μm in length (Fig. 2). When placed on the skin, their sharp tips allow easy insertion into the stratum corneum, while the short length ensures adequate penetration into the skin without disrupting nerves in deeper skin tissue. Microneedles have been used extensively for the delivery of NAs. Mikszta et al. used microneedle arrays to deliver plasmid DNA encoding a hepatitis B surface antigen for immunization [10], and they showed extensive immunological response in mice. Antibody titers following application of microneedle arrays were significantly higher and less variable than when delivered using either intradermal or intramuscular injection. Chabri et al. used microneedles to deliver cationic lipid-DNA complexes (~100 nm diameter) into the skin [11]. Ding et al. demonstrated successful immunization of mice with co-administration of diphtheria toxoid and CpG oligonucleotide delivered by microneedle array [12], and Gonzalez-Gonzalez et al. demonstrated effective delivery of anti-luciferase siRNA and gene silencing in luciferase expressing transgenic mice [13].

Microporation—Microporation is another technique that employs physical disruption of the SC for delivery of large therapeutics or therapeutic carriers. An array of resistive elements can be placed on the skin. An electric current pulsed through the array results in localized ablation of corneocytes in contact with the array [14]. Alternatively, erbium:yttrium-aluminum-garnet (Er:YAG) laser arrays can be used for localized ablation of the SC and epidermis [15]. Similar to microneedle arrays, microporation has gained

considerable interest over the last decade. For example, Lee et al. used laser microporation to deliver antisense oligonucleotide as well as plasmid DNA into the skin [16]. Delivery of antisense oligonucleotide was enhanced 3–30 fold compared to intact skin *in vitro*. In addition, expression of GFP in nude mice was enhanced 160 fold after application of GFP plasmid DNA. The amount of enhancement correlated with both the laser fluency as well as the size of oligonucleotide. The same group also showed enhanced delivery of siRNA [17]. siRNA delivery into skin was enhanced 3.5 fold and localized mainly in the dermis. Hessenberger et al. used laser microporation to deliver CpG oligonucleotides into the skin and successfully protected against immune response to grass pollen in mice [18]. They used the Precise Laser Epidermal System (Pantec Biosolutions) which creates well-defined arrays of micropores in the skin, and allows precise control over the number, density, and depth of the micropores giving the user unprecedented tuneability over the amount and localization of therapeutic delivered [19].

NA delivery using active methods

Electroporation—Electroporation can be used to permeabilize the skin and enhance passive diffusion of drug. The mechanism of electroporation is quite different from that of electrically-induced microporation. Electrically-induced microporation utilizes electric fields to induce thermal ablation of SC microstructure creating pores in the skin [14]. On the other hand, electroporation is the application of short duration (< 0.5 s) and high intensity (< 100 V) electric pulses to the skin [20] which result in transient permeabilization of the lipid bilayers in the skin and concurrently permeabilize cell membranes of epidermal keratinocytes. Electroporation is also expected to create aqueous pores through the skin. However, electroporation, unlike microporation, acts through non-thermal mechanisms as lipid rearrangement, reduced skin resistance, and enhanced transdermal transport are observed in the absence of a significant temperature rise in the pulse medium [20]. Using electroporation, Regnier et al. showed enhanced permeability of the stratum corneum to a phosphorothioate antisense ODN [21]. The permeability enhancement lasted up to 1 hr post-electroporation in rat skin *in vitro*. Specifically, transport was enhanced > 4-fold into the SC and > 3-fold into viable skin tissue when oligonucleotide solution was applied immediately following electroporation. Further, Zhang et al. demonstrated effective gene transfection and expression in the epidermis of human skin [22].

Iontophoresis—Iontophoresis can be used to drive transport of charged drugs like NAs. Applying a continuous low intensity (< 10 V) electric field at a constant current [20] has been used extensively to deliver a wide range of charged therapeutics including calcitonin, luteinizing hormone-releasing hormone, and dexamethasone [5]. In contrast to electroporation which acts primarily on the skin structure, iontophoresis is not believed to cause major changes to the skin. Minor structural effects can be observed and may partially contribute to delivery enhancement through sweat ducts and hair follicles [20, 23]. Nevertheless, iontophoresis is believed to primarily act on the drug itself, driving transport of a charged molecule by means of an applied electric field. Using iontophoresis, Kigasawa et al. demonstrated successful delivery of anti-IL-10 siRNA [24]. Specifically, using an atopic dermatitis model in rats they showed a 73% reduction in IL-10 mRNA levels after treatment with anti-IL-10 siRNA and iontophoresis. The same group later extended the

technique for vaccination as well as treatment of melanoma tumors [25]. Kigasawa et al. reported enhanced delivery of CpG oligonucleotides into the epidermis and dermis using iontophoresis compared to that by free diffusion. Further, for the treatment of melanoma tumors in hairless mice, CpG oligonucleotides delivered using iontophoresis resulted in tumor reduction comparable to subcutaneous injection. Abu Hashim et al. also demonstrated the ability to deliver NF- κ B decoy ODN into skin using iontophoresis for atopic dermatitis treatment [26]. While passive diffusion of FITC-NF- κ B decoy ODN was negligible for all concentrations tested, ~ 100 pmol/cm² was delivered after 6 hours of iontophoresis using 0.5 mA current density. In addition, the authors observed a linear dependence between the flux of ODN into the skin with both current density and concentration of drug. When the technique was applied for the treatment of atopic dermatitis in mice, the authors noted significant reduction in both tissue swelling and TNF α expression. In comparison, treatment with buffer alone, as well as treatment with a scrambled ODN sequence, resulted in no significant reduction in either ear swelling or TNF α . Iontophoresis has also been used to deliver NA complexes into the skin. Brus et al. achieved enhanced delivery of polyethylenimine (PEI) complexed with ODN [27]. Negatively charged ODN was electrostatically complexed with positively charged PEI at a charge ratio of 1:13.3 to create positively charged complexes. Complexing was shown to protect oligonucleotides from enzymatic degradation and enhance intracellular delivery in epidermal keratinocytes. Complexes did not show any measureable passive diffusion, however, under constant electrical current the complexes did passage into the skin primarily via the shunt pathway.

