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THE ROLE OF MBTPS2 IN EPIDERMAL PROLIFERATION AND DIFFERENTIATION

by
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My journey to fulfill this thesis work arose from a strong belief in the well-known motto “When there is a will, there is a way”.

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AN ABSTRACT OF THE THESIS OF

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Ichthyosis ("fish scales") and ichthyosiform disorders are inherited skin disorders for which no ideal therapy exists. These disorders are mainly caused by disturbances in epidermal differentiation and can present either as isolated skin diseases or as part of syndromes. Clinically, there is a spectrum of phenotypic features ranging from mild skin dryness to complete disruption of the skin barrier resulting in severe complications and death at times.

Advances in molecular techniques in the past few years played a substantial role in the identification of genetic alterations involved in the pathogenesis of ichthyosis/ichthyosiform disorders. We have recruited multiple families with different types of ichthyosis/ichthyosiform disorders during the past four years. We identified several mutations in different genes related to these disorders. MBTPS2 is a gene mutated in ichthyosis follicularis with atrichia and photophobia (IFAP). The function of MBTPS2 in epidermal physiology is unknown.

IFAP syndrome is a severe form of syndromic ichthyosis, which is clinically characterized by nonscarring hair loss, hyperkeratotic psoriasiform plaques, follicular ichthyosis, photophobia and at times ectrodactyly.

Therefore, IFAP syndrome at the molecular level could have disturbances in epidermal proliferation and differentiation. Among the major pathways involved in proliferation is Tp63, which is also associated with syndromes involving ectrodactyly when mutated. On the other hand, NOTCH and the more recently investigated matriptase, are involved in skin differentiation.

In this study, we investigated whether and how MBTPS2 is involved in the processes of epidermal proliferation and differentiation under normal and disease processes.

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LIST OF ABBREVIATIONS

ARCI	Autosomal recessive congenital ichthyosis
ARIH	Autosomal recessive ichthyosis with hypotrichosis
DMEM	Dulbecco's Modified Eagle Medium
HEK	Human embryonic kidney cells
IFAH	Ichthyosis follicular atrophoderma, hypotrichosis, and hypohidrosis
IHC	Immunohistochemistry
IFAP	Ichthyosis follicularis, atrichia, and photophobia
IRF	Interferon-responsive genes
MBTPS2	Membrane-bound transcription factor site 2
NF-B	Nuclear factor-B
OL	Oligomerization domain
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
SAM	Sterile α motif
TA	Transactivation domain
TID	Transactivation inhibitory domain
Tp63	Tumor protein p63

CHAPTER I

INTRODUCTION

A. The human skin

1. Skin anatomy and physiology

The skin is the largest organ of the body that is responsible for protection against external pathogens, prevention of excess water loss and thermoregulation. Skin is composed of three different layers: epidermis, dermis and subcutaneous tissue (Figure 1) (Kanitakis, 2002).

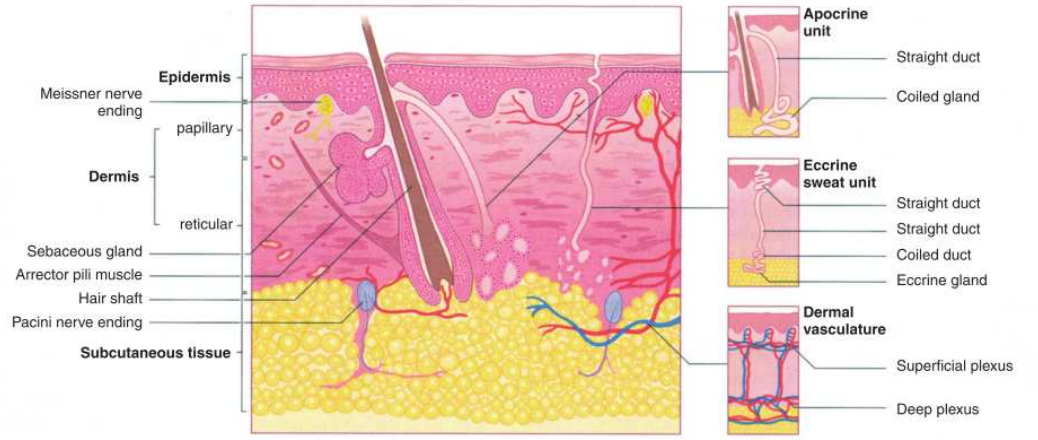

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Figure 1: Anatomy of normal human skin

2. Histology

The outermost layer, the epidermis, is a stratified squamous epithelium that contains two main cell types: keratinocytes and dendritic cells in addition to other types of cells including melanocytes, Langerhans cells, and Merkel cells (Kanitakis, 2002).

The epidermis is divided into four layers: basal cell layer (stratum germinativum), squamous cell layer (stratum spinosum), granular cell layer (stratum granulosum), and cornified cell layer (stratum corneum) (Murphy, 1997). The basal cell layer contains column-shaped keratinocytes that attach to the basement membrane and form a single layer. Cells of the basal layer are mitotically active and give rise to cells of the outer epidermal layers (Jones, 1996; Lavker & Sun, 1982). Overlaying the basal layer is the squamous cell layer, which is made up of different cells that range from polyhedral cells at the Suprabasal spinous cells to larger and flatter cells at the upper spinous layers as they are pushed toward the surface of the skin (Murphy, 1997; Chu, 2008). The granular layer is made up of flattened cells containing keratohyaline granules, the enzymatic activity of which produces the “soft keratin” (Chu, 2008). In contrast, absence of these granules in hair and nail produces the “hard keratin”. Lack of the granular layer leads to parakeratosis in which the keratinocytes nuclei are retained in the stratum corneum, resulting in psoriasis. The outermost layer, the cornified layer, consists of large, flat, polyhedral-shaped corneocytes that have lost their nuclei and are thus considered dead cells. This layer provides mechanical protection to the epidermis and prevents invasion by foreign substances (Jackson, Williams, Feingold, & Elias, 1993; Chu, 2008).

The epidermis is a continuously regenerating tissue; hence, it must maintain cells homoeostasis as well as regulate the interactions and junctions between epidermal cells. The regulation of epidermal proliferation and differentiation is regulated in part by the underlying dermis as well as the epidermal-dermal interface, which is formed by a porous basement membrane zone that allows the exchange of cells and fluid and holds the two layers together (James et al., 2006). Maintenance of epidermal thickness

depends on one of cells intrinsic properties known as apoptosis, which is regulated by a number of signaling molecules including hormones, growth factors, and cytokines (Haake & Hollbrook, 1999).

Epidermal Appendages include eccrine and apocrine glands, ducts, and pilosebaceous units that originate as downgrowths from the epidermis.

- Eccrine sweat glands are involved in the regulation of heat and are most abundant on the soles of the feet (Murphy, 1997; Sato & Dobson, 1970). The eccrine sweat gland is made up of three parts: the intraepidermal spiral duct, the straight dermal portion, and the coiled secretory duct (James et al., 2006; Mauro & Goldsmith, 2008).

- Apocrine Sweat Glands are involved in scent release (Murphy, 1997). Apocrine glands are present mainly in axillae and perineum and become active just before puberty.

- Apoeccrine Sweat Glands is found in the adult axillae and like eccrine gland, it opens directly to the skin surface. It is thought to contribute to axillary hyperhidrosis, a condition characterized by increased rates of perspiration (Mauro & Goldsmith, 2008).

