Activation of p38 and Erk Mitogen-Activated Protein Kinases Signaling in Ocular Rosacea

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Purpose. Rosacea-related cutaneous inflammation is a common cause of ocular surface disease. Currently, there are no specific pharmacologic therapies to treat ocular rosacea. Here, we aimed at determining the differences in intracellular signaling activity in eyelid skin from patients with and without ocular rosacea.

Methods. This was an observational, comparative case series including 21 patients undergoing lower lid ectropion surgery at one practice during 2013 and 2014 (18 patients with rosacea, 13 control patients), and 24 paraffin-embedded archival samples from Albany Medical Center, selected randomly (12 patients with rosacea, 12 control patients). Cutaneous biopsies resulting from elective lower lid ectropion surgery were analyzed by Proteome Profiler Human Phospho-Kinase Array, Western blot, and/or immunohistochemistry.

Results. Samples derived from ocular rosacea patients showed increased levels of phosphorylated (active) p38 and Erk kinases. Phosphoproteins were mainly localized to the epidermis of affected eyelids.

Conclusions. This finding provides a novel potential therapeutic target for treatment of ocular rosacea and possibly other forms of rosacea. Further testing is required to determine if p38 and Erk activation have a causal role in ocular rosacea. The selective activation of keratinocytes in the affected skin suggests that topical pathway inhibition may be an effective treatment that will ultimately prevent ocular surface damage due to ocular rosacea.

Keywords: ocular rosacea, p38, Erk, phosphorylation, MAPK

Acne rosacea is a highly prevalent disease,[1] and 58% to 72% of patients that suffer from this cutaneous ailment develop ophthalmic manifestations of their disease.[2] These disorders are characterized by cutaneous irritation and cosmetic deformity, and the ocular variant of rosacea results in clinically significant surface disease, with subsequent dry eye syndrome, pain, blurred vision, tearing, and photophobia.[3–9]

Several treatment options have been designed to address rosacea, and the multiplicity of therapies underscores the lack of efficacy of any particular one. Lifestyle modifications, eyelid hygiene, topical and oral medications, laser and light-based therapies, and surgical interventions have all been employed in the management of rosacea,[10] although the results of these treatments have not been uniformly effective and this ailment remains incurable. In fact, our current modalities either address inflammation in a very general sense (i.e., corticosteroids, dietary modifications, nonsteroidal agents, antibiotics, and so on) or attempt to reverse existing damage distal to the site of the pathology (i.e., meibomian gland probing, corneal surgery to address perforations, and so on). Consequently, our current therapeutic armamentarium fails to tackle the immunologic and cellular aberrancies of this disease and thus cannot suppress rosacea in a highly targeted, specific fashion.

Recently, several studies have advanced our comprehension of the biologic aberrancies inherent to rosacea.[10–12] In order to characterize the molecular biology of ocular rosacea, we previously assessed the concentrations of 48 individual cytokines, chemokines, and angiogenic factors in cutaneous biopsies of the disease and in control patients, and identified statistically significant enrichments of interleukins-1β and -16, stem cell factor, monocyte chemoattractant protein-1, and the monokine induced by interferon gamma.[13] Given that these molecules have been previously associated with the innate immune system, follow-up studies were performed to better understand this process. We previously demonstrated an enrichment of Toll-like receptors in cutaneous biopsies of ocular rosacea[14]; these proteins provide constant surveillance against invading pathogens, and, upon stimulation, coordinate an innate immune response.[15] Perhaps most excitingly, the number of Toll-like receptors correlated with the presence of the vascular abnormalities CD105 and intercellular-adhesion molecule-1 in cutaneous preparations of ocular rosacea, further implicating this variant of immunity in the disease.[14]

The current study sought to further refine our understanding of the cellular biology of rosacea. In essence, previous

studies have identified the role of Toll-like receptors in governing this disorder and have implicated key effector molecules that lead to its clinical manifestations. Careful analysis of the alteration of cell-signaling pathways that facilitate the development of rosacea may lead to the identification of discrete targets for highly specific therapeutic intervention in the management of rosacea and will ideally enhance our comprehension of the mechanisms that potentiate it.

