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Use of primary skin fibroblasts to understand the etiology of Disabling Pansclerotic Morphea

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The Thesis of Vivian K. Hua is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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LIST OF ABBREVIATIONS

3D	three-dimensional
α-SMA	alpha smooth muscle actin
APECED	autoimmune polyglandular endocrinopathy with candidiasis and ectodermal dysplasia
CWCA	circular wound closure assay
DPM	Disabling Pansclerotic Morphea
ECM	extracellular matrix
EF	eosinophilic fasciitis
ELISA	enzyme-linked immunosorbent assay
FAP	fibroblast activating protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GWAS	genome-wide association study
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IL	interleukin
JAK	Janus kinase
JLS	Juvenile Localized Scleroderma
LIF	leukemia inhibitory factor
p-STAT4	phosphorylated STAT4
PDGF	platelet-derived growth factor
PIAS	protein inhibitor of activated STAT
PUVA	psoralen plus UVA
PVDF	polyvinylidene fluoride
RT-qPCR	real-time quantitative polymerase chain reaction
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor- β
TNF-α	tumor necrosis factor-α
UVA	ultraviolet A

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ABSTRACT OF THE THESIS

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by

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Disabling Pansclerotic Morphea (DPM) is a rare fibrotic skin disorder that is characterized by rapid sclerosis in all skin layers, fascia, muscle, and bone. This disease primarily affects children under 14 years of age and has an unknown etiology. Diagnosis of DPM is largely dependent on recognition of clinical features and current treatment options are non-specific. To determine the current state of knowledge of DPM, we conducted a literature review

to identify all published cases of disease. This is the first study to summarize all DPM patients reported in the literature and the collective information will function as a valuable resource for both clinicians and scientists who are seeking more information about this disease. Our findings revealed that patients typically present with prominent skin findings accompanied by various extracutaneous complications which contribute to morbidity and mortality. Additionally, patient responses to current treatment options are inconsistent. Given the rarity of the disease and limited understanding of its pathophysiology, we attempted to define the etiology underlying DPM through use of primary human cell lines, and evaluation of wound healing by an *in vitro* scratch assay. Results from our optimization experiments show that scratching at a 45° angle decreases interexperiment variability. Finally, we have proposed experiments that will examine the inherent disease features on elements of fibroblast biology including inflammatory gene transcription and cell morphology. We believe results from these experiments will help unveil the molecular basis of DPM and aid in the identification of a more specific therapy for these patients.

INTRODUCTION

Overview of the Immune System

The immune system is the body's defense mechanism against perceived danger signals, including foreign particles, potentially pathogenic microbes, and cell metabolites. Traditionally split into innate and adaptive immunity, the immune response is a series of carefully orchestrated molecular interactions that maintain host homeostasis by balancing host defense and self-tolerance mechanisms. Errors in any stage of the immune response, may be detrimental to the host from failing to eradicate infection (immunodeficiency), perpetuating inflammation (autoinflammation) or generating self-reactive cells and autoantibodies (autoimmunity).

Autoimmune Diseases

Autoimmune diseases result from an erroneous immune response to self-antigens characterized by a disruption of immune regulatory mechanisms, including failure to delete self-reactive clones, or clonal anergy (1). The spectrum of autoimmune disorders ranges from localized, organ-specific inflammation to systemic disease (2). Despite being individually rare, autoimmune diseases collectively represent one of the ten leading causes of death among women under the age of 65 years (3). While women are disproportionately affected, autoimmune disease can manifest in anyone, regardless of sex, age, or race. The chronic nature of this type of disease leads to patient morbidity and mortality, as well as high economic costs (4). For many autoimmune diseases, the precise etiology remains unknown, but multifactorial models involving genetic susceptibility, loss of regulatory cells, hormonal factors, and exposure to environmental triggers have been proposed (1,2,5).

Skin manifestations of autoimmune diseases

As one of the largest organs of the human body, the skin functions as the body's first line of defense as both a mechanical and an immunological barrier to external pathogens. Mechanically, the skin's extensive surface area covers and protects the body from external harm. Immunologically, the skin provides a complex microenvironment where a variety of cell populations can interact and participate in both the initiation and regulation of an effective immune response (6). Due to its involvement in maintaining immune homeostasis, the skin is widely recognized as a reflection of internal pathological conditions, including autoimmunity (7). Defects in immune regulation, including within the skin, have been linked to the development of both organ-specific and systemic autoimmune diseases accompanied by prominent skin findings. For example, hypopigmented spots, erythematous plaques, and blisters can be found on the skin of patients with vitiligo, psoriasis, and autoimmune blistering diseases, respectively. Skin manifestations of systemic autoimmune diseases vary depending on the specific disease, but the development of nodules and lesions are the most common cutaneous findings in these patients (7).

Scleroderma is an autoimmune skin disorder characterized by an accumulation of extracellular matrix (ECM) proteins, vascular injury, and immunological abnormalities which results in fibrosis (8). Similar to many autoimmune disorders, scleroderma exists on a spectrum ranging from the less severe localized scleroderma to the most severe form known as systemic sclerosis. Localized scleroderma, or morphea, is the most common subtype among the pediatric population, and may be further classified by the depth and distribution of lesions (9). The juvenile localized scleroderma (JLS) spectrum consists of six different subtypes: plaque, generalized, bullous, linear, deep morphea and mixed (10,11). Sixty-five percent of JLS patients

have linear morphea, while the deep subtype is only 2% of reported cases (12). Disabling Pansclerotic Morphea (DPM), is the most severe subtype of deep morphea and is characterized by rapid sclerosis in all layers of the skin, fascia, muscle, and bone (Figure 1). This disease is a life-threatening condition for patients and, partially due to its rarity, the exact mechanism behind this disease is not well understood. Lack of knowledge of the underlying pathophysiology adds to the diagnostic challenges, as well as inhibiting systematic treatment trials, and as a result, a consistent effective therapy has not been established for patients with this disease (13).

Molecular and genetic etiology of autoimmune diseases

Antigens are foreign or self-molecules that evoke specific immune responses. Consequently, the expression of self-antigens in the thymus and bone marrow facilitates clonal deletion of self-reactive B- and T-lymphocytes. Clonal deletion is an important central tolerance mechanism that prevents autoreactive progenitor cells generated during lymphocytic development from entering peripheral circulation (2,4). Due to the lack of organ-specific antigen expression within the thymus and bone marrow, a small number of autoreactive lymphocytes may escape the clonal deletion process and enter the periphery. Peripheral tolerance mechanisms, such as cell anergy, immunological ignorance, and immune response regulation, ensure that the activity of the escaped autoreactive cells is controlled so unnecessary tissue damage may be prevented (4). Defects in these tolerance mechanisms lead to an autoimmune pathology. Environmental triggers may also contribute to autoreactivity. For example, organ-specific autoimmune diseases are frequently associated with history of trauma, which results in the release of isolated autoantigens followed by their recognition by autoreactive lymphocytes

present in periphery (2). This mechanism is particularly relevant to autoimmune skin diseases as the skin is an organ that is frequently injured.

Beyond environmental influences, genetics are also frequently implicated in the etiology of autoimmune disease. A few autoimmune diseases are caused by single gene mutations such as autoimmune lymphoproliferative syndrome and syndrome of autoimmune polyglandular endocrinopathy with candidiasis and ectodermal dysplasia (APECED, reviewed in (5,14)). In families, disease prevalence in first degree relatives is approximately five times higher than the 0.1-1% frequency observed on the general population, with an additional fivefold increase in monozygotic twins of affected individuals (15). However, these rare cases suggest that most autoimmune diseases are multigenic and require multiple susceptibility genes working together to produce the disease phenotype. For example, although many autoimmune diseases are linked to specific class I or class II human leukocyte antigen (HLA) molecules, these associations may only be valid with linkage to polymorphisms in other genes, such as those encoding tumor necrosis factor-α (TNF-α) or other complement proteins (reviewed in (5)). It is likely that different susceptibility genes confer variable levels of risk and the predisposition to autoimmune disease is determined by the different combinations of susceptibility and protective genes present in any given individual (reviewed in (5)).

Likelihood of a genetic link in morphea

The likelihood of a genetic link in morphea is high. Up to 46% of morphea patients have either a personal or family history of autoimmune disease, indicating that a there may be a common susceptibility locus for these disorders (reviewed in (16)). Specific HLA subtypes, such as HLA-B*37 and HLA-DRB1*04:04, have been shown to increase susceptibility to morphea

(17). Furthermore, cases of familial morphea can be found within in the literature (18–22). However, despite these findings, the specific genetic cause for this disease has not been identified.

Our lab recently identified patients with familial DPM, and a heterozygous mutation in *STAT4*, p.A635V (Broderick *et al*., in preparation). STAT4 is a member of a family of latent transcription factors that are heavily involved in JAK-STAT signaling, an intracellular signal transduction pathway that is critical for immune regulation. In addition to its involvement in regulating immune pathways, STAT4 may also play a crucial role in regulating cell division in non-immune cells. High levels of serine phosphorylated STAT4 have been reported in mitotic cells of the skin (23). Furthermore, STAT4 is involved in an autocrine loop with leukemia inhibitor factor (LIF) and LIF receptor to drive the production of IL-6 in fibroblasts (24). Together, these findings suggest that STAT4 activity and related pathways may contribute to the hyperproliferative and inflammatory phenotype seen in patients with various autoimmune and autoinflammatory skin diseases, including DPM.

Proposed mechanisms of morphea

The diversity of autoimmune disorders in addition to the differences in clinical manifestations between patients cannot be explained by a single theory or mechanism (1). Therefore, the proposed pathophysiological mechanisms underlying morphea closely follows the multifactorial model of autoimmune disease (Figure 2). Morphea patients are believed to be genetically predisposed to the disease (16,25), with disease processes thought to be initiated upon vascular injury and damage to endothelial cells. Endothelial cell injury subsequently stimulates the upregulation of adhesion molecules and the release of cytokines from the injury

site (16,25,26). These changes activate the immune response by facilitating the recruitment of antigen presenting immune cells such as eosinophils, CD4+ T cells, and macrophages to the injury site. It is proposed that during the recruitment process, these antigen presenting cells have an opportunity to present autoantigens that were released during injury, resulting in the formation of autoantibodies in morphea patients (reviewed in (25)). In addition to promoting an immune response, the cytokines released from the injury site can also promote fibroblast activation with increases in collagen and other ECM proteins (16,25,26). The imbalance between increased collagen production and reduced matrix metalloproteinases that mediate collagen degradation is the hallmark of the fibrosis seen in morphea patients (25). The mechanism leading up to this imbalance, however, remains unknown.

Role of fibroblasts in inflammation, wound healing, and fibrosis

Fibroblasts are mesenchymal cells derived from mesoderm tissue and they are the most common cell type found in connective tissues throughout the body. The principal role of fibroblasts is to produce, secrete, and maintain ECM components. However, fibroblasts also play pivotal roles in processes beyond ECM production and maintenance such as angiogenesis, cancer progression, inflammation, and wound healing (27). Upon wound injury, fibroblasts can produce and respond to proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-13, IL-33, and transforming growth factor-β (TGF-β) in a paracrine and autocrine manner (27). The cytokines secreted from fibroblasts promote the migration of various immune cells to the site of injury. In response to the secreted cytokines, fibroblasts differentiate into myofibroblasts that have an upregulated rate of ECM production and enhanced contractility that facilitates wound contraction at the injury site (27). In an effectively regulated immune response, the inflammation process

resolves with the clearance of recruited immune cells and fibroblasts reverting to a resting state (28,29). Dysregulation of the immune response promotes overactivation of fibroblasts, continual secretion of proinflammatory cytokines, and the persistence of an inflammatory infiltrate that results in chronic inflammation and fibrosis (27–29). Taken all together, fibroblasts are key mediators in the wound healing process, and dysregulation of the immune response by fibroblasts will likely lead to the manifestation of disease.