Sonophoresis—Low-frequency ultrasound has also been demonstrated to transiently permeabilize the SC lipid bilayers, facilitating the delivery of a large number of macromolecules including insulin, bovine serum albumin, and heparin [5]. Tezel et al. first reported delivery of therapeutic quantities of NAs into the skin using sonophoresis [28]. Delivery of ODNs into porcine skin *in vitro* was significantly enhanced by applying 2.4 W/cm² ultrasound for 10 min (Fig. 3). Interestingly, enhancement seemed to occur in localized regions of the skin (Fig. 3), however, these regions were not observed to be hair follicles as is commonly reported for iontophoresis-mediated delivery of NAs. Instead, localized penetration zones most likely correspond to localized areas of acoustic cavitation inherent to this technique, and occupied $\sim 5\%$ of skin surface area. In addition, the authors showed no observable effect of ultrasound on skin histology. NA delivery with ultrasound was also shown to treat skin disease *in vivo*. Tran et al. demonstrated $\sim 30\%$ reduction in melanoma tumor size when an anti-B-Raf siRNA liposome formulation was delivered into skin using sonophoresis [29]. The liposome was postulated to protect siRNA from enzymatic degradation in skin, as well as aid intracellular uptake by melanoma cells.

NA delivery using passive methods

Nanoparticles—Nanoparticles are promising due to their high loading capacity, ability to shield enzymatic degradation, and reduce immunogenicity. Due to their size, nanoparticles are expected to require physical perturbation of the SC and underlying epidermis for effective delivery. Recently, however, functionalized nanoparticles have been shown to passively diffuse through the skin and elicit a therapeutic response. For example, Siu et al. demonstrated effective delivery of functionalized carbon nanotubes for the treatment of

melanoma *in vivo* [30]. Single-walled carbon nanotubes functionalized with PEI were shown to deliver anti-B-Raf siRNA and result in B-Raf silencing and attenuation of tumor growth in a mouse melanoma model. Ozbas-Turan et al. delivered chitosan complexed with β -galactosidase plasmid DNA into mouse skin resulting in significant expression of β -galactosidase after 7 days of treatment [31]. Similarly, Cui et al. showed delivery and expression of luciferase plasmid DNA when complexed with chitosan [32].

Liposomes—Liposomes have also been studied extensively for nucleic acid delivery for the treatment of skin disease. For example, Desai et al. used cationic lipid nanoparticles complexed with anti-TNF α siRNA and capsaicin to treat psoriasis *in vivo* [33]. Treatment resulted in significantly reduced expression of a number of inflammatory cytokines including TNF α , IL-17, IL-23, and NF- κ B. Bracke et al. used ultraflexible liposomes loaded with anti- β defensin-2 to treat psoriasis *in vivo* [34]. Plasmid DNA can also be delivered using liposomes. Li et al. delivered IL-4 encoding plasmid DNA into mouse skin *in vivo* for the treatment of psoriasis [35]. Enhanced expression of IL-4 led to suppressed hyperplastic and inflamed vessels in the skin. Similarly, Kim et al. used liposomes complexed with antisense ODN for IL-13 mRNA to treat atopic dermatitis *in vivo* [36]. Treatment with liposome formulation resulted in a significant reduction in skin thickness and inflammatory cell infiltration.

Spherical NAs—Highly ordered spherical complexes of nucleic acids (spherical nucleic acids) have shown potential for treating skin disease due to their enhanced delivery into skin, internalization into skin cells, and protection of NAs from degradation (Fig. 4) [37]. Further, several different sequences of NAs can be incorporated into a single construct for multifactorial diseases. Imaging agents like quantum dots can be used as the core. Alternatively, hollow spherical nucleic acids can be prepared for incorporation of additional drug. Zheng et al. successfully delivered anti-EGFR (epidermal growth factor receptor) siRNA into mouse skin using gold-core nanoparticles [38]. Specifically, gold nanoparticles coated with a dense layer of highly-ordered and covalently bound siRNA resulted in passive transport through intact mouse SC and localized exclusively in the dermis and epidermis. After 3 weeks of treatment the authors observed nearly complete knockdown of EGFR as well as repression of downstream phosphorylation, and reduction in epidermal thickness. Spherical nucleic acids also demonstrated the ability to stifle nuclease degradation and efficiently internalize into a large variety of cell types to stimulate gene silencing [39]. Further, spherical nucleic acids have been used to treat psoriasis [40] as well as aid wound healing [41] *in vivo* through knockdown of TNF α and ganglioside GM3 synthase, respectively.