METHODS

Patient Demographics

For the immunohistochemistry (six women and six men in each group), the average age for rosacea patients was 57.1 years (standard deviation = 6.9 years) and the average for controls was 56.4 years (standard deviation = 7.3 years). For the Western blots, the rosacea average was 74 years (standard deviation = 20.4 years) and the control average was 74.3 years (standard deviation = 16.3 years). The rosacea group had eight women and six men, and the control group had seven women and three men. For the profiler, the rosacea average was 72.75 years (standard deviation = 11.7 years) and the control average was 75.4 years (standard deviation = 13.6 years). The average ages of patients and controls were not statistically significantly different.

Biopsies and Protein Extraction

We obtained patient informed consent to analyze cutaneous biopsies resulting from elective lower lid ectropion surgery. This protocol was reviewed and approved by the Albany Medical College Institutional Review Board, and adhered to the tenets of the Declaration of Helsinki. Immediately after resection of skin from patients with rosacea and from age- and sex-matched controls, the specimens were frozen at $-80°C$ for later retrieval. A total of 31 samples were obtained. All patients underwent eyelid tightening, and patients with extensive lower eyelid malposition were specifically excluded, with the intent of eliminating potentially confounding chronic irritation and cicatrization.

Each sample was thawed, weighed, and processed for protein extraction. For that, 300 μL ice-cold lysis buffer containing 1% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO, USA) in phosphate-buffered saline (PBS) containing protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN, USA) and 50 mM pervanadate (Sigma-Aldrich Corp.) was added to the sample together with 250 mg zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA). Then samples were homogenized through three cycles of 1 minute each on a Mini-Beadbeater-96 (BioSpec Products). Samples were centrifuged at >12,000g for 2 minutes at 4°C and the supernatant was cleared by a second centrifugation for 15 minutes at >12,000 for 2 minutes at 4°C. Lysates were aliquoted. Some aliquots received 1× volume of 2× Laemmli buffer and were boiled for 5 minutes. Then, samples were immediately stored at $-80°C$ until use. Sample identity was masked during all experimentation and unmasked only after all samples were quantified.

Protein Arrays and Western Blot

Detection of 46 phosphoproteins was performed on Proteome Profiler Human Phospho-Kinase Array Kit membranes (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions using 100 μg total protein. Western blots were performed using 10 μg/lane of total protein and detected using

the primary antibodies detailed in Supplementary Table S1. Horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). Signal was detected with West Pico or West Femto reagents (Pierce, Waltham, MA, USA) and a FujiFilm LAS-3000 imager (Tokyo, Japan). Band quantification was performed using FujiFilm MultiGauge software from raw image files according to manufacturer's instructions.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and rehydrated with sequential steps in xylene and ethanol solutions (100% to 95% to 70%). Endogenous peroxidase activity was blocked with 0.5% H_2O_2/MeOH for 10 minutes at room temperature (RT) and antigen retrieval was done for 30 minutes at 100°C in 10 mM citrate buffer, pH 6. Samples were blocked with 5% normal goat serum (Vector S-1000, Burlingame, CA, USA) for 1 hour at RT. Primary antibodies were incubated at a dilution 1:80 in PBS overnight at 4°C. Biotinilated anti-mouse secondary antibodies (Vector ba-9200) 1:500 in PBS were incubated for 1 hour at RT. Then, samples were incubated with avidin/biotin peroxidase (Vector ABC kit Elite PK-6100) in the dark for 30 minutes at RT and signal was detected with 3,3′-diaminobenzidine (Immpact DAB; Vector SK-4105) and counterstained with hematoxylin (Vector H-3404) prior to dehydration and mounting with VectaMount (Vector H-5000). Positively stained cells were quantified per ×1000 field. Sample identity was masked during all experimentation and unmasked only after all samples were quantified.

Statistical Analysis

Ocular rosacea versus control samples were compared by Student's *t*-test. A $P < 0.05$ was considered statistically significant.

RESULTS

Rosacea is a cutaneous ailment. The involvement of meibomian glands and ocular surface observed in ocular rosacea may be secondary to the effects of the cutaneous inflammation. In order to identify the mechanisms underlying ocular rosacea, we sought to compare the level of activation of multiple signaling pathways in diseased and control skin tissue by analyzing cutaneous biopsies resulting from elective lower lid ectropion surgery from patients with or without rosacea. The diagnosis of rosacea was made clinically through slit-lamp investigation. All patients in the rosacea group had evidence of bilateral chronic blepharitis, eyelid margin telangiectases, and meibomian gland clogging, in addition to the characteristic external cutaneous findings of the disease. All patients showed mild to moderate ocular rosacea; no severe cases of ocular rosacea were analyzed for this study.