Primary dermal fibroblasts as a cell model system to study skin disease

Primary dermal fibroblasts have been used extensively to study fibrotic disorders of the skin and have provided valuable insight into the underlying mechanisms of disease. In autoimmune disorders of the skin, these studies have implicated abnormal activation of the Wnt signaling pathway and increased production of profibrotic cytokines such as TGF-β and platelet-derived growth factor (PDGF, reviewed in (16,30,31)). These *in vitro* studies have formed the basis of many ongoing clinical trials, emphasizing the importance of these models on translating basic science findings to treatment of patients (32). For example, *in vitro* testing of TGF-β and PDGF pathway inhibitors on primary dermal fibroblasts led to the discovery of imatinib mesylate as a potential therapy for patients with autoimmune skin disease including scleroderma (reviewed in (31)). The use of fibroblasts in the study of various skin disorders strongly suggests that utilization of the same *in vitro* cell model system may be useful in defining the mechanisms that are involved in DPM.

Use of a fibroblast model to identify therapeutic targets for the treatment of DPM

Beyond understanding the mechanisms of disease progression in DPM, identification of a candidate gene enables an opportunity to study targeted therapies in our *in vitro* system. STATs are a family of latent cytoplasmic transcription factors that play a critical role in facilitating cellular responses to cytokines and are involved in a variety of processes including immune responses, cell growth and differentiation, cell survival, apoptosis, and oncogenesis (33–35). Phosphorylation of STATs by Janus kinases (JAKs) in response to cytokines results in the formation of homo- or heterodimers that can translocate into the nucleus, bind DNA, and regulate the expression of many cytokine-responsive genes (33). An understanding of the extensive involvement of JAK-STAT signaling immunity and inflammation has led to the identification and implementation of JAK inhibitors as a treatment for many inflammatory disorders, including autoimmune disease. The first FDA-approved JAK inhibitor was a JAK1/JAK2 specific inhibitor named ruxolitinib, developed for the treatment of myeloproliferative disorders. Subsequently, tofacitinib, a JAK1/3 inhibitor was developed to treat patients with rheumatoid arthritis (36). The success of these JAK inhibitors has led to the development and testing of a new generation of JAK inhibitors that are even more selective for various rheumatological disorders and autoimmune skin diseases (37,38). Our fibroblast model allows for pre-clinical testing of these new inhibitors as potential therapies for DPM.

Aims of Research

In the field of immunology, studies in single patients have paved the way for the establishment of a causal relationship between genotype and phenotype (39). Consequently, in rare disease research, a review of clinical case reports is essential as they provide the necessary

background for clinicians and scientists to postulate molecular and genetic mechanisms implicated in disease pathogenesis (40). Prior to the establishment of a causal relationship, an understanding of the phenotype of the patient is necessary. However, an abundance of rare disease related phenotypic information is thought to be buried amongst the growing amount of medical literature (41). Therefore, to better recognize the phenotype of patients and to better understand the current state of knowledge of DPM, this study aims to identify all published cases of DPM in addition to identifying common characteristics and symptoms by conducting a literature review. Attempted therapies and outcomes will also be identified and summarized. Compilation of all DPM relevant information will serve as a valuable resource for clinicians and scientists who are seeking background information, common symptoms, and trialed therapies for this rare disease. This study also aims to determine whether the novel *STAT4* variant discovered in our patient is a likely gene candidate for DPM by conducting a literature review on studies that implicate *STAT4* variants in the development of various autoimmune diseases. Finally, we describe the development of an *in vitro* assay to study fibroblast biology in this syndrome.

We hypothesize that we can use patient-derived dermal fibroblasts to characterize the differences in fibroblast biology in DPM. An *in vitro* wound healing assay (scratch assay) will be used to investigate the variant's effect on wound healing. Initial experiments will focus on optimizing the conditions of the scratch assay with a focus on scratch angle in two different cell lines. In addition, we propose future experiments that can be conducted to examine differences in inflammatory gene transcription and cell morphology between patient and control dermal fibroblasts. Our goal is that these experiments will serve as the foundation for the discovery of the molecular basis of DPM and pave the way for the identification of a more specific and effective therapy for DPM patients.

MATERIALS AND METHODS

Identification of published DPM cases

A literature search was conducted using the search terms ‘disabling pansclerotic morphea of childhood’ and ‘pansclerotic morphea’ on Google scholar and PubMed. All original articles, case reports, abstracts, and letters to the editor written in English were included regardless of publication date. Additional publications were identified by performing a general Google search using the same search terms or by directly searching for the title of publications cited within articles that were already obtained.

Identification of STAT4 variants related to autoimmune disease

A literature search was conducted using the search terms ‘STAT4,’ ‘STAT4 SNP’ and ‘STAT4 and autoimmune disease’ on Google scholar and PubMed. English publications that implicated STAT4 in the development of autoimmune disease were included regardless of publication date or study type. Additional information about SNP type, chromosome 2 location, associated alleles, and sequence change was obtained from a combination of details found on the gnomAD website and the SNP database on the NCBI website (see URLs).

Fibroblast cell culture

Primary dermal fibroblast cell lines were isolated from consented donors and were previously established in culture in the Broderick lab under a UCSD IRB approved protocol. Human primary skin fibroblasts were cultured from skin biopsy samples as previously described (42). Briefly, 1mm skin biopsy pieces were placed in a 6-well plate coated with 0.1% gelatin and DMEM with 20% FBS and 1% Antibiotic-Antimycotic (Gibco). Media was replaced every 2-3

days, and cell cultures were confluent after 2-3 weeks. After passage 2, cells were maintained in DMEM/F12 with 10% FBS and 1% antibiotic-antimycotic at 37°C and 5% CO_2. All cells were used between passages 4 and 9.

Optimization of in-vitro wound healing assay (scratch assay)

Primary dermal fibroblast cells from independent donors were seeded at a density of 2 x 10^5 cells/mL onto a 6-well plate in DMEM/F12 with 10% FBS and 1% antibiotic-antimycotic (Gibco), and incubated at 37°C and 5% CO_2 until a confluent monolayer was formed. A linear wound was induced by gently scratching the monolayer at a 90° angle or at a 45° angle with a P-200 pipette tip. Images of the wound area were taken using a ToupCam camera (ToupTek Photonics) at 4X objective every 7 hours, 3 times a day until complete closure of the wound was observed. A total of 3 images corresponding the top, middle, and bottom of the well were taken per condition per timepoint to account for unevenness of the induced scratch throughout the well. Wound width was measured using ImageJ software (Version 1.52a, Java 1.8.0_112) by overlaying and centering a grid. Wound width measurements were taken by measuring the distance between each side of the induced scratch. A total of 4 wound width measurements were recorded in pixels per image. The average of the 4 width measurements at 0 hours post-scratch was used to normalize all wound width values at each timepoint to 1. The average of the normalized values obtained from the 3 separate images per each timepoint was used to represent the normalized wound width of the entire scratch for each condition.

URLs

gnomAD: https://gnomad.broadinstitute.org/

SNP database: https://www.ncbi.nlm.nih.gov/snp/

Statistical analysis

Data are expressed as means and standard deviation. Statistics and graphing were performed using Microsoft Excel and GraphPad Prism (Version 8.4.3, Graph Pad Software Inc., CA). Statistical significance was determined using a student's *t* test. A *p* value of less than 0.05 was considered statistically significant.

Planned Experiments

RNA Extraction

Total RNA will be homogenized and extracted from patient and control cells at the same timepoints as the scratch assay using the Trizol Reagent protocol per the manufacturer's instructions (Life Technologies). First, RNA will be isolated from the samples by phenol-chloroform extraction. RNA will be located within the upper layer within the aqueous phase while proteins and DNA are in the lower layer within the organic phase. Following extraction of the aqueous phase, RNA will be precipitated with isopropanol and dissolved in RNase free water. RNA concentration will be determined via NanoDrop and samples will be stored at -80°C.

RT-qPCR

Isolated RNA will be diluted to 25 ng/µl and used to generate cDNA using the High Capacity cDNA Reverse Transcription Kit per the manufacturer's instructions (Applied Biosystems). Primers were designed using the Realtime PCR Tool on https://www.idtdna.com by selecting for only exons and coding regions. Primer sequences to be used are listed in

Supplementary Table 1. The qPCR reactions will be carried out in a SYBR green system. Samples will be run in triplicate in a thermocycler (Bio-rad CFX96) and the results will be analyzed using CFX Manager. Relative gene expression will be determined by the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of the controls. GAPDH will be used as the housekeeping gene for both control and patient samples.

Enzyme-linked Immunosorbent Assay (ELISA)

Secreted proteins will be measured in the cell culture supernatants by ELISA. Secreted IL-6, pro-collagen I alpha-1 and fibronectin, etc. will be accessed using the Duo-Set ELISA kits (R and D Systems, Inc.) per manufacturer's protocol. ELISA absorbances will be measured on an EnSpire Plate Reader.

Western-Blot

Changes in levels of STAT4, PIASx, SOCS3, fibroblast activation protein (FAP), vimentin, and alpha-smooth muscle actin (α-SMA) will be visualized by Western-blot. Amount of phosphorylated STAT4 (p-STAT4) will be used to quantify the magnitude of signaling via the JAK-STAT pathway. Detection of p-STAT4 will be achieved with an anti-p-STAT4 (Tyr693) antibody. Fibroblast cells will be lysed at corresponding timepoints during the scratch assay in Lysis Buffer (50 mM Tris pH 7.8, 50 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10% glycerol, and 1 tablet of Roche Complete Mini without EDTA). The soluble proteins will be run on a pre-made PAGE gel (4-15%, Bio-rad) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-rad). GAPDH will be used as a loading control. Protein will be detected

via chemiluminescent- HRP substrates (ThermoScientific) and exposed onto radiography film. ImageJ will be used to quantify and compare the density of the protein bands.

Immunocytochemistry (ICC)

To examine morphological differences, patient and control fibroblast cells will be grown on glass coverslips and fixed with 4% paraformaldehyde prior to staining for F-actin filaments with Alexa Fluor 488 phalloidin (ThermoScientific). Staining for vimentin and FAP will also be performed. Nuclei will be stained with DRAQ5 (Invitrogen). All staining procedures will be performed according to the manufacturer's instructions and images will be collected on a Lecia confocal microscope. Patient and control fibroblasts will be stained both pre- and post-scratch.

Treatment of fibroblasts with ruxolitinib

To determine if JAK inhibition can alter fibroblast biology and whether the observed changes are dosage dependent, patient and control fibroblast cells will be treated with either 0.1 µM or 1 µM of ruxolitinib. Cells will be treated for a total of 5 days prior to the start of the scratch assay and sample collection. 24 hours prior to the beginning of the scratch assay, the cells will be given a fresh dose of ruxolitinib and will remain in the media throughout the duration of the scratch assay experiment.