- Hair Follicles provide protection and distribute sweat gland products. It originates from basophilic cells in the basal cell layer of the epidermis and grows at a downward angle into the dermis (Murphy, 1997). The hair follicle is composed of dermal papilla, hair matrix, root sheath (inner and outer sheath), bulge, arrector pili muscle, sebaceous glands containing abundant lipid droplets known as sebum (Figure 2). Hair grows in cycles of various phases: anagen is

the active growth stage, catagen is the involuting or regressing phase, and telogen is the resting phase (James et al., 2006).

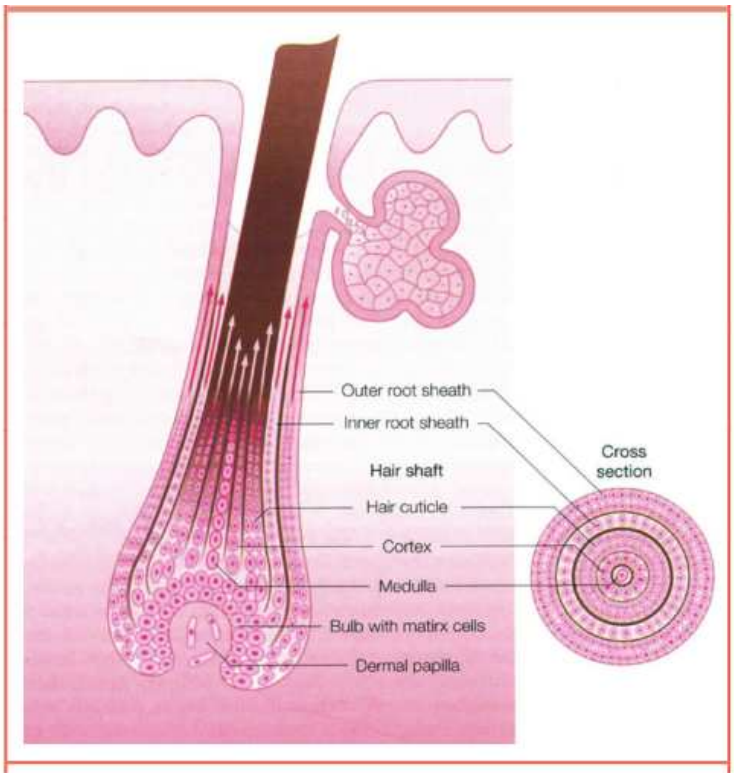


Figure 2: Hair follicle structure

The dermis is the bulk of the skin responsible for flexibility, elasticity and strength. It accommodates different types of cells that enter in response to various stimuli such as nerve and vascular networks. These cells include fibroblasts, macrophages, mast cells, lymphocytes, plasma cells, and other leukocytes. The matrix of this rich connective tissue is made up of collagen and elastic fibers. The main type of collagen present is collagen type I, which is responsible for stress resistance. On the other hand, elastic fibers are responsible for elasticity but do very little to resist shearing of the skin (James et al., 2006).

The subcutaneous tissue is considered an endocrine organ in which fat cells or lipocytes start to develop by the end of the fifth embryonic month. Given that, the subcutaneous tissue provides resilience to the body and serves as an energy storehouse (James et al., 2006).

3. Skin Development

Skin is made up of two major layers: the epidermis and the dermis. The epidermis is derived from the surface ectoderm while the dermis is derived from mesenchyme. Epidermal development begins by division of single layered ectodermal cells covering the embryos surface to a layer of simple squamous epithelial cells known as periderm. Cells in this layer are replaced by cells originating from basal layer through continuous keratinization and desquamation. This continues until week 21 after which the periderm disappears. The desquamated cells in addition to sebaceous gland secretions and hairs form the whitish protective substance that covers fetal skin at birth, which is known as vernix caseosa. By week 11, the basal layer or stratum germinativum forms an intermediate skin layer and by the end of 4^{th} month, the adult epidermis will be arranged into four successive layers as follows (from bottom to top):

- Basal layer: continuously produce new cells that replenish the above layers
- Spinous layer: made up of layer of polyhedral cells joined by fine fibrils
- Granular layer: made up of cells containing small keratohyaline granules
- Horney layer: upper most layer, loaded with keratin and forms the hard surface of the epidermis

Skin pigmentation is caused by melanin, a substance synthesized by melanocytes, which arise from the neural crest migration and invasion of the epidermis during the first 3 months of development to form melanoblasts then melanocytes.

As for the dermis development, during 3rd and 4th month collagen and elastic fibers are formed, in addition the corium, superficial dermal layers, forms dermal papillae that project into epidermis where some papillae contain small capillaries and others contain sensory nerve endings. On the other hand, the subcorium, deep dermal layer, contains mainly fat cells (Figure 3).

Abnormalities in any of these processes lead to impaired skin development associated with variety of conditions one of which is ichthyosis resulting from excessive cornification in superficial layers of the skin.