Peptides—Peptides hold potential as drug delivery vehicles owing to their simplicity, diversity, biocompatibility, and potential for multi-functionality. In addition, peptides can be easily screened and selected from phage-display libraries for various functions (Fig. 5). Phage-display screening offers a powerful tool for high-throughput discovery of novel peptide sequences that can enhance penetration of large cargos into skin. Moreover, peptide sequences that localize in unique regions of the skin can also be selected to target specific cell types and minimize side effects. Over the last ten years, several peptides have been

identified which possess the ability to enhance transport of NAs into the skin and elicit a therapeutic response. The first of these peptides discovered using phage-display screening was TD-1 (ACSSSPSKHCG) [42]. Lin et al. showed TD-1 could enhance transport of GAPDH siRNA into viable tissue in the skin, as well as subcutaneous tissue, and silence GAPDH expression [43]. Co-incubation of GAPDH siRNA with TD-1 resulted in similar GAPDH expression levels as intradermal injection, while expression levels for both methods of application were significantly less than siRNA applied on the skin without peptide [43]. Further, application with TD-1 resulted in significantly reduced levels of target mRNA in the skin for up to 3 days and target protein in the skin for up to 7 days. Hsu and Mitragotri identified another peptide using phage-display screening, SPACE peptide (ACTGSTQHQC), with the ability to not only enhance delivery of siRNA across the skin but also enhance intracellular uptake [44]. *In vivo* application of SPACE peptide conjugated to IL-10 siRNA or GAPDH siRNA resulted in ~30% or ~45% knockdown in protein expression, respectively. This result is in contrast to the control formulation containing siRNA alone which showed negligible gene silencing. Further, SPACE peptide was shown to be non-toxic at concentrations as high as 10 mg/mL [45]. Uchida et al. used a dual-peptide system with both Tat peptide (GRKKRRQRRRCG) and AT1002 (FCIGRLCG) to enhance gene silencing in the skin [46]. Tat peptide and siRNA were complexed via electrostatic interactions. Complexes were then co-incubated with AT1002 and applied on the skin. Here, Tat peptide was used as a cell-penetrating peptide to enhance intracellular delivery, and AT1002 was used as a tight-junction modulator to enhance permeation into the epidermis and dermis. Tat peptide has also been shown to enhance macromolecule transport across the SC [47, 48]. The system was later applied to treat atopic dermatitis in mice [49]. Application of anti-RelA siRNA complexed with Tat peptide and co-incubated with AT1002 led to reductions in ear thickness, clinical skin severity, topical cytokine levels, and serum IgE production. Yi et al. successfully delivered anti-microphthalmia-associated transcription factor (anti-MITF) siRNA conjugated to TD-1 R8 peptide (ACSSSPSKHCGRRRRRRRR) for the treatment of patients with melisma [50]. Treatment with a cream formulation containing TD-1 R8 peptide co-administered with anti-MITF siRNA resulted in reductions in tyrosinase, tyrosinase-related protein 1, and melanocortin 1 receptor leading to measurable inhibition of melanin production and melanocyte apoptosis. In fact, after 4 weeks application patients demonstrated a significant lightening of facial hypermelanosis lesions and almost completely restored dark lesions to normal skin color after 12 weeks.

Dendrimers—Although less studied than peptides for delivering NAs into skin, dendrimers are similarly advantageous due to their diversity, ease of synthesis, and functional group density. Bielinska et al. demonstrated the use of dendrimers for topical gene delivery [51]. Dendrimer complexes resulted in measurable gene expression of chloramphenicol transacetylase that was ~7-fold higher than with naked plasmid. In addition, Venuganti et al. used a similar dendrimer in combination with iontophoresis to deliver antisense oligonucleotide [52]. Treatment with antisense oligonucleotide targeting the anti-apoptotic protein, Bcl-2, resulted in significantly enhanced apoptosis and reduction in tumor size in mice.

Potential Impact

The burden of skin disease is alarming and growing fast [53]. 1 out of 3 individuals are estimated to have a skin disease at any given time, resulting in direct healthcare costs over \$30 billion annually in the US alone [54]. Symptoms from the ~3000 different skin diseases range from itching, redness, and irritation to physical disfigurement, or death. Furthermore, skin diseases manifest externally leading to severe and even debilitating emotional distress and social prejudice [54]. Dermatological drugs aimed at treating skin disease reap an estimated \$24.4 billion in annual revenue [55]. In addition, many other skin conditions, not considered disease, are of major concern. These include cosmetic conditions like wrinkling, cellulite, discoloration, and skin pliancy. Growing demand for more effective treatments of cosmetic conditions is evidenced by the emergence and growth of the cosmeceutical market which was estimated to be ~\$35 billion globally in 2013 [56]. Topical NA therapies have exciting potential to reduce this burden and be transformative in the way we deal with and treat skin disease (Table 3). Examples are discussed in detail below.

Targeting orphan disease can help speed translation by allowing fast-tracked regulatory processing from orphan drug designation. There are several orphan skin diseases with recognized NA targets. Hidradenitis suppurativa (Hurley disease) is an orphan disease that affects over 100,000 people in the US [57], most commonly women [58]. It is a chronic and debilitating disease characterized by painful abscesses, redness, and scarring, leading to significant physical and psychological impairment and reduced quality of life [59]. Currently, the most common form of treatment is surgical excision of diseased areas of the skin [60]. For severe forms of Hurley disease there is no FDA approved treatment. Studies into the aetiology and pathogenesis of Hurley disease suggest it is a multifactorial inflammatory disease characterized by overexpression of TNF α [61] as well as other inflammatory cytokines like IL-17 and IL-23 [62]. Treatment of Hurley disease with anti-TNF α monoclonal antibodies or other biologics has been shown to be effective [60, 63]. TNF α promotes inflammation through recruitment of vascular endothelial cells and immune cells. Further, it is overly expressed at sites of inflammation while repression generally results in alleviation of inflammation [64]. While there is no FDA approved drug indication for treating moderate to severe Hurley disease, several anti-TNF α biologics are already on the market including etanercept and adalimumab. In fact, the anti-TNF α monoclonal antibody, Humira (adalimumab, Abbvie Inc.), was recently granted orphan drug designation by the FDA to start clinical trials on moderate to severe Hurley disease patients [65]. On the other hand, these drugs can only be administered by injection or infusion and systemic side effects limit their long-term use. Alarmingly, the most common side effect of systemic delivery is the development of other autoimmune diseases as a direct result of treatment due to increased expression of IL-17 and IL-23 as well as other inflammatory cytokines [64]. Therefore, topical delivery of anti-TNF α siRNA or antisense oligonucleotide is an attractive platform to limit off target effects, improve patient compliance, and reduce hospital-related costs. Even more impactful, a cocktail of topical anti-TNF α , anti-IL-17, and anti-IL-23 NA therapeutics may prove synergistic at alleviating Hurley disease symptoms while avoiding adverse reactions.