RESULTS

Patient characteristics and symptoms

We identified a total of 48 reports comprising 67 patients published between February 1980 and March 2020. With less than 70 reported cases within the last 40 years, our results support the rarity of DPM. A small number of adult-onset DPM cases have been reported in literature in addition to juvenile-onset cases. Juvenile-onset DPM cases and adult-onset DPM cases are summarized in Table 1 and Table 2, respectively. Among juvenile-onset DPM patients, females were more frequently diagnosed, with the age of disease onset ranging from 5 months to 14 years. In contrast, males were predominately affected among adult-onset patients with age of disease onset ranging from 18 to 76 years.

In patients with DPM, rapid and deep fibrosis of the skin results in chronic ulcerations, atrophy, and painful joint contractures that interfere with mobility (13). Fibrotic conditions promote the formation of pigmented sclerotic plaques which predominately affected the scalp, trunk, face, and exterior surfaces of the limbs (43). Although the extremities were reportedly involved during the initial stages of disease, they were usually spared during the later stages (44). Hardening of the skin is one of the most common initial symptoms observed in patients. Alopecia, ectropion of the eyelid, and squamous cell carcinoma were also observed (Table 3). Extracutaneous complications including hair loss, gastrointestinal involvement, vascular involvement, neoplasia, bone abnormalities, restrictive pulmonary disease, and cardiomyopathy have also been reported. Histological examination of skin biopsies from patients with DPM commonly revealed a thickened dermis and dense collage bundles. Calcification of tissues were also present in some cases. Notably, signs of a lymphohistiocytic infiltrate suggest that an overactive immune response may be involved. This is further supported by laboratory findings

including elevated C-reactive protein and erythrocyte sedimentation rates. Except for anti-nuclear antibody, autoimmune markers were often absent in patients with DPM. Table 4 summarizes the most common histopathological and laboratory findings among DPM patients reported in literature.

DPM as diagnostic challenge

Due to a lack of a definitive test for DPM, diagnosis is largely dependent on recognition of clinical features. Traditionally, DPM is distinguished from generalized morphea via involvement beyond the skin layer and from systemic sclerosis by the absence of Raynaud's phenomenon (13). Additionally, the presence of "tank top sign," a pattern of skin fibrosis of the torso that makes the patients appear to be wearing a tank top, can help distinguish DPM from other fibrotic skin diseases (45). However, the diagnosis of DPM remains difficult due to the overlap of symptoms from other diseases. For example, a patient with simultaneous presentation of symptoms of eosinophilic fasciitis (EF) and DPM has been noted within the literature (46). In addition, while histopathology can help support DPM diagnosis, the findings are largely indistinguishable from other forms of morphea (13). In a few cases, patients have been misdiagnosed with rheumatoid arthritis, EF, or ulcerative dermatitis, before receiving a final diagnosis of DPM (47–49). These delays in diagnosis and subsequent morbidity and mortality further underscore the needs for greater knowledge about DPM.

Treatments and outcomes of DPM

Available treatment options for both juvenile and adult patients integrate multiple drug therapies that target the pathogenic mechanisms that are thought to be involved in disease

manifestation and progression (Figure 2). Suppression of the immune system is achieved by various immunosuppressive drugs including methotrexate, mycophenolate mofetil, and corticosteroids (50). In the most severe cases, autologous stem cell transplant has been reported to be effective in halting disease progression, but it has not been curative (51). Phototherapy involving ultraviolet A (UVA) 1 or psoralen plus UVA (PUVA) is commonly implemented in an attempt to degrade the excess ECM components contributing to fibrosis and cases of successful treatment of DPM patients with both types of phototherapy have been reported in literature. More recently, bosentan, imatinib mesylate, and anti-thymocyte globulin have been reported to be effective in treating DPM (52–54).

While some patients exhibit a partial response to treatment, responses are overall inconsistent as subsets of patients show no improvement in symptoms despite being treated with the same or similar therapies. Table 5 provides a detailed summary of the various attempted treatments and outcomes of DPM patients reported in literature. In some cases, a single therapy was clearly responsible for the improvement of symptoms and these treatments are bolded within the body of the table. However, despite multiple therapeutic attempts, the survival time of DPM patients after diagnosis remains less than 10 years (13). The most common cause of death is cachexia, although other complications such as pneumonia, sepsis, and gangrene also contribute to patient mortality (13).

Genetic etiology of DPM

The familial autoimmune background of morphea patients in addition to the ineffectiveness of available treatment options in curing disease, suggests that there is likely an underlying genetic component in DPM pathogenesis (9,25). The lab recently identified a variant

in *STAT4* as a putative cause for DPM. The *STAT4* gene, encoding the STAT4 protein, is believed to have arisen due to a tandem gene duplication along with *STAT1* (55). *STAT4* is expressed in myeloid cells, testis, thymus, heart, brain, and skin (55–57) and is predominately activated by IL-12, although other cytokines such as type I interferons, IL-23, IL-2, IL-27, and IL-35 can also induce its activation. (58). STAT4 has been shown to be crucial for T helper type-1 cell function, development, and interferon-γ production (59). To determine the relationship between *STAT4* variants and disease, we conducted a literature review on studies that implicated single nucleotide polymorphisms (SNPs) in the *STAT4* locus in various diseases. A total of 44 studies published between September 2007 and June 2020 were identified. Various single nucleotide polymorphisms (SNPs) in the *STAT4* locus have been associated with an increased risk of developing a wide variety of immune-related diseases (Figure 3). All reported SNPs were intronic and the most reported *STAT4* SNP is rs7574865, with most studies being genome-wide association studies (GWAS) (Supplementary Figure 1). Table 6 details the various *STAT4* SNPs reported in literature with the associated disease and the population of interest. The variant discovered in our patient has not been reported in any database, further supporting that this is may be likely gene candidate for DPM.

Optimization of scratch assay set-up: scratching at a 45° angle improves consistency between scratch assay experiments

A causal relationship between candidate genotype and clinical phenotype must be established via a relevant cellular phenotype (39). Based on clinical observations, DPM patients have an impaired wound healing ability. In the normal wound healing process, fibroblasts are first recruited to the wound site and then become activated to produce collagen and ECM

components. In the later stages of wound healing, fibroblasts are involved in wound contraction and remodeling of ECM (reviewed in (60)). Given the extensive involvement of fibroblasts in multiple stages of this process, we sought to characterize the phenotype of DPM fibroblasts by first examining for differences in wound healing ability *in vitro*. Wound healing was assessed via an *in vitro* "scratch" assay by quantifying fibroblast migration rate, as shown in Figure 4. The assay's compatibility with microscopy and low cost of materials make it an ideal method to measure cell migration *in vitro*. In addition, fibroblast migration patterns observed in this assay closely mimics migration patterns *in vivo* (61). Because wound width measurements will be obtained from a picture of a specific area of the wound, it is important to establish a method to ensure that the same location is photographed each time to reduce variability and ensure accurate results. Published protocols have recommended marking a point of reference directly on the plate with either a marker or a razor blade (61,62). However, we opted to mark reference points using a paper set-up to avoid tampering with the plate and to allow more freedom to select which area will be photographed (Figure 5). While the paper set-up enabled the same area to be photographed each time, we noticed that our experimental repeats were inconsistent (Figure 6A).

Several factors could contribute to the variability of the results, including differences in pressure, tip angle (62), and utilization of different cell lines. Because pressure and tip angle are factors that are controlled by the individual conducting the assay, this led us to realize the scratch assay is largely operator dependent. Therefore, we sought to optimize the variable factors involved in this assay, beginning with the tip angle. Our results show that migration rates were more consistent when scratches were made at a 45° angle rather than at a 90° angle (Figure 6B), and this may be attributed, at least partially, to a notable decrease in clumps of cells located within the wound area (Figure 6C). Next, we sought to determine whether the same decrease in

variability that resulted from changing the tip angle can be observed across different cell lines. Using two independent fibroblast cell lines, we compared the migration rate of the cells (indicated by the slope of the plotted line) when scratching at a 90° angle to the migration rate when scratching at a 45° angle. Our results indicate that tip angle modification reduces interexperimental variability across all cell lines tested (Figure 7).

DISCUSSION

This study aims to establish an association between a candidate genotype and a clinical phenotype by establishing a model to study rare skin diseases. A thorough review of the literature is an important component of the research process as it not only provides the necessary background for high quality research but also maximizes relevance and originality (63). In rare disease research, a review of clinical case reports is essential as the phenotypic information described within the reports forms the foundation for future research projects on a specific disease. DPM is an ultra-rare and severe form of deep morphea and its effects extend beyond the layers of the skin. It is known for following an aggressive course of disease progression that leads to patient immobility and ultimately death. Details regarding the underlying etiology remain largely unknown.

To begin to understand the current state of knowledge of DPM, we conducted a literature review on all published cases of this disease. Our findings revealed that while both juvenile-onset and adult-onset cases have been reported, there are fewer adult-onset cases compared to the number of juvenile-onset cases. The differences in average age of disease onset and male-female distribution suggest that adult-onset patients may be suffering from an entirely different disease. However, it currently remains unclear if adult-onset disease is an extreme of the same disease or represents an independent pathophysiological entity. Whether adults and juveniles have an equivalent version of the disease is an important clinical distinction that needs to be established to ensure accurate diagnoses. The misdiagnoses of DPM as other diseases is likely attributed to lack of familiarity with this rare disease (50). This is the first study to summarize all DPM patients reported in literature, their associated symptoms, clinical test results, and attempted treatments. However, we recognize that the number of reported cases included within this study

may not accurately reflect the number of reported cases worldwide. Because this study only synthesized information from case reports published in English and there have been cases of DPM reported in other languages, the total number of DPM cases reported in this study may underestimate disease prevalence. However, despite this limitation, all DPM relevant information summarized in this study will be useful in increasing knowledge and clinician familiarity with DPM. Additionally, the summarized findings will be a useful resource for scientists who are interested in studying this specific disease.

Our literature review findings also show that available therapies are non-specific for DPM and patient responses to treatment have been inconsistent. The inconsistencies in responses to treatment suggest that identification of a genetic cause could lead to a more specific and targeted therapy for DPM patients. We have identified a novel variant of *STAT4* in a family with DPM. To determine whether this could be a likely gene candidate for disease, we conducted a literature review on *STAT4* variants and their relationship to autoimmune disease development. Our findings revealed that *STAT4* variants are highly associated with development of various autoimmune disorders, suggesting that this is a likely gene candidate within the family. Furthermore, based on all the information gathered from our literature review, we reasoned that an *in vitro* fibroblast cell model system would be useful in characterizing the effect that this variant has on disease pathogenesis. Based on this cellular model, we have planned experiments that utilize patient derived fibroblasts to determine the variant's effect on wound healing, inflammatory gene transcription, and cell morphology.