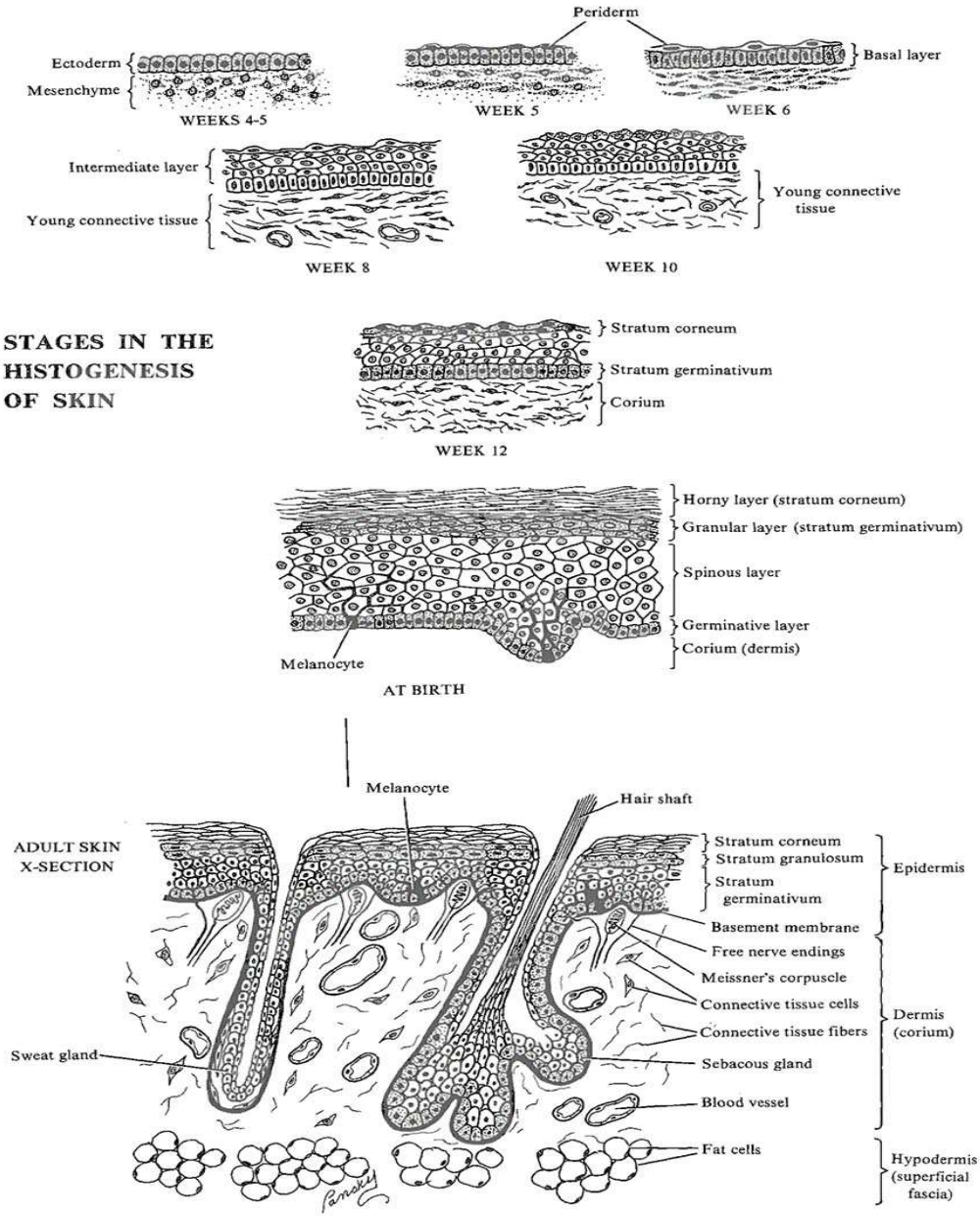


Figure 3: Skin Development

B. Ichthyosis/Ichthyosiform disorders

1. Definition

Ichthyosis is derived from the Greek root *ichthys*, meaning fish. It is a descriptive name for a group of inherited disorders of keratinization (cornification) in which the skin encase a large amounts of scales.

2. Etiology and prevalence

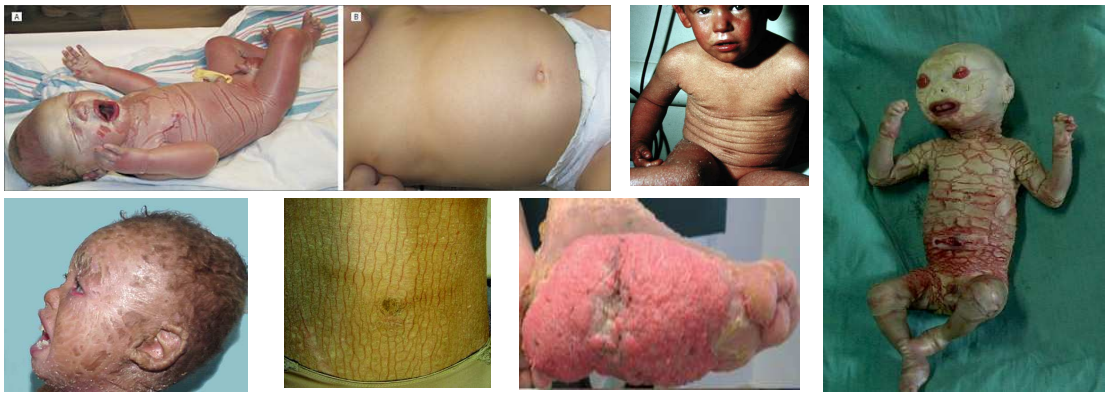
Ichthyosis can be hereditary or acquired. Hereditary forms can present at birth or occur later in life and they include syndromic and non-syndromic subtypes. Acquired ichthyosis usually occurs in adults and manifests as small, white, fishlike scales that are frequently concentrated on the extremities but may be seen in a generalized distribution. This form of ichthyosis may be associated with internal neoplasia (eg, Hodgkin lymphoma, leukemia), systemic illness (eg, sarcoidosis, HIV infection, hypothyroidism, chronic hepatitis, malabsorption), bone marrow transplantation, or the intake of certain medications that interfere with sterol synthesis in epidermal cells (eg, nicotinic acid).

In total, the incidence of the ichthyosis/ichthyosiform disorders is around 1/100,000 worldwide. Among the most encountered forms of ichthyosis in Lebanon is a subgroup referred to as autosomal recessive congenital ichthyosis (ARCI) (Table 1) with an incidence of 1:300,000. Most ARCI affected individuals are born with a colloidon membrane, an enclosing body case that is shed over several days. Some patients will go on to develop mild skin diseases while on the other end of the spectrum, some cases maybe fatal (harlequin ichthyosis) (Figure 3), indicating a genotype-phenotype correlation (Baden et al., 2013).

Table 1: The spectrum of autosomal recessive congenital ichthyosis can range from self-resolving ichthyosis to the lethal harlequin ichthyosis

	Disease	Characteristic Clinical Features		Mutated Gene	Protein	Function
HARLEQUIN	HARLEQUIN	MOST SEVERE	Premature birth, severe ectropion/ eclabium, thick armor-like skin with deep fissures.	ABCA12	ATP-binding cassette sub-family A member 12	ATP-binding cassette transporter; transport molecules across extra- and intracellular membranes
COLLOIDON	LAMELLAR ICHTHYOSIS (LI)		Ectropion/eclabium at birth. After shedding of collodion membrane skin develops large, brown, plate-like scale.	TGM1 in most; ABCA12 in some	Transglutaminase 1	Formation of cornified envelope
	INTERMEDIATE PHENOTYPES BETWEEN LI AND CIE *		Variable congential presentation (collodion or only erythroderma) and subsequent skin manifestations	CYP4F22	Cytochrome P450 family 4, subfamily F, polypeptide 22	A member of the cytochrome P450 family of monooxygenases
	CONGENITAL ICHTHYOSIFORM ERYTHRODERMA (CIE)*		After shedding of collodion membrane skin appears red (erythroderma) with fine, white scale.	ALOXE3 & ALOX12B in some	Arachidonate lipoxygenase 3 & arachidonate 12-lipoxygenase;	Lipoxygenases;
				NIPAL4 (ICHTYN) reported	Ichthyin	Mg(2+) and divalent cation transporter
				TGM1 reported	Transglutaminase 1	Formation of cornified envelope
	SELF HEALING COLLODION BABY	MILDEST	Erythroderma or collodion membrane at birth. Clears, often within weeks leaving minimal redness and scaling	ALOXE3; ALOX12B I	Arachidonate lipoxygenase 3 & arachidonate 12-lipoxygenase;	Lipoxygenases;
				TGM1	Transglutaminase 1	Formation of cornified envelope

Figure 4: The figure below demonstrates the wide variety in clinical presentations of patients with ichthyosis and ichthyosiform disorders ranging from mild to debilitating patterns



3. Classification

The approach to diagnostically focus down toward a specific type of ichthyosis involves consideration of a number of factors including: clinical dermatological features, age of onset, family history, and associated abnormalities.

Five distinct types of inherited ichthyosis are noted, as follows: ichthyosis vulgaris, lamellar ichthyosis, epidermolytic hyperkeratosis, congenital ichthyosiform erythroderma, and X-linked ichthyosis (Table 2).