Epidermolysis bullosa (EB) is another rare skin disease with few effective treatments. EB affects roughly 30,000 people in the US and ~500,000 people globally [66]. EB symptoms typically include severe skin fragility to the point where large segments of skin can be removed from simply scratching or rubbing skin [66]. Alarming, few patients with severe EB survive past the age of 30 due to extensive wounds and infection. There is no FDA approved drug indication for EB. Management of EB consists of limiting blister formation from scratching and friction [66]. For more effective treatment, studies suggest IL-1 β and its receptor may be a valuable target. Indeed, overexpression of IL-1 β has been detected in patients with EB and treatment of EB in mice with anti-IL-1 β or IL-1 β receptor antagonist resulted in improvement of symptoms [67]. Further, TWi Biotechnology Inc. was recently granted orphan drug designation for its anti-IL-1 β and anti-IL-1 β receptor small molecule drug AC-201 [65]. EB can also be targeted at the genetic level. One subtype of EB (epidermolysis bullosa simplex), is predominantly caused by dominant-negative mutations in the genes encoding keratin-5 and keratin-14 (K5 and K14). siRNA therapies that specifically knockdown mutant keratin without affecting wild-type keratins have shown promise [68, 69]. A second subtype of EB (dystrophic epidermolysis bullosa) is caused by mutations in collagen VII and gene delivery of wild-type collagen VII has shown promise as a therapeutic in phase I/II clinical trials [70].

Pachyonychia congenita (PC) is a rare genetic autosomal dominant skin disorder that affects 5,000 to 10,000 people [71]. PC may be caused by mutations in either K6a, K6b, K6c, K16, or K17 [66]. The disease typically manifests with calluses and blisters on the feet and palms, as well as discoloration, cysts, and hyperkeratosis of the hair follicles [72]. Moreover, patients typically live in constant pain. Due to the large number of different mutations that can result in PC, topical application of personalized NA therapies holds the most potential. In fact, treatment of patients with topical siRNA therapies are showing promise both in mouse models as well as in humans [73–77].

Netherton syndrome is an autosomal recessive disorder estimated to affect 1/50,000 individuals [78]. Symptoms present at birth or shortly after and include painful peeling and scaling of skin. More alarmingly, Netherton syndrome manifests significant impairment of skin barrier function leading to life-threatening dehydration and frequent systemic infections. Netherton syndrome is caused by a mutation of the serine protease inhibitor Kazal type 5 (SPINK5) gene leading to serine protease hyperactivity and excessive protein degradation in skin. Essentially, protease hyperactivity results in excessive and premature epidermal desquamation [79]. Netherton syndrome may be treated by down-regulating production of serine proteases in the skin, or alternatively, inducing expression of wild-type serine protease inhibitor [80]. Due to the significant reduction in skin barrier function, initial treatment with topical NAs may not require dermal enhancement. Instead, technologies to localize drug in the skin such as cell-targeting or skin targeting peptides may be required. However, as skin integrity improves more significant enhancement may be required.

Mainstream disease would also benefit from topical NA therapies. For example, psoriasis is estimated to affect nearly 4.5 million adults in the US alone [81]. Children are also commonly affected by the disease. It is a chronic skin disease that presents as severe lesions, redness, and itching and poses a significant burden on patients' quality of life. About 20–

25% of patients are estimated to be dissatisfied with their current treatment [81]. Of even more concern, for patients suffering the most severe form of psoriasis, erythrodermic psoriasis, there is no standard treatment regimen [82]. Due to the multifactorial nature of psoriasis, NA therapies may be advantageous because NA cocktails targeting a number of different targets can be formulated and delivered leading to down/up regulation as needed. Psoriasis is one of the more exhaustively studied forms of inflammatory skin disease and numerous therapeutic targets have been proposed; a subset is described here. Similar to Hurley disease, TNF α is perhaps the most established drug target for treating severe forms of psoriasis [64]. Topical delivery of anti-TNF α siRNA as well as a siRNA cocktail including anti-TNF α and anti-STAT3 was shown to be effective at alleviating psoriasis-like symptoms in mice [33, 83]. Activated T helper cells have also been shown to play a major role in the manifestation of psoriasis, and selective skewing from Th1 phenotype to the IL-4 producing Th2 phenotype can alleviate psoriasis symptoms [84]. Further, selective differentiation to the Th2 phenotype can be simply induced by subcutaneous injection of IL-4 [84]. As an alternative to injection, psoriasis may be alleviated through topical delivery of plasmid DNA to induce expression of IL-4 in the skin. Proof-of-concept has been demonstrated in a K14-VEGF transgenic mouse model of psoriasis [35].