To examine wound healing, we attempted to quantify fibroblast migration rate using an *in vitro* scratch assay. However, we noticed that our initial experimental repeats were inconsistent. These inconsistencies led us to realize that slight variations in factors such as tip angle could

greatly affect result consistency and accuracy, making this assay largely operator dependent. To maximize consistency and accuracy of results, we sought to determine the optimal tip angle to use for this assay. Our results suggest that scratching at a 45° angle is optimal for maintaining consistency between scratch assay experiments across different cell lines. However, variability between experimental repeats still exists (Figure 6). This variability could be attributed to the difference in pressure applied between experimental replicates. Because the scratches were made manually using a pipette tip, the amount of pressure applied when scratching may be difficult to control. This issue could be addressed by switching to performing a circular wound closure assay (CWCA), an alternative version of the current scratch assay method. Unlike the scratch assay, the CWCA utilizes a pipette tip attached to a vacuum that facilitates the removal of a small circular area of cells (64). This set-up is suggested to enable the creation of consistent sized wounds while controlling for pressure. In addition, the circular wound eliminates the need to take multiple pictures per timepoint, making this assay more efficient than the current method. Wound closure rates can still be quantified using freely available imaging analysis software such as ImageJ.

Regardless of the type of migration assay used, we anticipate the migration rate of the patient fibroblasts to be slower than that of control fibroblasts based on the impaired-healing phenotype observed in DPM patients. If the results from our fibroblast migration assay match what we expect, we can conclude that patient fibroblasts have an impaired rate of migration and that this impairment may be influenced by the novel *STAT4* variant. We hypothesize that JAK-STAT signaling dysregulation in patient fibroblasts will result in persistent signaling down this pathway and the overproduction of proinflammatory mediators. Therefore, we anticipate higher levels of p-STAT4 in patient samples compared to controls. STAT4 signaling may be induced by

several cytokines including IL-12, IL-23, and IL-2 (58), but not all of these pathways are present in skin fibroblasts. Therefore, experiments will focus on examining levels of IL-6 and STAT4 due their involvement in autocrine signaling in fibroblasts (65). We expect to see increased expression of both IL-6 and STAT4 at the RNA and protein level in patient samples at baseline (pre-scratch) compared to control samples. In addition, we would also expect these high expression levels to be maintained in the patient samples throughout all timepoints of the scratch assay. In contrast, in control fibroblasts, we anticipate the levels of inflammatory transcripts and protein to initially rise following disruption of the cell monolayer via scratching, but then drop back to baseline levels towards the end of scratch assay as the wound approaches full closure.

Reduced efficacy of regulatory factors may also contribute to JAK-STAT dysregulation. Suppressors of cytokine signaling (SOCS), and protein inhibitor of activated STATs (PIAS) are two families of proteins that negatively regulate JAK-STAT signaling. SOCS protein expression is induced by cytokines and they exert their inhibitory effects by either directly binding to JAKs, binding to receptors to interfere with JAK binding, competing with STATs to bind to activated receptors, or by targeting signal transducers for proteasomal degradation (66–69). In contrast, PIAS proteins are constitutively expressed and they exert their inhibitory effects by directly interacting with STAT dimers in the nucleus (67,69,70). Depending on how our identified variant interacts with signaling receptors and/or the regulatory proteins, we may expect to see increased or decreased expression of these transcripts compared to controls.

Upon activation, fibroblasts acquire a different morphology than their quiescent counterparts (reviewed in (60)). Therefore, we anticipate seeing a difference in the expression of structural elements related to fibroblast activation between patient and control cells. FAP, vimentin, and α-SMA are all markers that are associated with fibroblast activation and we expect

to see low expression levels of all three markers in control samples at baseline. During the scratch assay, we would expect to see increased levels of all three expression markers followed by a decrease in expression towards the end of the assay. In patient cells, we would expect to see high expression of FAP and α-SMA, but lower levels of vimentin at all timepoints. Vimentin deficient fibroblasts have been shown to have impaired rates of motility and this is aligned with our hypothesis of patient fibroblasts having an impaired rate of migration compared to control fibroblasts (47).

Finally, this system may be useful to study potential therapeutics for DPM. Due to the ability of JAK inhibitors to block signaling down the JAK-STAT pathway, we would expect to see decreases in levels of proinflammatory transcripts and cytokines in patient fibroblasts following treatment with ruxolitinib. We hypothesize that limiting JAK-STAT signaling will lead to improvements in migration rates and cell morphology in patient fibroblasts. Specifically, we would expect both migration rates and morphology to resemble that of control fibroblasts following JAK inhibition. We do not anticipate significant changes in control fibroblasts following JAK inhibition due to integration of JAK-STAT regulatory mechanisms within these cells.

As with many experiments, there will be instances in which the results that were obtained do not match what we expect. In preparation for this, we have considered alternative experimental outcomes and have planned future experiments that could contribute to further characterization of the *STAT4* variant's effect on fibroblast biology. In terms of wound healing, if the results indicate that there is no difference between patient and control fibroblast migration rate, this would suggests that the impaired wound healing ability of DPM patients may be due to a defect in fibroblast contraction rather than migration. Differences in fibroblast contraction can

be examined by performing a collagen gel contraction assay. Cells in contact with a 3D environment behave differently than cells in conventional tissue culture so usage of a 3D collagen matrix may better mimic fibroblast behavior *in vivo* (71).

Regarding expression of regulatory factors, one possible outcome would be that there is no difference in expression levels of the factors involved in regulating JAK-STAT signaling. In this case, the results would suggest that pathway dysregulation may be due to a defect in regulatory factor binding rather than expression and that we should shift our focus to examining protein-protein interaction. Initial experiments would focus on examining PIASx due to its ability to interact directly with phosphorylated STAT4 in the nucleus (72). PIASx-STAT4 interaction can be evaluated via immunoprecipitation of p-STAT4 followed by Western-blot with an anti-PIASx antibody. The role of JAK-STAT regulatory proteins in fibroblasts has not been explored and the results of these experiments may help characterize their specific functions in the context of DPM pathogenesis. Finally, if the results from our immunocytochemistry experiments indicate that there are minimal differences between structural elements of patient and control fibroblast, this would suggest that a fibroblast cell model may not have been a good model to use to examine mechanisms implicated in disease pathogenesis and that we should consider utilizing a different cellular model. Several proinflammatory and profibrotic cytokines involved in the development of dermal fibrosis can be produced by keratinocytes. Furthermore, highly proliferative keratinocytes have been found within the epidermis of systemic sclerosis patients (reviewed in (16)), suggesting that we could use keratinocytes as an alternative cellular model to characterize the molecular differences between patient and control cells.

By synthesizing all the information obtained from performing a literature review, we were able to hypothesize that a novel association exists between a specific *STAT4* genotype and

the phenotype of DPM. In addition, the information from the literature enabled us to deduce that primary dermal fibroblasts would be useful cellular model to study rare skin diseases. The experiments described in this study aims to characterize the effects of a novel *STAT4* variant on DPM pathogenesis in addition to evaluating the therapeutic potential of JAK inhibition for this specific disease. If the results align with our expectations, it will provide support for our hypothesis that DPM is driven by persistent JAK-STAT signaling and that dysregulation of the signaling pathway may be driven by the novel *STAT4* variant found in our patient. More importantly, it will provide evidence that JAK inhibition is effective in altering the skin phenotype associated with DPM and should be further explored as a potential therapeutic option. It is our hope that the results from these experiments will provide more insight into the mechanisms behind DPM pathogenesis and pave the way for the implementation of a more specific and targeted therapy for these patients. Finally, we believe that all the information pertaining to DPM and *STAT4* variants summarized in this study will serve as the foundation for other research studies in the future.

Table 1. Summary of juvenile-onset DPM cases.

First Author	Number of Patients	Sex	Age (years)	Age of Disease Onset (years)	Age of Death (years)	Ref.
Diaz-Perez	14	10F, 4M	≤14	≤14	14, 16 (2F)	(47)
Kweon	1	M	27	4	27	(48)
Scharfetter-Kochanek	1	F	8	4		(73)
Gruss	1	M	16	6		(74)
Todd	1	F	7	7		(75)
Wollina	1	M	11	7		(76,77)
Mihailov	1	F	13	11		(78)
Chakravarty	1	F	9	NR		(79)
Parodi	1	M	20	2	20	(80)
Wollina	1	M	16	8	16	(81)
Nguyen	1	F	9	6.5		(82)
Devidayal	1	F	1.5	0.67	1.5	(44)
Doede	1	M	16	5	16	(83)
Yildirim	1	F	8	NR		(84)
Padmavathy	1	F	20	5		(85)
Nandy	1	M	12	11		(86)
Roldan	1	F	4	4		(52)
Surjushe	1	M	6	1		(87)
Iqbal*	1	F	9	4		(88)
Wollina	2	M	17	6		(89)
		F	19	12	19	
Gauthier	1	M	18	10		(90)
Forsea	1	F	19	12		(91)
Petrov	1	F	19	12		(92)
Tekin	1	F	27	15	NR	(93)
Pan	1	M	11	4		(43)

Asterisks denotes a patient with a mixed phenotype. Blank spaces denote no indication of patient death in respective publication. Ref: Reference; F: Female; M: Male; NR: Not Reported.

Table 1. Summary of juvenile-onset DPM cases, continued.

First Author	Number of Patients	Sex	Age (years)	Age of Disease Onset (years)	Age of Death (years)	Ref.
Moll	2	M	NR	6		(51)
		F	NR	6		
Marques	1	M	NR	7		(94)
Kura	1	F	7	0.42	7	(95)
Banks	1	F	7	7		(53)
Odhav†	1	M	4	4		(46)
Dasgupta	1	M	8	5		(96)
Grewal	1	M	25	10		(97)
Ruiz-Matta	1	M	23	5	NR	(98)
Martini*	2	F	16	4		(99)
		M	4	4		
Jamalpur	1	F	15	7		(100)
Zhang	1	F	6	NR		(49)
Soh	1	M	5	5		(101)
Kaddioui	1	F	4	2.58		(102)

Asterisks denotes a patient with a mixed phenotype. Blank spaces denote no indication of patient death in respective publication. Ref: Reference; F: Female; M: Male; NR: Not Reported.

Table 2. Summary of adult-onset DPM cases.

First Author	Number of Patients	Sex	Age (years)	Age of Disease Onset (years)	Age of Death (years)	Ref.
Cantwell	1	F	24	22		(103)
Peter	2	M	50	49		(104)
		M	40	37		
Maragh	2	M	26	23.5		(105)
		M	32	18		
Song	1	M	50	50		(54)
Sherber	3	3M	NR	NR		(45)
Singh	1	M	26	24		(106)
El Jouari	1	M	76	76		(107)
Abbas	1	F	60	42		(108)
Adil	1	F	52	52		(109)

Blank spaces denote no indication of patient death in respective publication. Ref: Reference; F: Female; M: Male; NR: Not Reported.

Table 3. Summary of common DPM symptoms.