Name	**Gene**	**Protein**
Ichthyosis vulgaris	*FLG*	Filaggrin
X-linked ichthyosis	*STS*	Steroid sulfatase
Congenital ichthyosiform erythroderma, Nonbullous (nbCIE)	*TGM1*, *ALOXE3*/*ALOX12B*	Transglutaminase 1 Arachidonate lipoxygenase 3 Arachidonate 12-lipoxygenase, 12R type
Epidermolytic hyperkeratosis (bullous ichthyosis, bCIE)	*KRT1*, *KRT10*	Keratins
Harlequin-type ichthyosis	*ABCA12*	ATP-binding cassette transporter 12
Ichthyosis bullosa of Siemens	*KRT2*	Keratin 2A
Ichthyosis hystrix, Curth-	*KRT1*	Keratin 1

Macklin type		
Hystrix-like ichthyosis with deafness	*GJB2*	Connexin-26 (Gap junction beta-2)
Lamellar ichthyosis, type 1	*TGM1*	Transglutaminase 1
Lamellar ichthyosis, type 2	*ABCA12*	ATP-binding cassette transporter 12
Lamellar ichthyosis, type 3	*CYP4F22*	Cytochrome P450, subfamily 4F, polypeptide 22
Lamellar ichthyosis, type 4	*LIPN*	Lipase family, member N
Lamellar ichthyosis, type 5	*ALOXE3*	Arachidonate lipoxygenase 3
Autosomal Recessive Congenital Ichthyosis	*CERS3*	ceramide synthase 3

Table 2: Genetics of Hereditary Ichthyosis

Multiple congenital ectodermal dysplastic syndromes are associated with scaling and other system defects (Table 3).

Name	Gene	Protein
CHILD Syndrome	*NSHDL*	NAD(P) dependent steroid dehydrogenase-

		like
Conradi-Hünermann syndrome	*EBP*	Emopamil binding protein
Ichthyosis follicularis with alopecia and photophobia syndrome	*MBTPS2*	Membrane-bound transcription factor peptidase, site 2
Keratitis-ichthyosis-deafness syndrome	*GJB2*	Connexin-26
Netherton syndrome	*SPINK5*	Serine peptidase inhibitor, Kazal type 5
Sjögren-Larsson syndrome	*ALDH3A2*	Fatty acid dehydrogenase

Table 3: Genetic diseases with Ichthyosis

4. Pathology

Pathologically, ichthyosis is a disease of inappropriate epidermal differentiation. It occurs secondary to a decreased rate of shedding of the stratum corneum, upper epidermal layer, which results in a very thick yet a dysfunctional skin barrier (Wajid et al., 2010). The thick layers that develop completely disrupt the skin barrier function leading to severe hypernatremic dehydration secondary to water loss from evaporation. Moreover, the thick keratotic skin disrupts the normal microbiome of the skin leading to recurrent bacterial and fungal infections.

One form of ichthyosis is IFAP syndrome, which is an X-linked genodermatoses characterized by a triad of ichthyosis follicularis, atrichia, and photophobia (Oeffner et al., 2011; Fong et al., 2015). Histopathologically, the epidermal

granular layer is generally well-preserved or thickened at the infundibulum. Hair follicles are poorly developed and usually surrounded by an inflammatory infiltrate. Given the X-liked mode of inheritance, males are mostly affected and have the IFAP triad of follicular ichthyosis, atrichia of the scalp, and photophobia from birth. Carrier females may present some clinical features including asymmetric distribution of body hair, patchy alopecia, and linear lesions of follicular ichthyosis following the lines of Blaschko (Oeffner et al., 2009). IFAP can occur with or without the BRESEK/BRESHECK syndrome, a multiple congenital malformations characterized by brain anomalies, intellectual disability, ectodermal dysplasia, skeletal deformities, ear or eye anomalies, and renal anomalies, with or without Hirschsprung disease and cleft palate or cryptorchidism.

5. Diagnosis and treatment

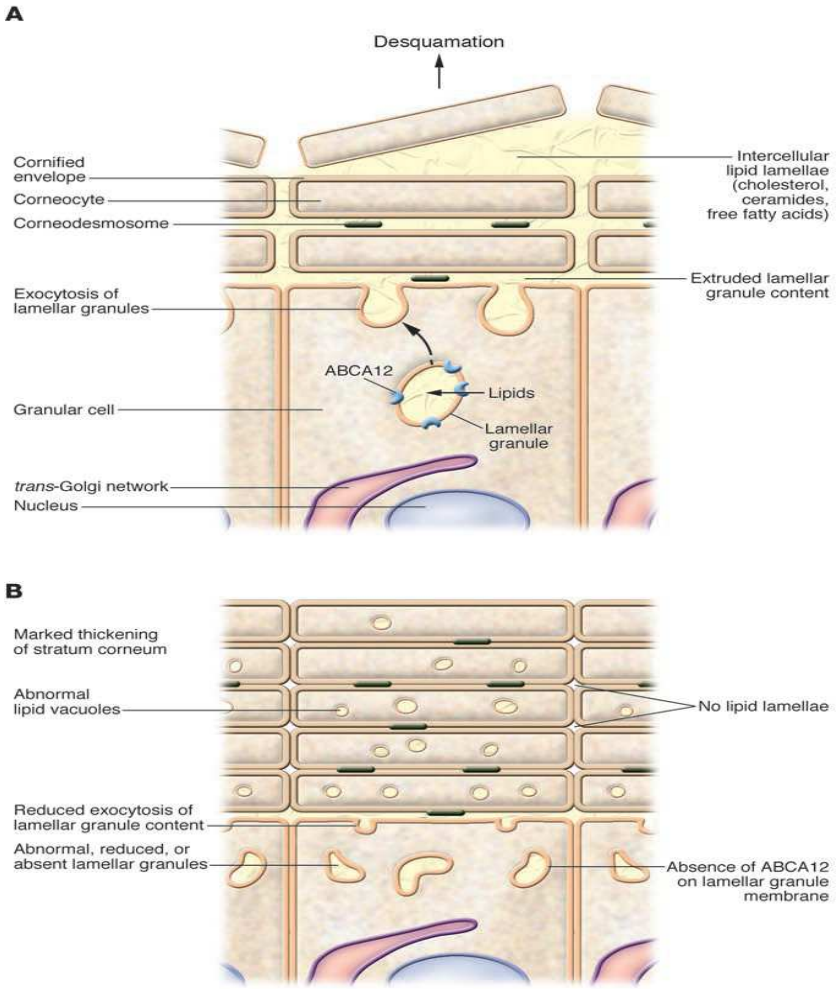
Diagnosis is based on patient's medical history and physical exam. Because there is no cure for ichthyosis, treatment is targeted at managing the signs and symptoms. Treatment may include creams, lotions, or ointments to relieve dryness. Lengthy bathing in salt water or preparations containing salicylic acid (aspirin) or urea may also ease scaling. For more severe cases, vitamin A derivatives called retinoids may be prescribed.

C. Players in skin development

The process of differentiation in the skin relies on the normal production of lipids, mainly cholesterol (Feingold et al., 2014). Disturbances in the cholesterol production/transport /delivery/function/modifications/metabolism are highly involved in the pathogenesis of skin differentiation leading to ichthyosis/ichthyosiform disorders

(Figure 4). Additionally, many metabolites accumulating in the cholesterol synthesis pathway have been shown to be highly toxic and associated with several forms of the ichthyosis/ichthyosiform disorders (Seeger et al., 2014). Interestingly, several genes underlying a big subset of ichthyosis/ichthyosiform disorders have been identified over the past several years and these include Filaggrin (FLG), ATP-binding cassette transporter 12 (ABCA12), Membrane bound transcription factor peptidase site 2 (MBTPS2), Steroid sulfatase (STS), and Keratin 1 (KRT1) (Rinne et al., 2007; Baden et al., 2013). As expected, a large number of these genes are implicated in the pathway related to formation of cholesterol either directly or indirectly. Identification of such genes will pave the way for targeted therapies that will hopefully provide better clinical results with fewer side effects.