Atopic dermatitis (AD) affects 10–20% of children and 1–3% of adults worldwide [85]. AD typically manifests with severe redness and itching, skin lesions, and papules resulting in significant impact on quality of life. Similar to psoriasis, the inflammatory nature of the disease makes topical NA treatment appealing. Although the exact cause of AD is not known, upregulation of inflammatory pathways such as the NF- κ B inflammatory pathway are typically observed, and inhibition of these pathways have shown promise. For example, delivery of anti-RelA, an important member of the NF- κ B inflammatory pathway, resulted in significant improvement in disease symptoms in mice [49]. In addition, knockdown of NF- κ B with decoy ODNs also showed therapeutic promise in mice [26]. Several cytokines are also believed to be associated with the pathogenesis of AD including IL-4, -5, -10, and -13. These cytokines are typically expressed in significantly higher quantities in AD lesions compared to healthy skin [85], and knockdown of these cytokines and others with topical NA therapies has shown promise in mouse models of AD [24, 36, 86, 87]. Further, suppressing the Th2-type dominated immunological response typical of AD may hold therapeutic promise. Indeed, delivery of CpG oligonucleotides was shown to significantly reduce AD lesions in mice by shifting from Th2-type dominant immune response to a more balanced Th1/Th2 immune response [88].

Cosmetic conditions would also benefit significantly from topical NA therapies developed as either pharmaceuticals or cosmeceuticals. However, cosmeceutical development may benefit from faster translation to the clinic due to reduced FDA regulations provided claims of the cosmeceutical are appropriately chosen. For example, cellulite manifests in 80–90% of post-adolescent women [89]. It is characterized by an “orange peel” appearance of the skin primarily in the thighs and buttocks. While physical burden is not associated with cellulite, significant emotional burden is typically associated with cellulite. Cellulite pathology is generally not well understood, however, a few NA targets that show promise are proposed in literature. For example, decreased microcirculation in subcutaneous adipose tissue is believed to play a role in cellulite formation. Specifically, overexpression of angiotensin-

converting enzyme has been linked to higher production of angiotensin II, a vasoconstrictive peptide, along with lower production of bradykinin, a vasodilative peptide [89]. Breakdown of the extracellular matrix (ECM) due to enzyme hyperactivity may also play an important role in cellulite [90]. Normal ECM may potentially be restored by silencing enzymes like collagenase, elastase, and lipase. Finally, fibrosis and localized inflammation may play an important role in cellulite formation [89]. Specifically, hypoxic inducible factor-1 (HIF-1) mutations that minimize its ability to induce fibrosis and inflammation have been shown to be associated with individuals who do not typically have cellulite, or have reduced severity of cellulite, while individuals with wild-type HIF-1 were shown to have a higher probability of more severe presentation of cellulite.

Skin wrinkling is another cosmetic condition that generates significant emotional burden. Wrinkling affects everyone as they age, however, treatment is most typically sought by post-adolescent females. Unfortunately, few effective treatments exist for the reduction of wrinkles. Currently, the most common treatment is intradermal injections of botulinum toxin A (Botox). Botox, however, is extremely toxic and results in temporary muscular paralysis at the site of injection. Further, Botox must be administered by a trained physician limiting its use and increasing its cost. Alternatively, topical NA therapies may be beneficial as both a treatment and prevention strategy. For example, elastase upregulation has been proposed as an important factor for ultraviolet irradiation induced wrinkle formation [91]. Inhibition of elastase with N-phenethylphosphonyl-L-leucyl-L-tryptophane resulted in reduced formation of wrinkles in mice exposed to daily doses of ultraviolet irradiation. Therefore, knockdown of elastase with topically applied NAs may be a viable option for the treatment and prevention of skin wrinkling, as well as provide patients with a safer and cheaper alternative to Botox injections.

Melasma is a hyperpigmentation disorder that manifests as darkening or browning of the skin, typically on the face [92]. Melasma disproportionately affects women and disproportionately affects ethnicities with darker skin color, however overall, melasma is generally estimated to account for 4–10% of all dermatological-related doctor visits [93]. Moreover, melasma is commonly reported to induce significant psychological burden, social impairment, and reduced quality of life [92, 93]. The most common treatments are skin lightening agents like tyrosinase inhibitors and spot removing agents like retinoic acid, kojic acid, and azelaic acid [50]. However, these are typically either ineffective or result in unsightly white spots when used in the clinic. Topical NA therapies may prove beneficial for treating melasma without adverse side effects associated with current treatments. Specifically, studies have shown silencing of the microphthalmia-associated transcription factor (MITF) gene could be an effective treatment strategy. MITF encodes for tyrosinase, tyrosinase-related protein 1, and melanocortin 1 receptor which are all involved in melanin synthesis. Topical delivery of anti-MITF siRNA has shown promise in human trials [50].

Remaining Challenges

Despite significant efforts to deliver NAs into the skin as well as identifying NA targets for treating skin disease, significant hurdles remain. Three major areas posed serious challenges in the past and still remain the bottleneck to successful translation of topical NA therapies:

(1) skin delivery, (2) cellular internalization, and (3) stability of NAs. These challenges must be addressed concurrently to develop successful NA topical delivery systems for use in humans.

Microneedles and other physical methods are the most effective at enhancing delivery into the skin, and the type and size of therapeutic is not restricted. Further, large depots can be easily incorporated for sustained release. However, significant questions remain in regards to their effectiveness at localized and homogeneous delivery into skin tissue. Specifically, physical methods inherently result in localized penetration zones. They do not address horizontal diffusion of NA which is retarded by cell-cell tight junctions in the epidermis [7, 8]. Active methods such as electroporation [94] and sonophoresis [28] may also be limited by localized penetration zones (Fig. 3). The effect of size of penetration area on clinical outcome needs to be determined. Combination therapies with co-delivery of NAs and, for example, tight junction modulators may be required for efficacy in humans.