The initial pathway characterization was performed in eight samples using an unbiased approach consisting in the simultaneous analysis of 46 proteins. Lysates were allowed to bind to Proteome Profiler Human Phospho-Kinase Array Kit membranes (R&D Systems). Results are summarized in the Table. Among other signals, the levels of activation of p38 and Erk1/2 were increased in samples from patients with ocular rosacea when compared to samples from control patients without ocular rosacea (Fig. 1).

To confirm the results obtained by the phosphokinase array, we tested the levels of pT180Y182-p38 (p-p38) and pT202Y204-Erk1 (p-Erk) in the proteins extracted from these and other samples by Western blot analysis. Figure 2A shows a

TABLE. Human Phospho-Kinase Array Comparison Between Control and Rosacea Samples

Phosphoprotein	Control, AU	Rosacea, AU	P Value
p38 alpha (T180/Y182)	214.6128	767.6414	0.004048
Erk1/2 (T202/Y204, T185/Y187)	301.6816	961.7494	0.091031
JNK 1/2/3 (T183/Y185, T221/Y223)	508.5686	334.213	0.335009
GSK-3 alpha/beta (S21/S9)	816.7295	396.0909	0.172968
EGF R (Y1068)	539.6214	275.2359	0.269187
MSK1/2 (S376/S360)	541.086	457.1573	0.552135
AMPK alpha1 (T183)	333.114	261.343	0.617421
Akt 1/2/3 (S473)	231.4213	284.4941	0.695334
TOR (S2448)	190.1555	594.5279	0.042689
CREB (S133)	240.4558	399.0289	0.001656
HSP27 (S78/S82)	532.9816	969.6496	0.174132
AMPK alpha2 (T172)	394.3459	717.9143	0.163357
beta-Catenin	292.4131	292.8966	0.997046
Src (Y419)	339.7041	302.9746	0.805285
Lyn (Y397)	398.7986	289.2499	0.304199
Lck (Y394)	385.5884	213.7605	0.125884
STAT2 (Y689)	879.1555	1417.081	0.105447
STAT5a (Y699)	472.9613	790.4458	0.040134
Fyn (Y420)	212.7338	391.9093	0.033901
Yes (Y426)	238.8639	339.5305	0.022155
Fgr (Y412)	198.5089	145.5631	0.133263
STAT6 (Y641)	364.3103	915.7491	0.028046
STAT5b (Y699)	321.626	701.1226	0.006747
Hck (Y411)	254.5958	453.9424	0.054826
Chk-2 (T68)	281.8076	357.9696	0.209134
FAK (Y397)	292.364	286.1925	0.9115
PDGF R beta (Y751)	262.0724	222.9519	0.522738
STAT5a/b (Y699)	301.6294	397.8481	0.283671
PRAS40 (T246)	735.7211	465.6334	0.315541
p53 (S392)	2171.036	2851.344	0.192892
Akt 1/2/3 (T308)	3989.069	4505.205	0.202167
p53 (S46)	3360.122	3813.075	0.089048
p70 S6 Kinase (T389)	728.0834	731.2244	0.979898
p53 (S15)	3581.397	3663.909	0.876948
c-Jun (S63)	3546.526	4461.907	0.100241
p70 S6 Kinase (T421/S424)	4266.741	4476.675	0.669157
RSK1/2/3 (S380)	5900.862	11343.56	0.040403
eNOS (S1177)	3175.718	3793.325	0.211349
STAT3 (Y705)	3331.626	4407.933	0.175331
p27 (T198)	923.5785	1238.758	0.079273
PLC gamma-1 (Y783)	2284.429	2417.716	0.584671
STAT3 (S727)	1733.881	2185.037	0.404835
WNK-1 (T60)	1845.118	2498.976	0.11562
Pyk2 (Y402)	2947.138	3233.06	0.614251
HSP60	6713.87	12461.38	0.03298

Presented as average of each condition and statistical significance of the difference of the mean (2-tailed Student's t-test, n = 4). AU, arbitrary units.