Cutaneous features				Atrophy			Extracutaneous complications			Other symptoms					
Tight Skin	Plaques	Ulcers	Contractures	Muscle	Skin	Soft Tissue	Bone	Pulmonary	CM	Alopecia	Ectropion	Mutilation	SCC	Disability	Ref.
+	+	+	+	+		+	+	+			+			+	(47)*
					+									+	(103)
	+	+												+	(104)*
	+	+	+				+			+			+	+	(48)
		+	+												(73)
	+	+	+								+				(74)
		+	+												(75)
	+	+	+					+	+	+				+	(76,77)
	+				+			+							(78)
	+		+											+	(79)
		+	+	+						+			+		(80)
		+	+				+		+	+	+	+	+	+	(81)
		+	+				+								(44)
	+	+	+									+	+	+	(83)
			+		+					+				+	(84)
+			+			+								+	(85)
+	+	+	+							+	+		+	+	(105)*
							+	+							(86)
	+	+	+					+						+	(52)
+	+				+									+	(87)
+			+				+							+	(88)†
	+	+	+	+	+		+	+	+	+	+	+	+	+	(89)*
	+	+												+	(90)
	+			+				+						+	(91)
		+	+		+					+	+		+	+	(92)
+			+											+	(54)
	+	+	+				+			+	+	+	+	+	(93)
	+	+	+											+	(43)
		+	+							+					(94)
+	+	+	+							+	+			+	(95)
			+					+							(53)
			+											+	(46)†

Blank spaces represent an absence of a specific symptom. A single asterisk denotes more than one reported patient. † denotes a patient with a mixed phenotype. ‡ denotes multiple patients reported with at least one patient having a mixed phenotype. Ref: Reference; CM: Cardiomyopathy; SCC: Squamous Cell Carcinoma

Table 3. Summary of common DPM symptoms, continued.

Cutaneous features				Atrophy			Extracutaneous complications			Other symptoms					
Tight Skin	Plaques	Ulcers	Contractures	Muscle	Skin	Soft Tissue	Bone	Pulmonary	CM	Alopecia	Ectropion	Mutilation	SCC	Disability	Ref.
					+	+									(96)
+			+			+	+							+	(106)
	+	+	+								+		+	+	(97)
	+	+						+			+		+	+	(98)
						+		+						+	(99)‡
+	+		+											+	(100)
			+	+										+	(107)
	+														(49)
		+	+		+						+				(101)
	+													+	(108)
			+												(109)
	+														(102)

Blank spaces represent an absence of a specific symptom. A single asterisk denotes more than one reported patient. † denotes a patient with a mixed phenotype. ‡ denotes multiple patients reported with at least one patient having a mixed phenotype. Ref: Reference; CM: Cardiomyopathy; SCC: Squamous Cell Carcinoma

Table 4. Summary of clinical test results of reported DPM patients.

First Author	Histopathology				Laboratory Findings					Ref.
	Calcification	LI	Thick Dermis	Dense Collagen Bundles (Collagenization)	HG	Elevated CRP	Elevated ESR	Eosinophilia	Positive ANA	
Diaz-Perez*	+	+			+		+	+		(47)
Cantwell								+		(103)
Peter*							+		+	(104)
Scharfetter-Kochanek		+	+	+	+			+	+	(73)
Gruss	+	+	+	+	+					(74)
Todd		+		+			+		+	(75)
Wollina		+		+	+	+		+		(76,77)
Mihailov					+		+		+	(78)
Chakravarty	+		+	+						(79)
Parodi	+									(80)
Wollina								+		(81)
Devidayal		+								(44)
Yildirim		+		+						(84)
Maragh					+		+	+		(105)
Nandy							+			(86)
Roldan			+	+	+			+	+	(52)
Surjushe			+							(87)
Iqbal†				+			+			(88)
Wollina*						+	+	+	+	(89)
Gauthier						+	+			(90)
Forsea					+		+	+		(91)
Petrov							+			(92)
Song		+	+	+						(54)
Tekin	+	+			+	+	+			(93)
Pan						+	+			(43)

Blank spaces represent an absence of a specific clinical result. A single asterisk denotes more than one reported patient and results shown are compiled from all patients reported in respective case report. † denotes a patient with a mixed phenotype. LI: Lymphohistiocytic infiltrate; HG: Hypergammaglobulinemia; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; ANA: Antinuclear Antibody; Ref: Reference

Table 4. Summary of clinical test results of reported DPM patients, continued.

First Author	Histopathology				Laboratory Findings					Ref.
	Calcification	LI	Thick Dermis	Dense Collagen Bundles (Collagenization)	HG	Elevated CRP	Elevated ESR	Eosinophilia	Positive ANA	
Kura		+		+					+	(95)
Banks		+	+	+				+		(53)
Dasgupta				+			+	+	+	(96)
Odhav†		+	+	+			+	+		(46)
Singh		+	+	+			+			(106)
Jamalpur		+		+						(100)
Zhang		+			+		+	+	+	(49)
Adil		+	+	+						(109)
Kaddioui									+	(102)

Blank spaces represent an absence of a specific clinical result. A single asterisk denotes more than one reported patient and results shown are compiled from all patients reported in respective case report. † denotes a patient with a mixed phenotype. LI: Lymphohistiocytic infiltrate; HG: Hypergammaglobulinemia; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; ANA: Antinuclear Antibody; Ref: Reference

Table 5. Summary of reported treatments and outcomes of DPM patients.

First Author	Treatments	Outcome	Ref.
Diaz-Perez*	cyclophosphamide, D-penicillamine, PT, anti-malarial drugs	2D, 2PR, 9PD	(47)
Peter*	intravenous penicillin, glucocorticosteroids, NSAID, **cyclosporine A**	2PR	(104)
Kweon	antibiotics, steroids, SCC excision, skin graft	D	(48)
Scharfetter-Kochanek	antibiotics, steroids, OISD, **PUVA**	PR	(73)
Gruss	cyclosporine, steroids, **UVA1**, PT	PR	(74)
Todd	penicillin, corticosteroids, penicillamine, PUVA	PD	(75)
Wollina	systemic antibiotics, iron and zinc supplements, **IVIG**, prednisolone, **PUVA**, ACEi	PR	(76,77)
Mihailov	**PUVA**	PR	(78)
Parodi	anti-septic medication, amputation	D	(80)
Wollina	acitretin, yogurt dressing, biosurgery with sterile maggots, amputation	D	(81)
Nguyen	arthrodesis, free-flap tissue coverage	Ankle stabilization	(82)
	MTX, prednisolone, D-penicillamine PT, OISD	PD	
Devidayal	antibiotics, oral antimicrobials	D	(44)
Doede	antibiotics, PUVA, biosurgery with sterile maggots, palliative ablation	D	(83)
Yildirim	**UVA**	PR	(84)
Padmavathy	PT	PD	(85)
Maragh*	MTX, prednisolone, penicillamine, OISD, narcotics, amputation, SCC excision, PT	1PR, 1PD	(105)
Nandy	D-penicillamine, PT	PR	(86)
Roldan	MTX, D-penicillamine, antibiotics, steroids, PUVA, PT, **bosentan**, CCB, ACEi	PR	(52)
Surjushe	**MTX**	PR	(87)
Iqbal†	prednisone, PT	PR	(88)
Wollina*	topical anti-infective solution, dry CO_2, PT, TENS, AHKT, **sildenafil**, RT	1PR, 1D	(89)
Gauthier	MTX, corticosteroids, intravenous antibiotics, wound care	PD	(90)
Forsea	MTX, prednisone, antibiotics, OISD, UVA, oral supplements, PT	PR	(91)

A single asterisk denotes more than one reported patient and treatments listed are a compilation of attempted treatments on all patients reported in respective case report. † denotes a patient with a mixed phenotype. ‡ denotes multiple patients reported with at least one patient having a mixed phenotype. Treatments that were clearly responsible for disease improvement are bolded. PT: Physiotherapy; SCC: Squamous Cell Carcinoma; PUVA: Psoralen plus UVA; OISD: Other Immunosuppressive Drugs; UVA: Ultraviolet A; IVIG: Intravenous immunoglobulin; RT: Radiation Therapy; ACEi: Angiotensin-converting enzyme inhibitor; MTX: Methotrexate; CCB: Calcium Channel Blocker; AHKT: Autologous Hair Keratinocyte Transplant; TENS: Transcutaneous Electrical Nerve Stimulation; MMF: Mycophenolate mofetil; ASCT: Autologous Stem Cell Transplant; NSAID: Nonsteroidal Anti-inflammatory Drug; HSCT: Hematopoietic Stem Cell Transplant; D: Death; PR: Partial Response; PD: Progressive Disease; Ref: Reference

Table 5. Summary of reported treatments and outcomes of DPM patients, continued.

First Author	Treatments	Outcome	Ref.
Petrov	D-penicillamine, cyclophosphamide, azathioprine, steroids, RT	PD	(92)
Song	MTX, MMF, prednisone, triamcinolone cream, methylprednisolone, IVIG, ciclosporin, **anti-thymocyte globulin**	PR	(54)
Tekin	narcotics, systemic antibiotics, amputation	D	(93)
Pan	MTX, prednisolone, azathioprine, cyclosporine, intravenous ampicillin, cloxacillin	PD	(43)
Moll*	MTX, MMF, methylprednisolone, steroids, PUVA, **ASCT**	2PR	(51)
Marques	MTX, MMF, D-penicillamine, calcipotriene, PUVA, **bosentan**	PR	(94)
Kura	MTX, methylprednisolone, PUVA, blood thinners	D	(95)
Banks	MTX, cyclophosphamide, prednisone, UVA, oral supplements, PT, **imatinib mesylate**	PR	(53)
Odhav†	MTX, MMF, methylprednisolone, steroids, ASCT, IVIG	PR	(46)
Dasgupta	steroids, oral supplements, PT, anticonvulsant	PD	(96)
Singh	MTX, steroids	PR	(106)
Grewal	MTX, MMF, **prednisone**, narcotics, amputation, oral supplements, bosentan, etanercept, **hydroxyurea**, citalopram	PR	(97)
Ruiz-Matta	antibiotics, narcotics	D	(98)
Martini‡	MTX, MMF, **tocilizumab**, antibiotics, prednisone, imatnib mesylate	2PR	(99)
Jamalpur	MTX, prednisone, phototherapy	NR	(100)
El Jouari	MTX, corticosteroids	PR	(107)
Zhang	MTX, MMF, methylprednisolone, **tocilizumab**	PR	(49)
Soh	MTX, MMF, OISD, NSAID, tocilizumab, prednisone, bosentan, IVIG, rituximab, ruxolitinib, HSCT	PD	(101)
Abbas	MTX, UVA1, hydroxychloroquine, steroids, PT, **hyaluronidase injections**	PR	(108)
Adil	MTX, MMF, prednisolone, methylprednisolone	PD	(109)
Kaddioui	MTX, oral corticosteroids, PT	PD	(102)

A single asterisk denotes more than one reported patient and treatments listed are a compilation of attempted treatments on all patients reported in respective case report. † denotes a patient with a mixed phenotype. ‡ denotes multiple patients reported with at least one patient having a mixed phenotype. Treatments that were clearly responsible for disease improvement are bolded. PT: Physiotherapy; SCC: Squamous Cell Carcinoma; PUVA: Psoralen plus UVA; OISD: Other Immunosuppressive Drugs; UVA: Ultraviolet A; IVIG: Intravenous immunoglobulin; RT: Radiation Therapy; ACEi: Angiotensin-converting enzyme inhibitor; MTX: Methotrexate; CCB: Calcium Channel Blocker; AHKT: Autologous Hair Keratinocyte Transplant; TENS: Transcutaneous Electrical Nerve Stimulation; MMF: Mycophenolate mofetil; ASCT: Autologous Stem Cell Transplant; NSAID: Nonsteroidal Anti-inflammatory Drug; HSCT: Hematopoietic Stem Cell Transplant; D: Death; PR: Partial Response; PD: Progressive Disease; NR: Not Reported; Ref: Reference

Table 6. Summary of various *STAT4* SNPs and their associated diseases.