Also of concern, physical methods do not enhance cellular internalization nor protect NAs from degradation in the skin. NAs need to enter into the cytoplasm or nucleus of cells to elicit a therapeutic response. Physical methods alone cannot facilitate this enhancement, and therefore, they must inherently be combined with other technologies to enable cell internalization. For example, microneedle arrays integrating active methods like electroporation [95–97] and sonophoresis [98] have been designed to facilitate NA delivery enhancement into cells. However, incorporating active methods for cell internalization further adds complexity and cost. Passive methods for cell internalization, for example, tagging NAs with cell-penetrating peptides, cell-penetrating dendrimers, or cell-penetrating aptamers have the potential to provide affordable means of permeation enhancement. In fact, several cell-penetrating peptides are currently in clinical trials which makes them an attractive option [99]. On the other hand, cell-penetrating peptides combined with physical or active dermal penetration enhancement still neglect the stability issues of NAs. siRNA, ODN, and plasmid DNA are susceptible to enzymes in the epidermis and dermis which can lead to significant degradation. To address this concern, packaging NAs in cationic lipid complexes or liposomes would both protect the NA from enzymatic degradation as well as aid cell internalization. In addition, chemical modifications of NAs like attaching charge neutral moieties via cleavable or reducible linkers or use of a phosphorothioate backbone has been shown to aid in internalization [100] and protect from degradation [101], respectively, without compromising function.

The regulatory hurdles for medical devices and drug-device combinations must also be addressed. Physical methods are sophisticated and will be highly regulated. Fabrication, sterilization, and implementation of the device must be demonstrated to result in safe and effective therapy. This regulation is in addition to any oversight process for the NA itself and further complicates translation of topical NA therapies. Along this line, a lot of effort has been spent over the years to address fabrication concerns. For example, methods have been devised to fabricate microneedle devices using steel and other strong materials [102]. Further, biocompatible and biodegradable microneedles have been proposed [103]. Many of these methods can be scaled using the same processes used for integrated circuits [104] which affords cost-effective manufacturing as well as the potential for outsourcing

manufacturing. Lacking, however, are studies assessing effects of sterilization and implementation protocols on patient safety. For example, all physical methods result in disruption of the skin barrier. This disruption will remain until re-epithelialization at the site of ablation can occur. While barrier function of the skin is diminished, pathogens may potentially enter the skin and cause infection. Therefore, the site of application may need to repeatedly be sterilized, or sealed with a sterile bandage, for days or weeks after application of the NA therapy. Depending on the frequency of application as well as the site of application (e.g. face, hands, feet), requiring a permanent or semi-permanent sterile bandage may not be tolerated by the patient. Prevalence of adverse events from physical ablation of the SC and epidermis must be determined before microneedle arrays and microporation devices see widespread use in the clinic.

Perhaps the most significant drawback of both physical and active methods, however, is limited application area. For diseases like Hurley disease, EB, moderate to severe psoriasis, skin wrinkling, and many others which can occupy a large percentage of skin surface area, physical and active methods may not be practical. In these cases, passive perturbation methods may be most effective.

Passive transport enhancers can be formulated as a cream and applied to all areas of the body, including the face, in a patient friendly manner. Further, formulations incorporating synergistic combinations of skin penetrating and cell internalizing transport enhancers can be easily envisaged. The main limitation of passive methods remains their low transport enhancement relative to physical and active methods. Further, passive transport enhancers like peptides and dendrimers may be limited to use with NAs of a certain size like antisense oligonucleotides and siRNA as opposed to plasmid DNA. Effort has been spent over the years to identify and understand passive transport enhancers. High-throughput screening for chemical enhancers is well-documented [105] and has proven useful for identifying synergistic combinations of chemicals that perturb the skin with minimal irritation [106]. Along the same lines, peptides can be screened in a high-throughput fashion using phage-display (Fig. 5). Phage libraries applied to the skin are selected based on their ability to transport through the skin. After only a few rounds, a library of $\sim 10^9$ peptide sequences can be screened. Particularly advantageous, this technique can be used to identify peptides that localize in particular layers of the skin e.g. epidermis or dermis. Delivery of NA therapies to specific locations in the skin could be beneficial to limit off-target effects. Phage screening is a powerful technique, and an extensive number of phage libraries exist. Effort should be placed on identifying novel peptides with powerful skin-penetrating ability. The same methodology could be used to screen for skin-penetrating aptamers, which to the authors' knowledge has not been attempted to-date, further broadening the space of chemically and structurally distinct passive transporters for delivering NAs.

As new transporters are identified, we can better understand how to overcome the skin barrier to deliver increasingly large cargos. For example, the number of skin-penetrating peptides identified thus far have afforded a better understanding of their mechanism of transport enhancement [107]. Interestingly, TD-1, Tat, and SPACE peptide all seem to bind and interact with keratin in the corneocytes in the skin facilitating transport of cyclosporine A through the transcellular pathway. As a result of this work, we posit screening of low

molecular weight ligands for affinity to keratin may help identify ligands with improved NA delivery. It is also conceivable that the mechanism of enhancement is dependent on the cargo delivered, however, similar studies with NA cargos are severely lacking.