sample of the band pattern obtained by the Western blots, while Figure 2B shows the quantification of the data. Western blot and band quantification was performed by a researcher masked to sample diagnoses. Confirming the results obtained previously, we observed a significant increase in the levels of p-p38 and p-Erk1/2 in rosacea samples. Other candidate signals were significantly increased in the proteome profiler array (CREB, Stat5a, Fyn, Yes, Rsk1/2/3, and HSP60), but subsequent analyses by Western blot were unable to confirm those results (Supplementary Fig. S1).
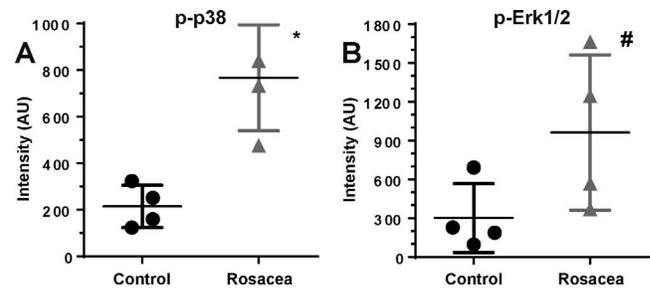
FIGURE 1. Phosphoprotein profiler array data showing increased phosphorylation of p38 and Erk kinases in eyelid biopsies from patients with ocular rosacea compared to age-matched controls. *$P < 0.05$; #$P < 0.1$ (2-tailed Student's t-test, n = 4 controls and 4 rosacea eyelids).

We then sought to confirm these results with a different technique and to determine which cell type(s) within the skin displayed increased p-p38 and p-Erk1/2 levels. Immunohistochemical analysis was performed on paraffin-embedded sections obtained from archived eyelid biopsies. As with previous assays, we compared lower eyelid ectropion biopsies from patients with or without ocular rosacea. Immunohistochemical staining and positive cell quantification were performed by two researchers masked to sample diagnoses. As shown in Figure 3A, the signal for these two activated protein species was almost exclusively located within the epidermis, suggesting that the keratinocyte is the main cell type responsible for the observed differences in the tissue lysates. Consistent with the overall increase of phosphorylated p38 and Erk observed in the protein array and Western blot assays, the numbers of epithelial cells that stained positively for these cell signals were statistically significantly higher in
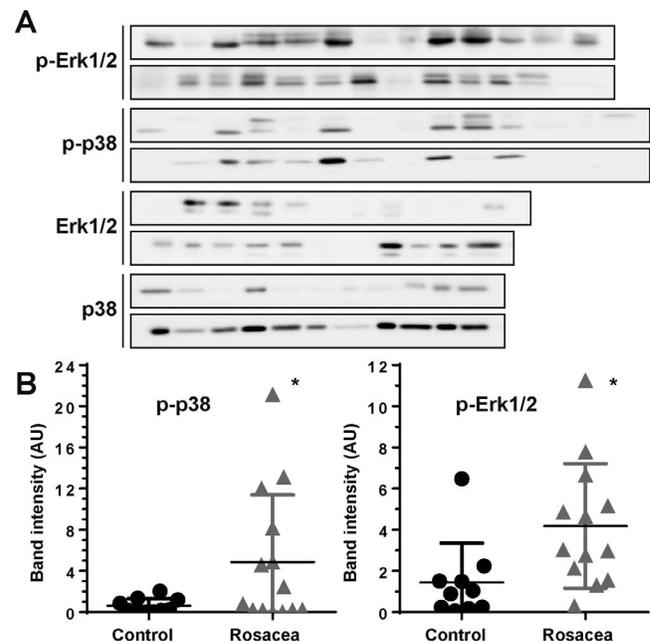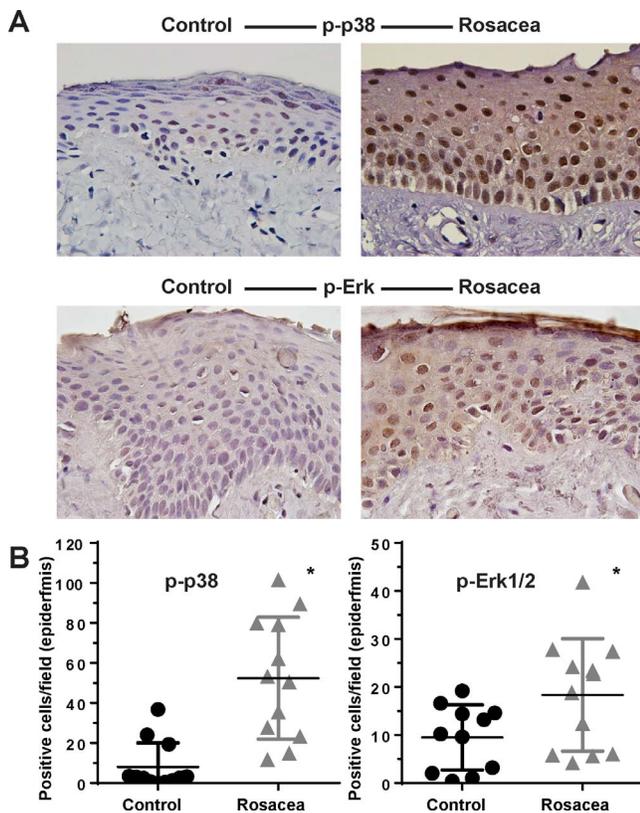