Figure 5: The normal differentiation of the skin requires appropriate levels of lipids and mainly cholesterol (A). The absence of cholesterol leads to overstacking of corneocytes and disrupted epidermal desquamation (B).



1. MBTPS2

MBTPS2 is a zinc metalloprotease essential for cholesterol homeostasis and endoplasmic reticulum stress response (Rawson, 2013). In normal skin, MBTPS2 is mainly expressed in the upper granular layer where transition from proliferation to differentiation usually takes place. Mutations in MBTPS2 lead to a severe ichthyosiform syndrome known as IFAP disease, an X-linked genodermatoses characterized by a triad of ichthyosis follicularis, atrichia, and photophobia (Oeffner et al., 2011; Fong et al., 2015). This occurs due to deficiency in function of either sterol or ER homeostasis that lead to disturbed differentiation of epidermal structures evoking the triad of IFAP phenotype (Wang et al., 2014; Mégarbané et al., 2011). Interestingly, IFAP disease has features of both ichthyosis (differentiation abnormalities) and over proliferation suggesting a role of MBTPS2 in both differentiation and proliferation.

2. Tp63

Different genes regulate differentiation and proliferation processes among which, Tumor protein p63 (TP63) is a main factor involved in proliferation, especially in basal epidermal layer. TP63 is a transcription factor belonging to the p53 gene family. TP63 encodes two protein isoforms encoded by two different promoters, one having an amino terminus-transactivating domain (TAp63) and the other lacking this domain (ΔNp63). TAp63 and ΔNp63 further undergo alternative splicing of the C-termini that generates α, β, and γ isotypes (Kanitakis et al., 2007; Jorge et al., 2002). Structurally, TP63 consists of transactivation domain (TA), DNA-binding domain and oligomerization domain (OL). The alpha isoforms contain, in addition, a sterile α motif (SAM) domain and a transactivation inhibitory domain (TID) at their C-termini. In skin,

ΔNp63α is the main isoform responsible for the action of TP63. Data from TP63 null mice shows that p63 is important for skin, limb and craniofacial development. These mice display single layered and translucent skin and die shortly after birth from dehydration due to lack of epidermal barrier (Chikh et al., 2007). TP63 exerts its function by transcriptionally activating or repressing various downstream targets. Many of these targets are involved in regulation of skin development and function such as initiating epithelial stratification, maintaining stratified epithelial stem cells, cell cycle regulation, epidermal lineage commitment, Keratinocyte adhesion, basement membrane formation, epidermal differentiation, barrier formation and appendage development, thus explaining the fact that Tp63 is often mutated in several genetic skin diseases (Koster MI., 2010; Ratovitski, 2013).

In normal skin, Tp63 is expressed in the basal and suprabasal epidermal keratinocytes and not expressed in the non-epithelial cells of dermis (Jorge et al., 2002). TP63, being a master gene in regulating genetic and epigenetic skin specific genes, interacts with several proteins and is subject to control by various factors, of particular interest NOTCH due to its role in differentiation.

3. NOTCH

NOTCH is a cell surface receptor that includes four genes, Notch1, Notch2, Notch3 and Notch4, functioning in a cell and context-specific manner. Notch signaling promotes commitment of keratinocytes to differentiation and suppresses tumorigenesis (Rangarajan et al. 2001, Sasaki et al., 2002). In mouse and human epidermis, Jagged 1/2 (encoding ligands for the Notch receptors), Notch1, and Notch2 are coexpressed in the supra-basal layers of differentiating keratinocytes. Mutations in Notch genes have been

associated with abnormalities in organ development and adult homeostasis (Wilson et al., 2006). Studies have shown that Notch promotes terminal differentiation through induction of keratin 1, involucrin and the cell-cycle inhibitor Waf1 and inhibiting the onset of fillagrin and loricrin expression until later stages of differentiation (Figure 6) (Nickoloff et al. 2002; Rangarajan et al. 2001). Moreover, Notch suppresses Tp63 in the upper epidermal layers through dwonregulation of interferon-responsive genes, including IRF7 and/or IRF3 and modulation of nuclear factor-B (NF-B) signaling. On the other hand, Tp63 counteracts the effect of Notch signaling in epidermal cells with high self-renewal potential (Nguyen et al., 2006). In parallel with these antagonizing effects, TP63 and NOTCH can also synergize in the early stages of differentiation through Tp63 mediated induction of Notch ligands, JAG1 and JAG2. The resulting increase in NOTCH signaling will then lead to downregulation of Tp63 in later stages of differentiation (Sasaki et al., 2002; Dotto GP., 2009). Altogether, these data suggest possible interactions or common signaling pathways between the different genes.

4. Keratin 1 and loricrin

Keratins are intermediate filaments expressed specifically by epithelia (Moll et al., 1982). They are subdivided into two subtypes: type I and type II keratins. Type I keratins are acidic and have low molecular weight, whereas type II keratins are basic or neutral and have high molecular weight. Keratins play a major role in maintaining the structural integrity and mechanical stability of cells as single-point mutations in keratin genes have been shown to be associated with keratinopathies. Examples of such skin disorders include epidermolysis bullosa simplex (mutations in keratin 5/14 genes), epidermolytic hyperkeratosis (mutations in keratin 1/10 genes) and epidermolytic

palmoplantar keratoderma (mutations in the keratin 9 gene) (Coulombe and Omary, 2002; Lane and McLean, 2004; McLean and Irvine, 2007; Uitto et al., 2007).

The keratin pair 1/10 belongs to type II and type I keratins respectively and is normally expressed by the suprabasal layers of keratinizing stratified epithelia (Moll et al., 1982). The process of skin development involves a switch from proliferation in the basal layers to differentiation in upper epidermal layers during which the expression of keratins changes from basal cell keratins, K5, K14 and K15 to the suprabasal epidermal keratins, K1 and keratin K10 (Moll et al., 2008). Hence, this pair is recognized as a marker of cellular differentiation.

Another marker of differentiation is Loricrin, a glycine-serine-cysteine rich protein, which is a major component of the cornified cell envelope compromising up to 80% of its proteins. Loricrin is synthesized in the granular layer and then migrates to cell periphery during terminal differentiation of keratinocytes where it deposits beneath the plasma membrane forming the cornified envelop, which provides protection against external injury. Mutations in the human loricrin gene have been associated with a dominantly inherited hyperkeratotic skin disorder known as loricrin keratoderma or Vohwinkel syndrome with ichthyosis (Schmuth et al., 2004).