Concurrently, definitive mechanistic studies to elucidate modes of transport enhancement of nanoparticles, liposomes, and spherical nucleic acids are lacking and studies that have been reported do not reach adequate consensus. Alvarez-Roman et al. have studied the distribution of polystyrene particles after topical application [108]. They observed almost exclusive uptake into the hair follicles. Follicular uptake was dependent on size of the particles with 20 nm particles accumulating to a larger extent than 200 nm particles. Similar localization in the hair follicles has been observed for liposomes [109–111] and ultradeformable liposomes [35], as well as titanium dioxide microparticles [112], micro/nanoemulsion droplets [113], lipoplexes [114], and solid lipid nanoparticles [112]. However, naked NAs have also been shown to localize in hair follicles when applied without any nanocarrier [115–117] which suggests nanocarriers may only hold potential as drug depots for controlled release topical formulations of NAs. Still others have demonstrated dispersed distributions of nanocarriers into viable epidermis and dermis. Verma et al. demonstrated enhanced drug delivery using liposomes and also concluded that size was the most important parameter [118]. However, it is important to note that these studies were performed with fluorescent drug and therefore do not distinguish between penetration of intact liposomes versus enhancement in drug delivery through simple fluidization of lipid bilayers. In fact, Kirjavainen et al. concluded that non-fusogenic fluorescently-tagged liposomes do not penetrate skin [119]. Instead, only liposomes that were fusogenic with SC lipids were able to enhance drug delivery. Moreover, fusogenic liposomes enhanced penetration of drug applied in free solution subsequent to application of liposomes. These data suggest liposomes may act through incorporation into and fluidization of the lipid bilayers to enhance drug delivery. Other factors of liposome design such as charge, amount of cholesterol, and acyl chain length did not appear to influence drug delivery. In contrast, Geusens et al. concluded liposomes can penetrate intact skin if flexible enough [120]. It is reasonable that liposomes that can penetrate via the multilamellar lipid bilayers in the SC. Others have also reached similar conclusions arguing ultraflexible liposomes create hydration-driven transport mechanisms through the skin [121]. However, since studies have shown localization of ultraflexible liposomes in hair follicles [35], another possibility is that flexibility does not aid penetration across multilamellar SC but instead enhances transport only across the unilamellar lining of hair follicles. If confirmed, this may preclude treatment of regions devoid of hair follicles like palms and foot pads. Interestingly, spherical nucleic acids, are not be expected to fluidize lipid bilayers, nor are they be expected deform to facilitate passive diffusion through tight intercellular lipid channels in the skin; therefore, we would expect them to localize in hair follicles like the majority of solid nanoparticles > 40 nm studied to date [122]. Yet, spherical nucleic acids exhibit extensive penetration homogenously into epidermal and dermal tissue [38, 40] How spherical nucleic acids penetrate skin has yet to be reported but should be of immediate interest given the significant potential this platform appears to have for treating skin disease. Although *in vitro* testing using Franz diffusion cells can be performed easily and quickly to screen large libraries of phage-displayed peptides and aptamers, testing individual formulations of nanocarriers

remains tedious. Thus, studies that continue to identify mechanisms of nanocarrier delivery and important design parameters for delivery enhancement are crucial to advance passive delivery methods.

On the other hand, passive enhancers can be significantly affected by the state of the SC. For example, a liposome formulation may be engineered to be non-toxic or non-irritating to intact skin. However, when applied on diseased skin, because barrier function is diminished, the same formulation may deliver an irritating or cytotoxic dose. In contrast, delivery using physical methods should be minimally affected by the diseased state of skin and active methods can be easily tuned to control the amount of dose delivered. Formulations of passive enhancers, especially complex formulations, are not as easily tuned. This must be considered during the design and implementation of passive enhancers.

Clearly, much is still unknown; however, translation of NA therapies appears to be close. Near-term challenges include validating safety of microneedle and microporation arrays. Establishment of regulatory guidelines will help on this front. In particular, we posit physical methods for delivering CpG oligonucleotides or other NA-based vaccine will be the first realization of topical NA therapy in the clinic. Indeed, the benefits of needle-free immunization are well-established [123]. However, despite their superior delivery efficacy, active and physical methods are severely limited by application area, and therefore, are not ideal for local delivery of NAs to skin tissue where disease symptoms manifest. To this end, long-term challenges include high-throughput screening of passive enhancers, followed by systematic studies to elucidate mechanisms of NA delivery. A synergistic combination of passive SC, tight junction, and cell membrane perturbers may be required to tackle most forms of skin disease and realize the full transformative potential of topical NA therapies in the clinic.

Conclusion

Topical application of NAs for the treatment of skin disease is an advantageous route for the translation of NA therapies to the clinic. Topical application offers many advantages over alternative methods of administration including avoidance of many challenges that are current roadblocks to systemic NA therapy implementation. Although the skin does pose a significant barrier to drug delivery, especially to large hydrophilic macromolecules like NAs, extensive effort has been spent to overcome this barrier. These efforts have been highlighted here, and include the use of microneedles, microporation, electroporation, iontophoresis, sonophoresis, nanoparticles, liposomes, spherical nucleic acids, peptides, and dendrimers. Translation of these technologies to the clinic is sure to have a transformative impact on the burden of both skin disease as well as cosmetic conditions. A large number of genetic and multifactorial inflammatory diseases result in chronic, physical and emotional burden to patients who have few treatment options other than repeated dosing by injection or infusion, or invasive surgery. Similarly, cosmetic conditions result in significant emotional burden with limited effective treatment options other than invasive surgery. Future efforts should focus on validating the safety of long-term physical disruption of the skin, better understanding the mechanisms NA enhancement into skin via passive methods, and

exhaustively screening for novel ligands and synergistic combinations of enhancers to realize the full potential of topical NA therapies in the clinic.

Acknowledgments

Conclusion

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Figure 1. Transport pathways into the skin

A: Intercellular pathway through lipid bilayers. **B:** Transcellular pathway through keratin-rich corneocytes. **C:** Shunt pathway through hair follicles and sweat ducts.

Figure 2. Microneedle arrays offer controlled length and sharp tips for easy insertion through the SC and into the epidermis

(a) Scanning electron micrograph of a 20×20 silicon microneedle array. (b) Scanning electron micrograph of a microneedle tip. Figure reproduced with permission from Henry S, et al. Microfabricated microneedles: A novel approach to transdermal drug delivery. *Journal of Pharmaceutical Sciences*. 1998;87:922–5.