STAT4 SNP	SNP Type	Chromosome 2 Position	Alleles	Coding Sequence Change	Associated Disease	Population Type	Ref.
rs7574865	Intronic	191099907	G/T	c.274-23582 A>C	JIA	Pediatric Population with JIA	(110)
						Han Chinese	(111)
					RA	Caucasian	(112)
						Colombian	(113)
						Japanese	(114)
						Spanish, Swedish, Dutch	(115)
						Korean	(116)
					SLE	Caucasian	(112)
						Colombian	(113)
						Japanese	(114)
						Iranian	(117)
						Spanish	(118)
						Han Chinese	(119)
					SSc	Spanish Caucasian	(120)
						French Caucasian	(121)
						Chinese	(122)
						Japanese	(123)
						European	(124)
					Primary SjS	Caucasian	(125)
					T1D	Cretan	(126)
						Asians, Caucasians	(127)
						Han Chinese	(128)
					T1D (Early Onset)	Korean	(129)

JIA: Juvenile Idiopathic Arthritis; RA: Rheumatoid Arthritis; SLE: Systemic Lupus Erythematous; SSc: Systemic Sclerosis; SjS: Sjögren's Syndrome; T1D: Type I Diabetes; UC: Ulcerative Colitis; NMOSD: Neuromyelitis Optica Spectrum Disorder; APS: Antiphospholipid Syndrome; HBV: Hepatitis B Virus; BD: Behçet Disease; PFAPA: Periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis; N/A: Not Applicable.

Table 6. Summary of various *STAT4* SNPs and their associated diseases, continued.

STAT4 SNP	SNP Type	Chromosome 2 Position	Alleles	Coding Sequence Change	Associated Disease	Population Type	Ref.
					Crohn's Disease	Caucasian	(130)
						Japanese	(131)
					Psoriasis	Cretan	(132)
					UC	Spanish	(133,134)
					Autoimmune Thyroid Disease (Graves' Disease)	Chinese	(135)
					NMOSD	Han Chinese	(136)
					Primary APS	Italian	(137)
					HBV related Hepatocellular Carcinoma	Chinese	(138)
						Vietnamese	(139)
						Asian	(140)
					Chronic HBV Infection	Korean	(141)
rs10181656	Intronic	191105153	G/C	c.274-28828 C>G	Autoimmune Thyroid Disease	Chinese	(135)
					SLE	Swedish	(142)
					NMOSD	Han Chinese	(136)
					RA	Korean	(116)
rs7582694	Intronic	191105394	C/G	c.274-29069 G>C	SLE	Swedish	(142)
						Polish	(143)
					Primary SjS	Swedish, Norwegian	(144)
					HBV related Hepatocellular Carcinoma	Han Chinese	(145)
rs11889341	Intronic	191079016	C/T	c.274-2691 G>A	RA	Korean	(116)

JIA: Juvenile Idiopathic Arthritis; RA: Rheumatoid Arthritis; SLE: Systemic Lupus Erythematous; SSc: Systemic Sclerosis; SjS: Sjögren's Syndrome; T1D: Type I Diabetes; UC: Ulcerative Colitis; NMOSD: Neuromyelitis Optica Spectrum Disorder; APS: Antiphospholipid Syndrome; HBV: Hepatitis B Virus; BD: Behçet Disease; PFAPA: Periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis; N/A: Not Applicable.

Table 6. Summary of various *STAT4* SNPs and their associated diseases, continued.

STAT4 SNP	SNP Type	Chromosome 2 Position	Alleles	Coding Sequence Change	Associated Disease	Population Type	Ref.
					HBV related Hepatocellular Carcinoma	Han Chinese	(145)
rs8179673	Intronic	191104615	C/T	c.274-28290 G>A	RA	Korean	(116)
					HBV related Hepatocellular Carcinoma	Han Chinese	(145)
rs3821236	Intronic	191038032	G/A	c.1434+1167 C>T	Primary APS	Italian	(137)
					SLE	Spanish	(118)
rs3024866	Intronic	191058115	G/A	c.1113-4 C>T	Primary APS	Italian	(137)
					SLE	Spanish	(118)
rs897200	Intronic	191153045	C/T	N/A	BD	Han Chinese	(146)
					Pulmonary Tuberculosis	Moroccan	(147)
rs10931481	Intronic	191090126	A/G	c.274-13801 C>T	Addison's Disease	European	(148)
					Polyarticular JIA	Han Chinese	(149)
rs10168266	Intronic	191071078	T/C	c.466-1307 G>A	NMOSD	Han Chinese	(136)
					Primary SjS	Han Chinese	(150)
rs7574070	Intronic	191145762	A/C	c.273+851 T>G	BD	Han Chinese	(146)
					BD/PFAPA	European	(151)
rs4274624	Intronic	191093930	C/T	c.274-17605 G>A	Addison's Disease	European	(148)
rs925847	Intronic	191032814	C/T	c.2044+144 G>A	UC	Korean	(152)
rs13426947	Intronic	191068528	A/G	c.544+1165 C>T	NMOSD	Han Chinese	(136)
rs10553577	Intronic	191090464	A/G	c.274-14139 T>C	Primary SjS	Caucasian	(153)
rs11893432	Intronic	191057148	C/G	c.1206+870 G>C	JIA	Han Chinese	(149)
rs1031509	Intronic	191145463	T/G	c.273+1150 A>C	Pulmonary Tuberculosis	Moroccan	(147)
rs7572482	Intronic	191150346	A/G	c.-2+601 T>C	Pulmonary Tuberculosis	Moroccan	(147)
rs10189819	Intronic	191144023	C/T	c.273+2590 A>G	Polyarticular JIA	Han Chinese	(149)

JIA: Juvenile Idiopathic Arthritis; RA: Rheumatoid Arthritis; SLE: Systemic Lupus Erythematous; SSc: Systemic Sclerosis; SjS: Sjögren's Syndrome; T1D: Type I Diabetes; UC: Ulcerative Colitis; NMOSD: Neuromyelitis Optica Spectrum Disorder; APS: Antiphospholipid Syndrome; HBV: Hepatitis B Virus; BD: Behçet Disease; PFAPA: Periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis; N/A: Not Applicable.

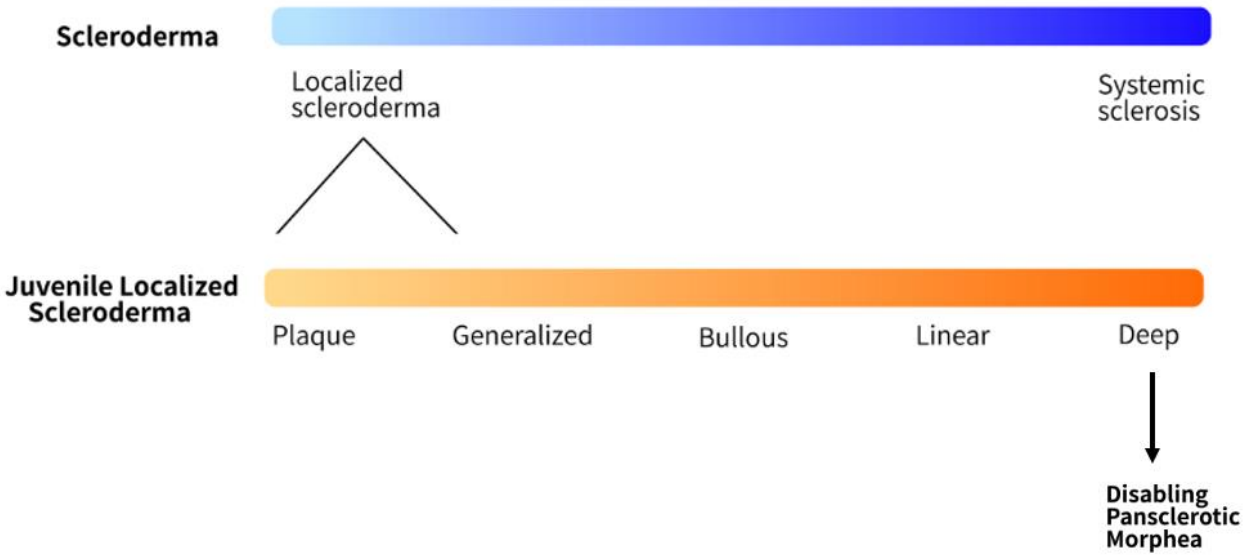


Figure 1. Disabling Pansclerotic Morphea is a severe form of juvenile localized scleroderma. Scleroderma spectrum disorders range from localized scleroderma to systemic sclerosis. The juvenile localized scleroderma (JLS) spectrum comprises of 6 different subtypes (mixed subtype not depicted). Deep morphea is located at the severe end of the JLS spectrum and DPM is the most severe subtype of deep morphea.

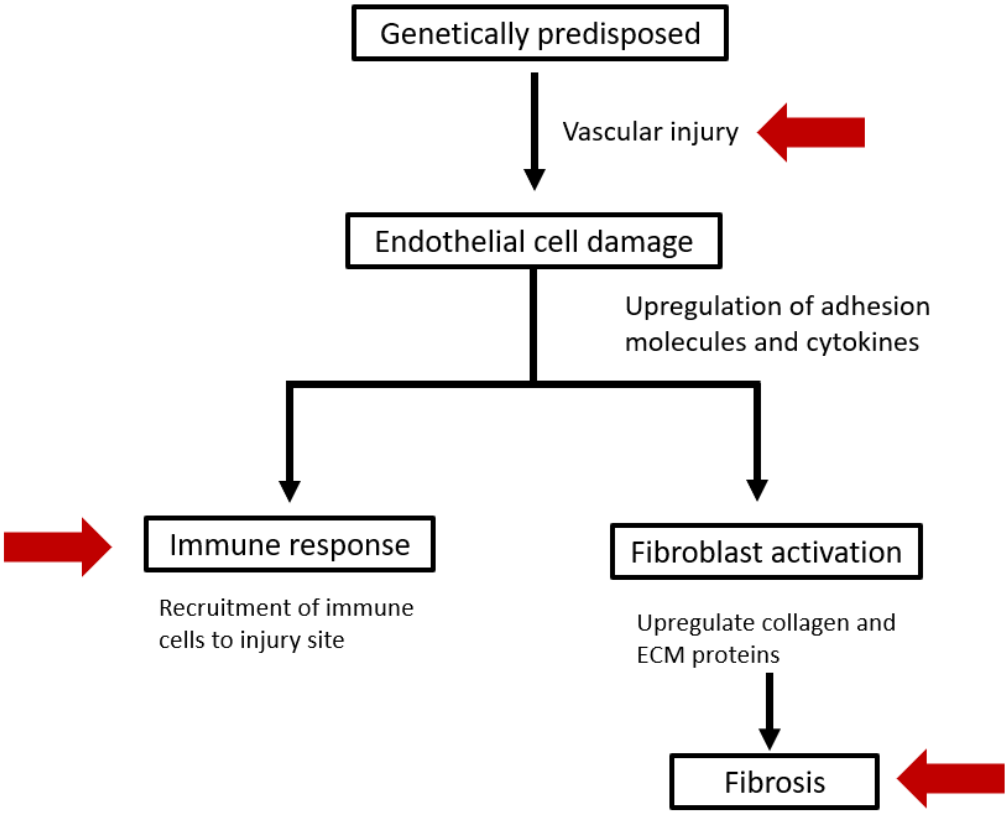


Figure 2. Proposed multifactorial mechanism of morphea. Morphea patients are thought to be genetically predisposed to the disease. Among genetically predisposed individuals, morphea is initialed upon vascular injury resulting in endothelial cell damage and the upregulation of both cellular adhesion molecules and proinflammatory cytokines. Upregulation of these molecules simultaneously triggers the activation of an immune response and activation of fibroblasts. Immune response activation will facilitate the recruitment of immune cells to the injury site while fibroblast activation will stimulate the upregulation of collagen and ECM proteins. Excess production of collagen and ECM proteins result in fibrosis. Red arrows indicate the three main areas of pathogenesis that are targeted by currently available treatments.