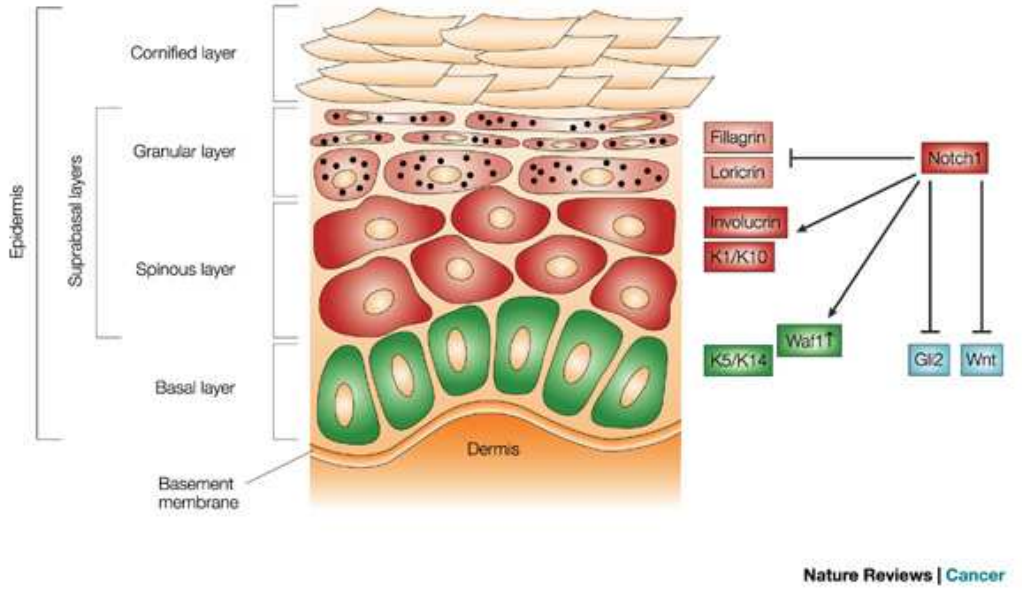


Figure 6: Representation of mammalian skin with several layers of differentiating cells, and some proteins that are expressed in specific cellular layers

5. Matriptase

In addition to MBTPS2, Matripase is another protease involved in skin development. Matriptase is a transmembrane serine protease that can undergo efficient autoactivation, and therefore has the capacity to initiate proteolytic cascade reactions. Studies have shown that human matriptase is highly expressed by the keratinocytes in the basal and spinous layers of the epidermis, but not in the granular layer. Moreover, studying the expression of matriptase during epidermal proliferation and differentiation suggested that human matriptase is likely to be involved in the regulation of keratinocytes proliferation and early differentiation.

Recently, mutations in matriptase have been associated with two congenital skin disorders, autosomal recessive ichthyosis with hypotrichosis (ARIH) and ichthyosis

follicular atrophoderma, hypotrichosis, and hypohidrosis (IFAH), which as the majority of congenital ichthyosis are characterized by defects in skin barrier. Epidermal development starts with the emergence of epidermal keratinocytes from proliferating basal cells followed by outwards differentiation to form the stratum corneum. Epidermal differentiation culminates by shedding (desquamation) of the outermost layer of corneocytes, which is accomplished through the physical degradation of adhesions between corneocytes (corneodesmosomes) in a regulated proteolytic process mediated by kallikrein serine proteases. However, dysregulation of these proteolytic procsses can lead to serious pathological consequences, a notable example of which is Netherton syndrome, a congenital disorder characterized by excessive proteolytic degradation of corneodesmosomes and cadherins, which leads to premature stratum corneum separation. Mutations in SPINK5 gene that encodes the lympho-epithelial Kazal-type-related inhibitor (LEKTI), a serine protease inhibitor that inhibits kallikreins, are associated with Netherton syndrome. Recent studies have shown that matriptase has an essential role in the premature desquamation by causing premature activation of epidermal pro-kallikreins. Furthermore, matriptase expression in Netherton syndrome changes to the suprabasal epidermis with the highest level of expression in the granular and transitional cell layers where it co-localizes with LEKTI (Ya-Wen Chen et al., 2014; Katiuchia Uzzun Sales et al., 2010).

D. Aim of the study

Ichthyosis and ichthyosiform disorders are rare genetic disorders for which no convincing therapy exists. These disorders are caused mainly by disturbances related to the differentiation of the epidermis. Our progressive understanding of the molecular and

signaling pathways involved in the pathogenesis of these diseases will contribute to the development of targeted therapies. The role of MBTPS2 in proliferation and differentiation and possible interaction with Tp63, NOTCH and matriptase have not been well investigated, yet mutations in MBTPS2 are associated with severe phenotypic features combining both proliferation and differentiation abnormalities. In our work here, we aimed to study the role of Membrane bound transcription factor peptidase site 2 (MBTPS2) in skin development, maintenance and interaction with other major players in skin formation.

CHAPTER II

MATERIALS AND METHODS

A. Cell lines and Cell cultures

HEK-293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Mediatech Inc., Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gemini, West Sacramento, CA), 1% penicillin streptomycin and 1% sodium pyruvate. Cells were incubated in a humidified incubator at 37° with 5% CO2.

B. Immunohistochemistry (IHC) staining

Tissue sections of paraffin-embedded normal and diseased human skin were deparaffinized in xylene and rehydrated through a graded series of decreasing ethanol concentrations. The slides were washed twice with PBS buffered solution. Heat mediated antigen retrieval with citrate buffer pH 6 was performed before commencing with IHC staining protocol. After antigen retrieval, the slides were blocked with 5% BSA for 1 h at room temperature. Slides were incubated with primary antibodies (Tp63, MBTPS2 and matriptase mAbs) overnight at 4^0C, and then washed in PBT three times. Fluorophore-conjugated secondary antibody (Rabbit Alexa 488) was used for the detection of positive staining. Cell nuclei were stained with DAPI. Negative controls were performed using PBS in place of the primary antibodies. Images were captured using a conventional fluorescent microscope. Exactly same exposure time was used for

the photographs of control and positive studies. Table 2 shows all antibodies, dilutions, and incubation times.

Antibody	Target	Source	Dilution	Incubation time
H-137	P63	Santa Cruz Biotechnology	1:75	Overnight at 4^0C
Anti-S2P	MBTPS2	Abcam	1:10	Overnight at 4^0C
Anti-Matriptase 2	Matriptase	Abcam	1:20	Overnight at 4^0C
Cyotokeratin 1 (E-12)	Keratin 1	Santa Cruz Biotechnology	1:20	Overnight at 4^0C
Loricrin (W-22)	Loricrin	Santa Cruz Biotechnology	1:20	Overnight at 4^0C

Table 4: Antibodies for IHC

C. Transformation of constructs into bacteria

The previously obtained constructs are then transformed into E.coli, XL1 blue strain bacteria initially stored at -80^0C. 1µg of the plasmid containing our DNA constructs is added to 100µL of bacteria. The mixture is placed 2 minutes on ice, 5 minutes at 37^OC, then 2 minutes on ice. The transformed bacteria is streaked on kanamycin selective agar plate, and then incubated at 37^0C overnight. Bacterial colonies incorporating the desired plasmid will grow on the plate. Bacterial colonies are picked with pipette tips, and transferred into 15-mL falcon tubes containing 3mL liquid broth and 3µl kanamycin. The tubes are incubated with shaking overnight at 37^0C, 200 rpm. Miniprep and maxiprep are performed using illustraTM plasmidPrep Midi Flow Kit by GE Healthcare according to the enclosed manufacturer's protocol.