FIGURE 2. Western blot analysis to confirm the initial results obtained with the protein profiler arrays. (A) Sample Western blot bands corresponding to the phosphorylated and total forms of each protein. (B) Band quantification expressed as a normalized result (p-protein/total protein). *$P < 0.05$ (1-tailed Student's t-test, n = 10 controls and 14 rosacea eyelids).

FIGURE 3. Immunohistochemical localization of p-p38 and p-Erk. **(A)** Representative fields showing the marked epidermal localization of the phosphorylated proteins in the rosacea samples. **(B)** Quantification of the number of positive cells/field. *$P < 0.05$ (2-tailed Student's t-test, $n = 12$ controls and 12 rosacea eyelids).

cutaneous biopsies of ocular rosacea than in age- and sex-matched controls (Fig. 3B).

DISCUSSION

Despite the epidemiologically rich nature of rosacea and its significant impact on the lives of patients that suffer from it, our current treatment options are woefully inadequate.[10] In fact, our treatment options are generally nonspecific and do not target the cellular and immunologic features that distinguish the disease from normal, healthy skin. As such, enhancements in our comprehension of the biology of rosacea will facilitate the development of new highly specific interventions in a highly translational manner. Ocular rosacea is a skin disease, and ocular surface and meibomian gland involvement are thought to be secondary to the inflammatory signaling emanating from the eyelid and periocular skin. Thus, we focused on our studies to assess the changes in eyelid skin intracellular signaling pathways to evaluate a potential cause, rather than secondary outcomes, of ocular rosacea.

The current study is the first to implicate mitogen-activated protein kinase (MAPK) activation in the pathogenesis of this disorder. Specifically, based on analyses that employed multiple different mechanisms to assess the enrichment of a wide array of individual kinases, activation of p38 and Erk was clearly increased in the epidermis of eyelids with ocular rosacea. Due to the study design, only mild to moderate cases were analyzed, and thus whether the intensity of activation correlates with disease severity is still an open question. A potential limitation of findings based on highly multiplexed assays is the increased

chance of false-positive and -negative results. Our findings that both p38 and Erk pathways are activated in rosacea are, however, highly unlikely to derive from nonspecific interactions, as they were consistently observed using three different methodologies: chemiluminescent membrane-based antibody array and Western blot of Triton X-100 lysates, as well as immunohistochemistry of paraffin-embedded tissue sections.

Due to the lack of available animal models of rosacea, it is very difficult to test a causal relationship for any intracellular signal in preclinical studies. However, our findings are consistent with the existing literature regarding cutaneous inflammation, and a few studies have explored the role that these proteins play in dermatologic disease. Yamasaki et al.[16] showed that an increase in the cathelicidin LL37, an antimicrobial peptide, promoted skin inflammation in rosacea. More recently, the same group reported that p38 inhibition selectively prevented the induction of IL-36γ in keratinocytes that were challenged in vitro with LL37.[17] Moreover, treatment with the anti-inflammatory agent azelaic acid (a known topical therapy for acne that has been also successfully used to treat some patients with rosacea[18]) reduced the expression of cathelicidin in mouse skin and serine protease activity in the skin of patients with rosacea.[19] It is well established that ultraviolet light (UVL), a trigger of rosacea flares, induces p38 activation in keratinocytes.[20] Importantly, Mastrofrancesco and colleagues[21] demonstrated that treatment with azelaic acid inhibited the UVL-induced increase in p38 phosphorylation in keratinocytes in vitro. Thus, the published literature in vitro suggests a role for p38 in the mechanisms of cathelicidin-mediated skin inflammation. Nonetheless, we are unaware of any previous studies that have directly assessed the levels of p38 and other MAPKs in cutaneous biopsies of rosacea; and, to our knowledge, this study is the first to directly implicate these proteins in the pathogenesis of this disorder.