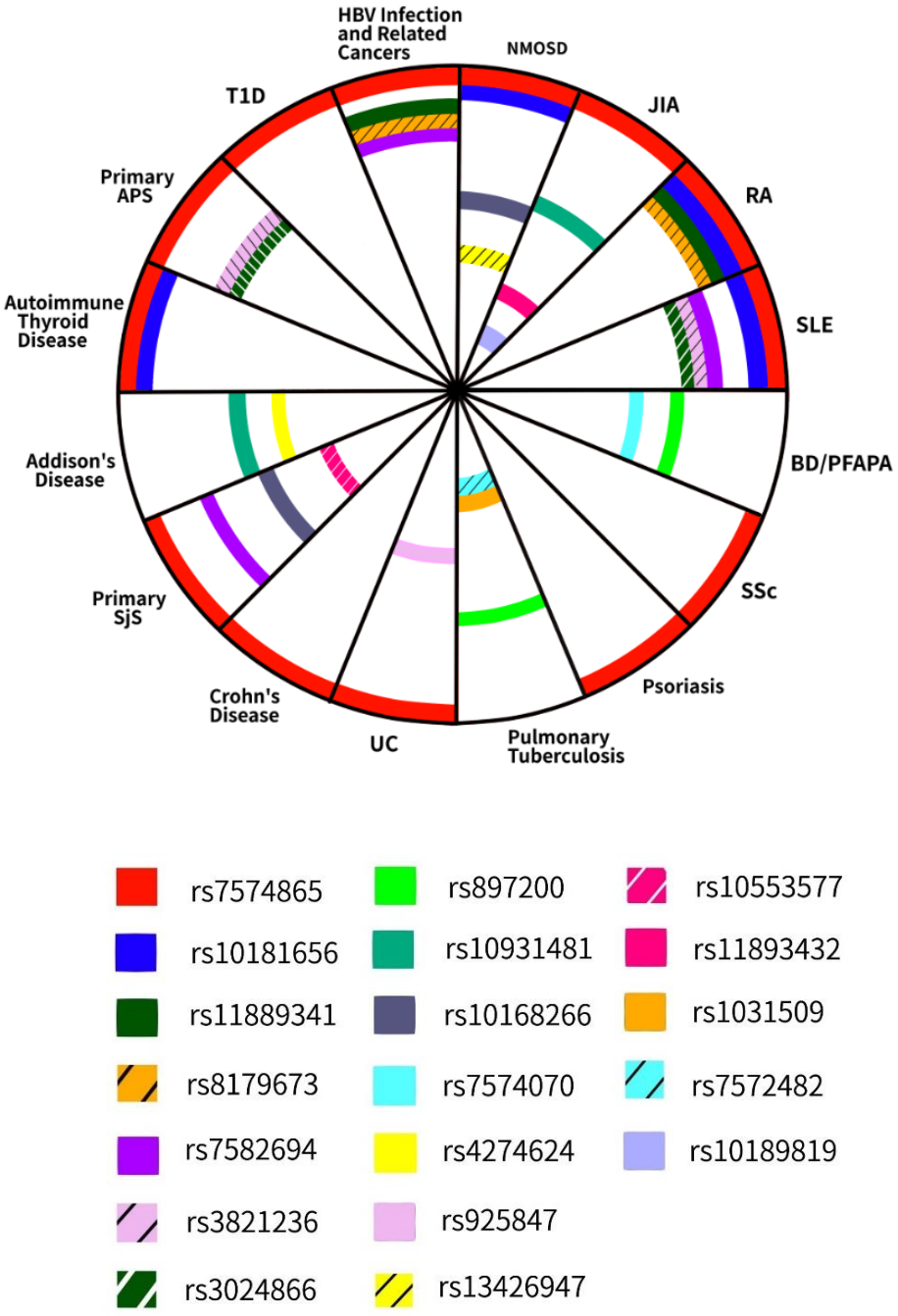


Figure 3. Various *STAT4* SNPs and their associated diseases. Each sector of the circle represents the 16 different diseases associated with SNPs in the *STAT4* gene. Colors represent different *STAT4* SNP numbers. Abbreviations: HBV: Hepatitis B Virus; NMOSD: Neuromyelitis Optica Spectrum Disorder; JIA: Juvenile Idiopathic Arthritis; RA: Rheumatoid Arthritis; SLE: Systemic Lupus Erythematous; BD: Behçet Disease; PFAPA: Periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis syndrome; SSc: Systemic Sclerosis; UC: Ulcerative Colitis; SjS: Sjögren's Syndrome; APS: Antiphospholipid Syndrome; T1D: Type I Diabetes

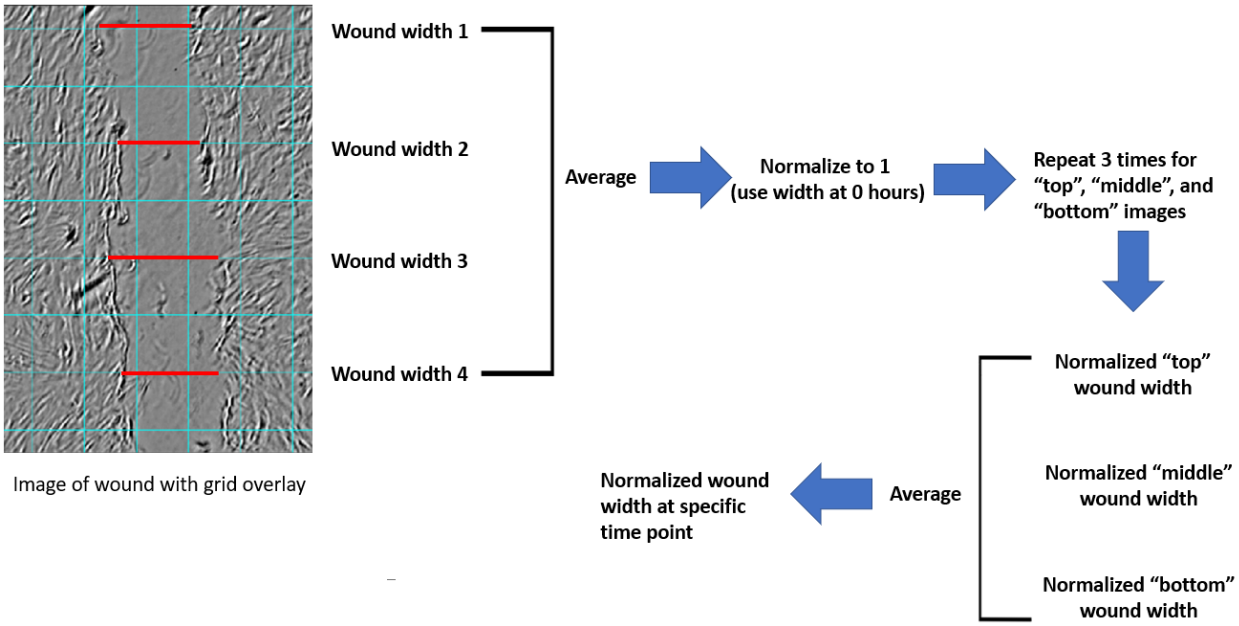


Figure 4. Schematic demonstrating analysis of scratch assay. For every image taken underneath the microscope, a grid was overlayed and centered using ImageJ software. Red lines indicate where the 4 different wound width measurements were taken (every other line). The average of the 4 wound width measurements at 0 hours post-scratch was used to normalize the wound width of every image to 1. The average of the normalized wound width for the “top,” “middle,” and “bottom” images was used to represent the normalized wound width at a specific timepoint.

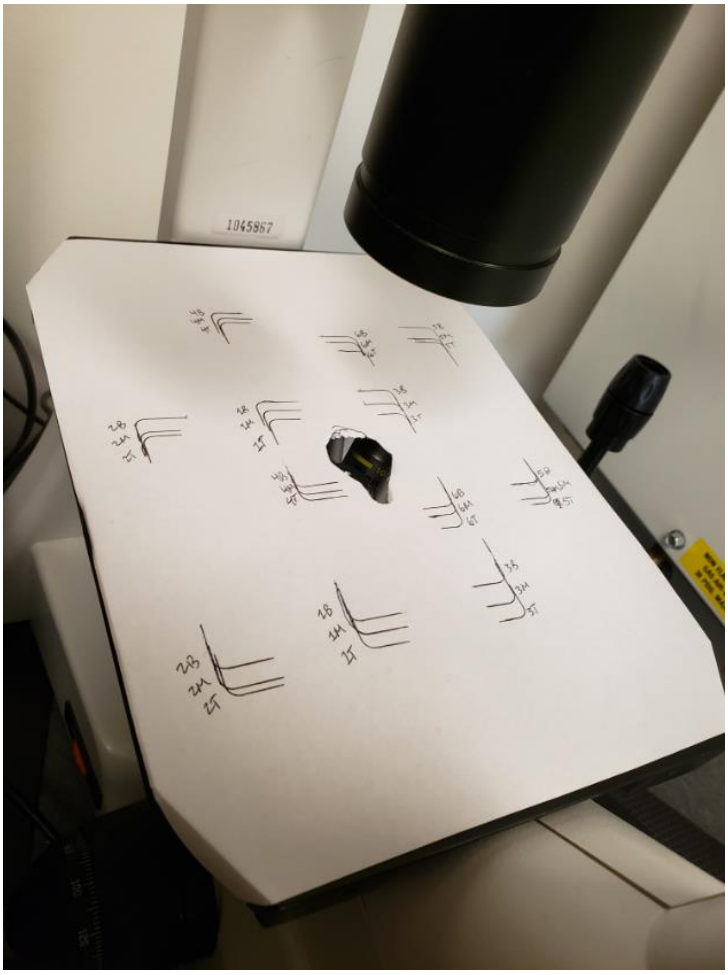


Figure 5. Use of a paper set-up to mark reference points for the scratch assay. The microscope stage was overlayed with a piece of blank paper. For each image taken, one upper and one lower corner of the plate were marked. Markings were used to align the plate for subsequent picture taking. New markings were created on a new sheet of paper for each experimental repeat.