D. Polyethyleneimine (PEI) transfection

HEK-293T cells are plated in 12-well Costar culture plates 24 hours prior to transfection with promoter and expression vectors (MBTPS2 wild type and mutant, p63, and NOTCH). The cells are transfected using the Polyethyleneimine (PEI) LIX transfecting reagent. 5µg of DNA per well is added to 350ul serum free media, then 10µl PEI is added to the mixture. The mixture is incubated for 20 minutes at room temperature. The mixture is then added drop by drop over the culture milieu. Media is changed one to two hours prior to transfection and three hours post transfection. After 24 hours, cell lysates were prepared and analyzed with a dual luciferase reporter assay system.

E. Luciferase Reporter assay

HEK-293T cells were transfected with promoter as well as MBTPS2 wild type and mutant, p63, and NOTCH using the PEI method described earlier. Controls were transfected with promoter/Luc only. The luciferase activity is measured 24 hours post transfection. The transfected cells are first rinsed with 1xPBS then lyzed with 1x 40 lysis buffer and left on the shaker for 10 minutes at room temperature. The cell lysate is then transferred into a 96 well plate (Costar) to which luciferin is added. Luciferin (Promega, Cat # E 1501) is prepared according to the manufacturer's protocol. The signal is read immediately using the Ascent Fluoroscan. The results are expressed as fold activation calculated by comparison with that of the promoter alone and the presented values are the mean +/- standard deviation of three independent experiments carried out in duplicates.

F. Nuclear Protein Extraction

Nuclear protein extracts from HEK293T cells were obtained according to the following protocol. The cells were first washed with 1X PBS. Then 2mL of trypsin-Ethylenediaminetetraacetic acid (EDTA) was added to the petri dishes to detach the cells. The petri dishes were then placed on the shaker for 20 minutes, to allow the detachment of the cells, and then the cells are harvested in eppendorf tubes and centrifuged for 90 seconds at 11000rpm. The supernatant is discarded, and the pellet is resuspended in 5x lamelli buffer then boiled for 3min. Protein concentration was measured at 280nm using nanodrop.

G. SDS-PAGE and Western blotting

Equal amounts of protein were resuspended in 5x lamelli buffer. The samples were boiled for 3min, loaded and electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE. The proteins were transferred to a Polyvinylidenedifluoride membrane (PVDF) membrane (Amersham), blocked with 5% fat free milk solution for one hour with shaking at room temperature. The membrane was then incubated overnight at 4^0C with the specific primary antibody diluted 1:200 in 1 % fat free milk. The membrane was then washed three times, 5 minutes each, with PBT (PBS, 0.05% Tween 20) and incubated with the corresponding secondary antibody at a dilution of 1:5000 for 2 hours at room temperature. Membranes were developed by autoradiography and ChemiDoc (Biorad).

CHAPTER III

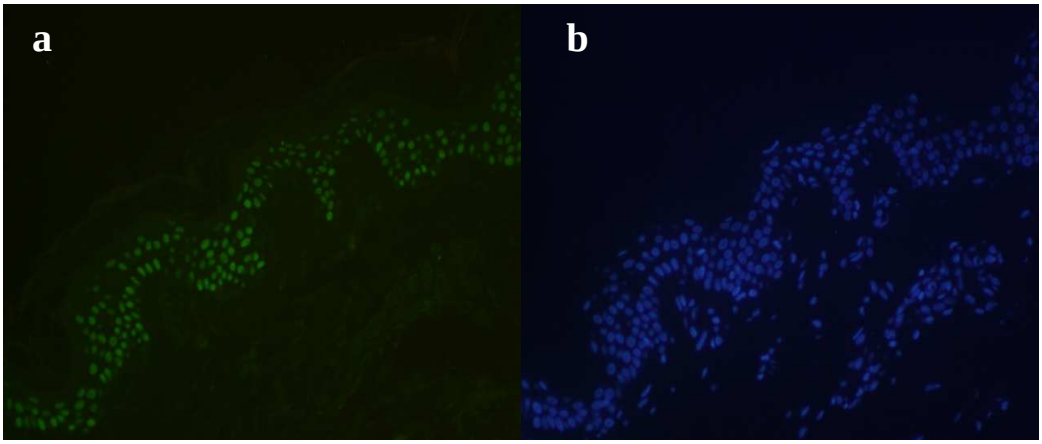
RESULTS

A. Tissue localization of Tp63, MBTPS2, matriptase, keratin 1 and loricrin

In order to detect Tp63, MBTPS2, matriptase, keratin 1 and loricrin expression in normal and IFAP skin, immunohistochemistry was performed. Paraffin embedded tissues were incubated with the specific primary antibody followed by the suitable secondary antibody and then visualized by fluorescence microscope.

1. TP63 expression in normal and IFAP skin

Normal



IFAP

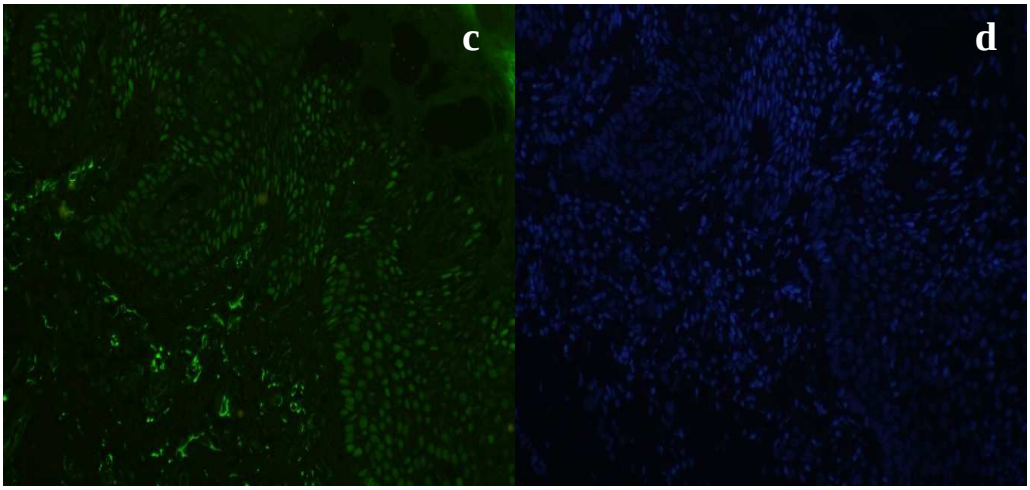
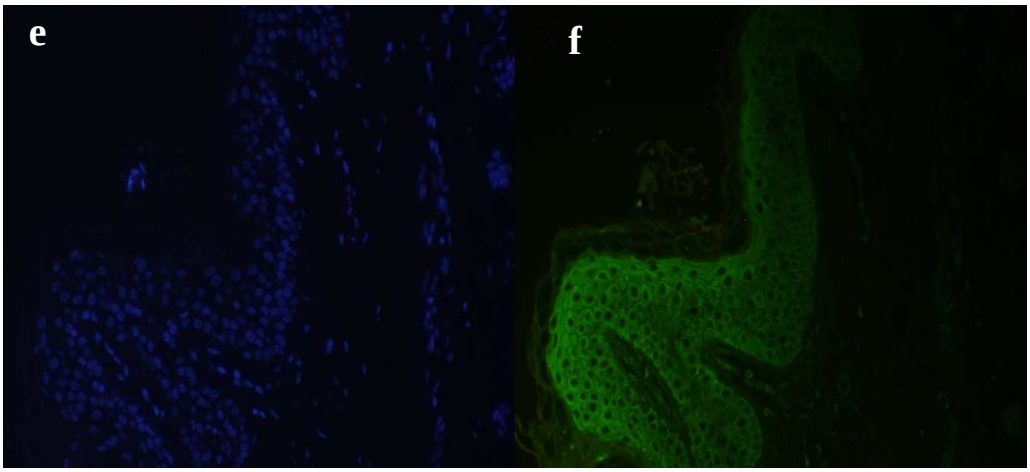