Along with other studies, our previous investigations have implicated Toll-like receptors (TLR) in the pathogenesis of rosacea.[14,22] These receptors are well known to promote p38 and Erk activation,[23,24] and these pathways have been shown to mediate multiple keratinocyte responses. For example, TLR2-mediated p38 and Erk activation was required for the inflammatory and antimicrobial responses of keratinocytes challenged with streptococcal M1 protein,[25] *Staphylococcus epidermidis* LP01 lipopeptide,[26] or *Candida albicans* phospholipomannan.[27] Moreover, the TLR4/p38 signaling axis is essential for normal cutaneous wound healing,[28] and expression of TLR2 and TLR4 was found to be altered in atopic dermatitis, contact dermatitis, and psoriasis.[29] The identification of the increased activation of p38 and Erk signaling in diseased skin suggests an active TLR/p38 axis as an important mechanism of ocular rosacea. This activation may ultimately yield increased cytokine levels, which then may result in the clinical manifestations of rosacea.

The activation of a cutaneous Erk pathway may also have a role in rosacea pathogenesis. For example, rosacea can promote a thickening of the epidermis. This is particularly evident in the phymatous subtype in which multiple layers of skin may lead to gross changes in cheeks and nose.[30] The Erk pathway has long been associated with epidermal proliferation and is an essential mediator of multiple growth factors, including epidermal growth factor.[31–33] Another typical hallmark of rosacea skin is the presence of multiple telangiectases.[30] Erk activation was shown to be critical in an in vitro model of activin receptor-like kinase 1 hereditary hemorrhagic telangiectasia (HHT).[34] Further, Erk is a key mediator of vascular endothelial growth factor,[35] another key factor in the pathogenesis of HHT.[36–38] It is unknown whether Erk activity is involved in the development of cutaneous telangiectasia observed in rosacea skin. The roles of Erk signaling in the

epidermis appear to be complex, as this pathway is required to prevent allergic skin disease in mice,[39] and a common side effect of the pathway inhibitor trametinib is skin rash.[40,41]

Selective inhibition of p38 and Erk pathways represents an intriguing possibility for the management of ocular rosacea. The absence of targeted remedies for this disease strongly indicates that cellular therapies would be a welcome addition to our interventional armamentarium, and mechanisms that target the disease at the site of its pathology (i.e., inflamed skin) may prevent and heal ocular surface damage. In fact, multiple clinical trials are currently active to test safety and efficacy of p38 inhibitors in Langerhans cell histiocytosis[42] as well as multiple inflammatory diseases,[43–45] including chronic obstructive pulmonary disease,[46] cardiovascular disease,[43] and rheumatoid arthritis.[47] Targeting MAPK signaling has been shown to have an acceptable safety profile. A recently completed large phase III trial to study the ability of Losmapimod (a selective p38 inhibitor) to reduce the incidence of cardiovascular events in subjects with acute coronary syndrome (LATITUDE-TIMI 60, NCT02145468) showed a good safety profile.[48] Similarly, MEK1/2 (the upstream activator of Erk1/2) inhibitors are being currently tested in phase III trials for the treatment of multiple oncologic pathologies,[49,50] and Trametinib, a MEK1/2 inhibitor, has been approved for the treatment of melanoma containing BRAFV600E or V600K mutations.[40,50] While other fields have adopted kinase inhibitors, the use of cellular therapies in the treatment of rosacea is a novel, highly exciting opportunity to enhance the lives of patients that suffer from this currently incurable disease. By documenting a constitutive increase of p38 and Erk activation in this disease, we therefore propose the possibility of the use of agents that inhibit these cell signals in the treatment of rosacea. Further studies are required to determine a possible involvement of these pathways in other aspects of this disease, such as meibomian gland dysfunction.

Specific activation of p38 and Erk pathways in the epidermis of eyelid skin with ocular rosacea suggests the possible involvement of these kinases in the pathogenesis of the disease. While further studies are required to determine a causal role for these kinases, the epidermal activation suggests that topical inhibition of these kinases might be a potential avenue to specifically treat ocular rosacea.

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