Figure 3. Sonophoresis enhances delivery of NA into skin through localized perturbation zones (a) Top view of skin exposed to sulfarhodamine B and ultrasound. (b) and (c) FITC-NA delivery into skin after ultrasound treatment. (b) Cross-section of skin corresponding to a localized perturbation zone. (c) Cross-section of skin corresponding to a non-localized perturbation zone. Figure modified with permission from Tezel A, et al. Topical delivery of anti-sense oligonucleotides using low-frequency sonophoresis. *Pharmaceutical Research*. 2004;21:2219–25.

Figure 4. The anatomy of spherical nucleic acid nanostructures

An inorganic core is densely functionalized with oligonucleotides containing three segments: a recognition sequence, a spacer segment, and a chemical-attachment group. Additionally, other functional groups such as dye molecules, quenchers, modified bases, and drugs can be attached along any segment of the oligonucleotide. Figure reproduced with permission from Cutler JI, Auyeung E, Mirkin CA. Spherical Nucleic Acids. *Journal of the American Chemical Society*. 2012;134:1376–91. Copyright 2012 American Chemical Society.

Figure 5. Experimental design to screen for skin-penetrating peptides using phage-display
The same strategy could be used to screen for aptamers.

Table 1

Advantages of NA topical delivery for the treatment of skin disease.

Advantages of NA Topical Delivery
• Targeted delivery into skin
• Large surface area for drug application
• Easily observe and excise sites of adverse reaction
• Easily monitor disease progress and adjust treatment
• Needle-free application
• Sustained and controlled delivery
• Avoidance of GI tract
• Limited to negligible systemic toxicity
• Limited to negligible clearance from diseased tissue
• User-friendly application

Table 2

Advantages and disadvantages of physical, active, and passive topical NA delivery methods.

Class	Methods	Advantages	Limitations
Physical	Microneedles; Microporation	<ul style="list-style-type: none"> • High enhancement • No therapeutic limitations • Easily incorporate large depot • Delivery not majorly affected by diseased state of skin 	<ul style="list-style-type: none"> • Cost • Limited application area • Slow return of skin barrier function • Potentially irritating • No direct cell internalization enhancement
Active	Electroporation; Iontophoresis; Sonophoresis	<ul style="list-style-type: none"> • Skin penetration and cell internalization • Controlled delivery • Easily incorporate large depot • Repeated application at the same site • Transient perturbation of skin 	<ul style="list-style-type: none"> • Cost • Limited application area • Localized penetration zones
Passive	Polymeric nanoparticles; Liposomes; Peptides; Dendrimers	<ul style="list-style-type: none"> • Low cost • Application to large skin surface • Can protect therapeutic from degradation • Multi-functional (SC perturbation, epidermal tight junction perturbation, cell membrane perturbation) • Targeting of specific cell-types 	<ul style="list-style-type: none"> • Nanoparticles, liposomes may localize in hair follicles • Peptides, dendrimers may be limited by size of NA • Lower enhancement • Delivery can vary depending on state of SC and epidermis

Table 3

Topical NA delivery can have a large impact in the clinic due to the large number of skin diseases with NA therapeutic targets responsible for their aetiology and pathogenesis.

Disease/ Condition	People affected	Causative molecule and NA target for therapy	Role in pathogenesis	Up/down regulation for therapy	Ref.
Hurley disease	100,000 in US	TNF α , IL- 17, IL-23	Promotes inflammation through activation of vascular endothelial cells and immune cells	Down	[61, 62]
Epidermolysis bullosa	500,000 globally	IL-1 β , IL-1 β receptor	Mutant K14 leads to upregulation of IL-1 β , which then results in a positive feedback signaling cascade that ends up further activating mutant K14 expression	Down	[67]
		Mutant K5 K14	Mutations result in keratin filament aggregation in keratinocytes leading to dysfunction	Down (mutant)	[68,69]
		Collagen VII	Mutation results in loss of anchoring-fibril formation	Up (wild- type)	[70]
Pachyonychia congenita	5,000–10,000 globally	Mutant K6a, K6b, K17	Mutations disrupt keratin filament formation resulting in skin cell fragility	Down (mutant)	[72]
Netherton syndrome	1/50,000 globally	Serine protease	Mutation in serine protease inhibitor Kazal type 5 leads to serine protease hyperactivity and excessive protein degradation in skin	Down	[79]
Psoriasis	4.5 million adults in US	TNF α , IL- 17, IL-23	Same as above	Down	[64]
		IL-4	IL-4 producing T helper cell population is repressed	Up	[84]
		β defensin-2	Highly upregulated in psoriatic lesions	Down	[34]
Atopic dermatitis	10–20% of children; 1–3% of adults	RelA	Important member of the NF- κ B inflammatory pathway	Down	[49]
		IL-4, -5, -10, -13	Overexpressed in AD lesions	Down	[85]
		Th2-type immune response	Th2-type dominated immunological response implicated in AD pathogenesis	Down	[88]
Cellulite	80–90% post- adolescent women	ACE	ACE has been linked to higher production of angiotensin II, a vasoconstrictive peptide, along with lower production of bradykinin, a vasodilative peptide	Down	[89]

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Disease/ Condition	People affected	Causative molecule and NA target for therapy	Role in pathogenesis	Up/down regulation for therapy	Ref.
		Collagenase, Lipase, Elastase	Enzyme hyperactivity leads to breakdown of subcutaneous and dermal ECM. Lipase suppression leads to build up of fatty tissue	Down (Collagenase, Elastase); Up (Lipase)	[90]
		HIF-1	HIF-1 induces fibrosis and inflammation which is implicated in the formation of cellulite	Down	[89]
Wrinkling	Universal with aging	Elastase	Breakdown of elastic fibers leads to reduced skin elasticity and wrinkle formation	Down	[91]
Melasma	4–10% of all dermatological visits	MITF	Inhibition of MITF gene leads to suppression of melanin production in dark facial lesions	Down	[50]