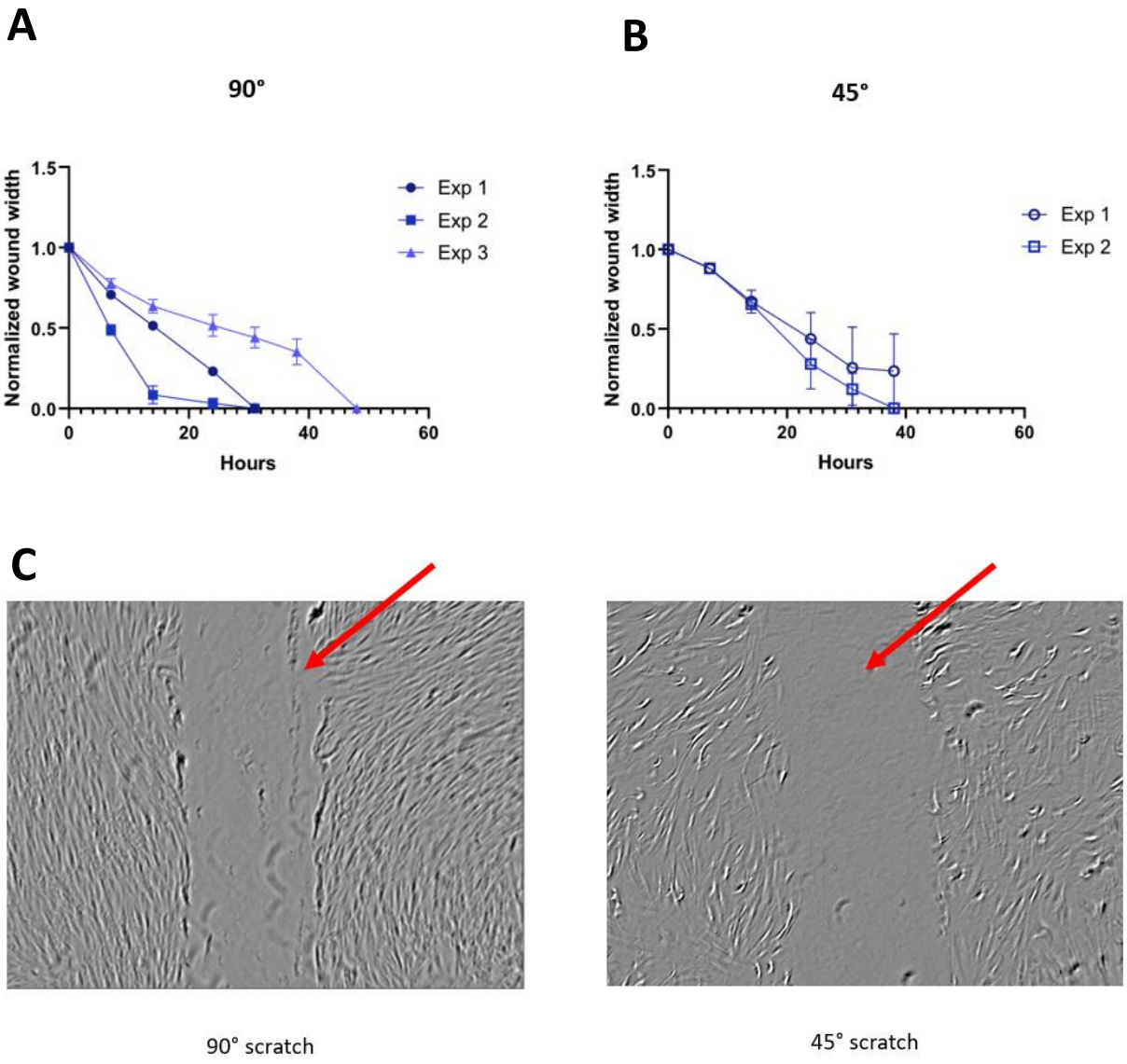


Figure 6. Modification of scratch angle from 90° to 45° leads to more reproducible migration rates in a single cell line. (A) Initial experimental repeats had inconsistent migration rates when scratching at a 90° angle. (B) Migration rates were more consistent when scratching at a 45° angle. (C) Scratching at 90° (left) produced more cell clumps within the wound area compared to scratching at 45° (right). Red arrows indicate presence of cell clumps when scratching at 90° and its absence when scratching at 45°.

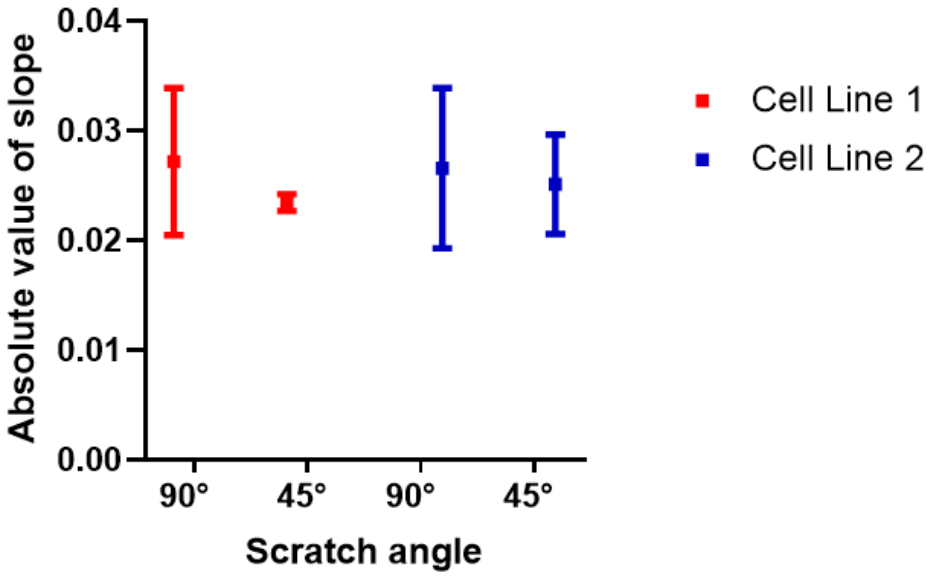
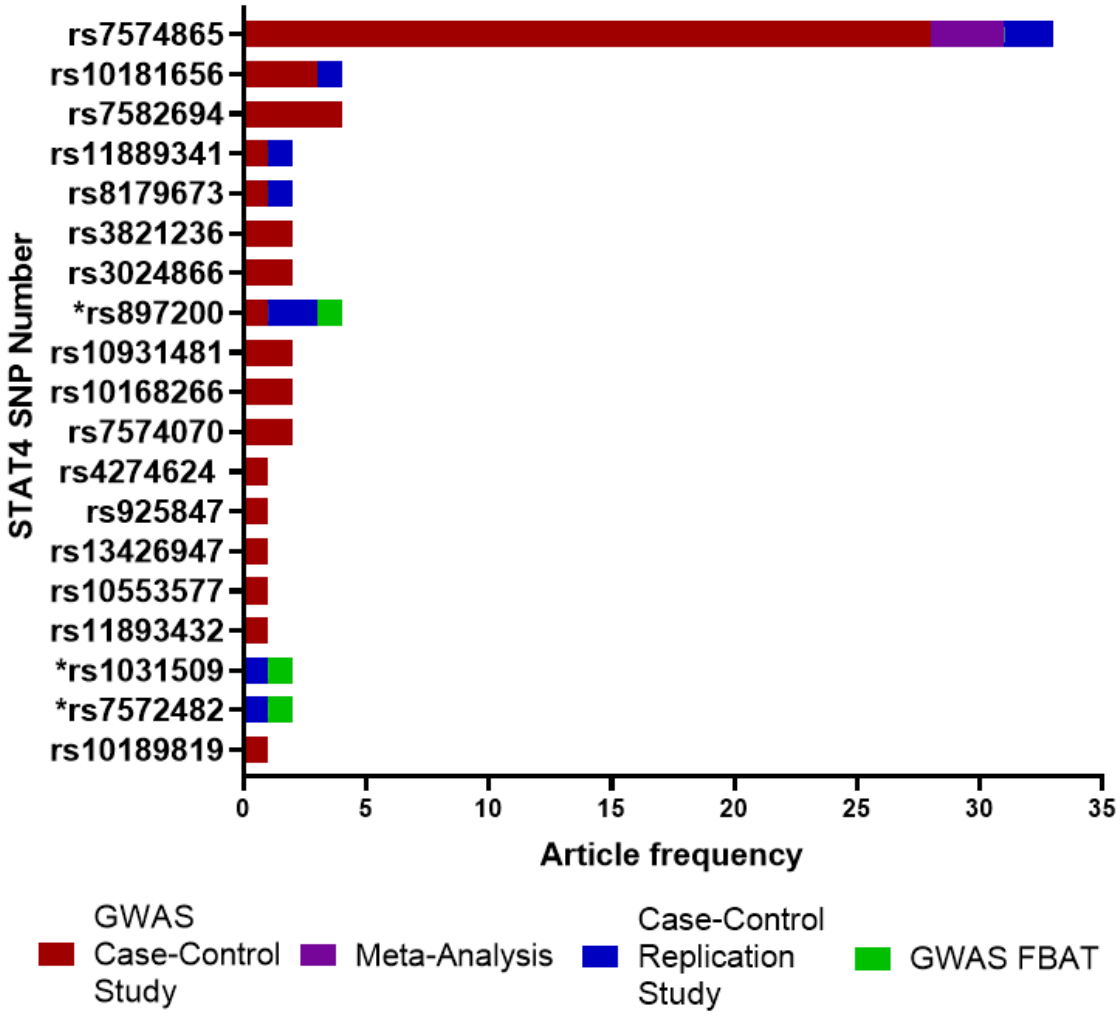


Figure 7. Tip angle modification reduced interexperiment variability in different cell lines. Normalized wound widths from 90° (n=3) and 45° (n=2) experiments were calculated at each scratch assay timepoint and plotted overtime. Magnitude of the slope of the resulting line was calculated by linear regression. Data are represented as means and standard deviation. Student's *t* test indicates no significant difference with *p* values > 0.05.

Supplementary Table 1. Sequence of primers to be used for RT-qPCR

Gene of interest	Primer sequence 5' → 3'
STAT4	TGGGTATGTCATGGGCTTTG
	TCACTTCCCCACTTTCAGAATG
IL6	CCACTCACCTCTTCAGAACG
	CATCTTTGGAAGGTTCAGGTTG
SOCS1	CTTCTGTAGGATGGTAGCACAC
	AGGAGGAAGAGGAGGAAGG
SOCS3	AGCCTATTACATCTACTCCGGG
	GCTGGGTGACTTTCTCATAGG
PIAS2	TGAAAGTATCCAGCCAACCG
	GGTCTTCCTCTTCGTCAGAAG
ACTA2	CCACAATGTCCCCATCTATGAG
	CGGACAATCTCACGCTCAG
FAP	TGTTTCGGTCCTGTCTATATGTG
	CCCATCCAGTTCTGCTTTCT
VIM	GTCAGCAATATGAAAGTGTGGC
	CTCAGTGGACTCCTGCTTTG
GAPDH	AATCCCATCACCATCTTCCAG
	AAATGAGCCCCAGCCTTC



Supplementary Figure 1. *STAT4* SNPs are largely reported by Genome-Wide Association Studies. The three SNPs marked with an asterisk were reported in the article by Sabri et al. in which a GWAS FBAT was conducted followed by a GWAS Case-Control study. All three SNPs were significantly associated with disease in GWAS FBAT. However, only rs7572482 and rs897200 were significant in the GWAS Case-Control study. SNP rs1031509 became significant only when FBAT and Case-Control data were combined under a recessive model. Abbreviations: GWAS: Genome-Wide Association Study; FBAT: Family Based Association Test

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