Figure 7: Tp63 expression in normal and IFAP skin using immunohistochemistry
TP63 (a, c) was found to be expressed in the nuclei of epidermal basal cells with more spread expression in IFAP (c) as compared to DAPI stain (b, d). The localization of Tp63 was visualized using anti-rabbit antibody (green color). Nuclei of the cells were visualized using DAPI dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

2. MBTPS2 expression in normal and IFAP skin

Normal



IFAP

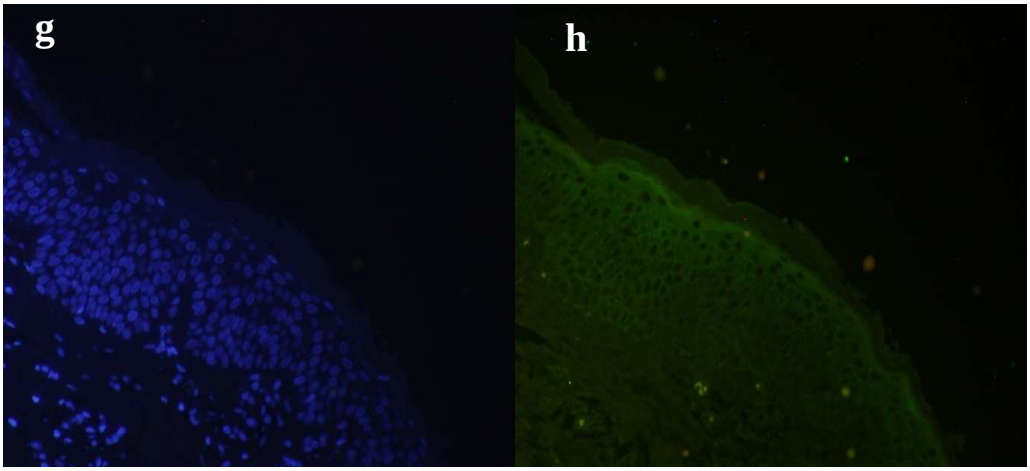
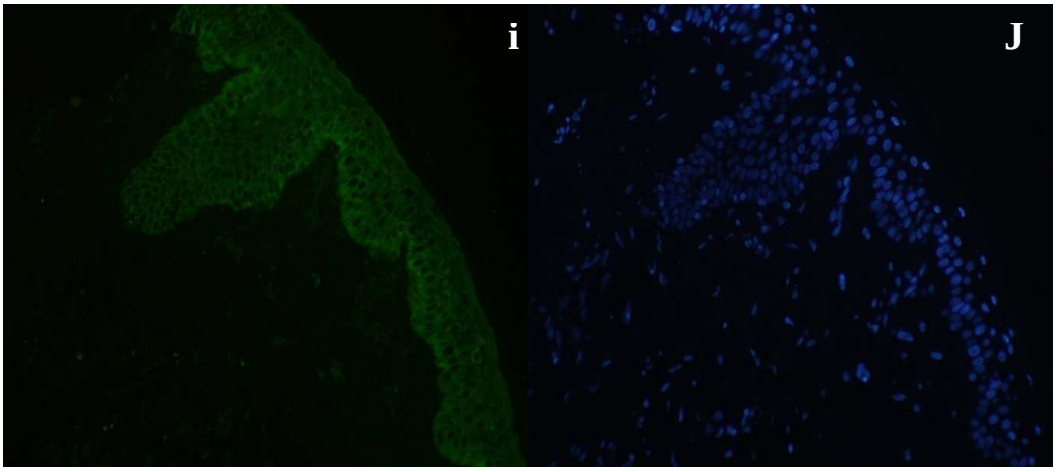


Figure 8: MBTPS2 expression in normal and IFAP skin using immunohistochemistry MBTPS2 (f, h) was found to be expressed in the cytoplasm of epidermal granular layer cells with decreased expression in IFAP (h) as compared to DAPI stain (e, g). The localization of MBTPS2 was visualized using anti-rabbit antibody (green color). Nuclei of the cells were visualized using DAPI dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

3. Matriptase expression in normal and IFAP skin

Normal



IFAP

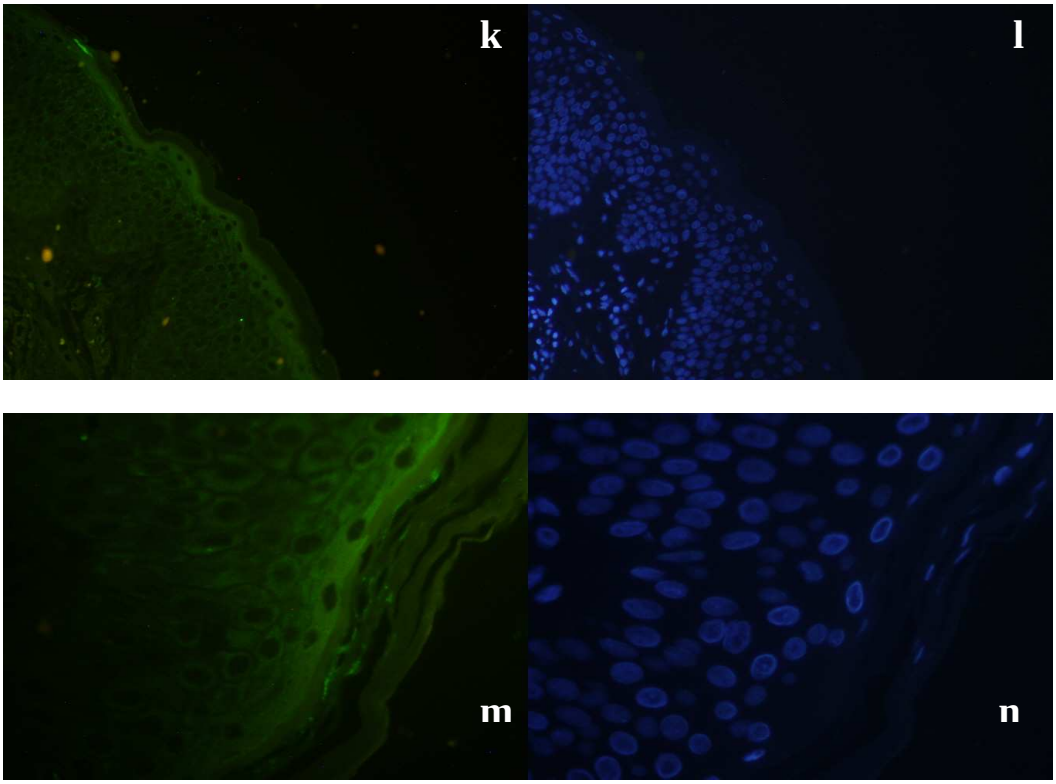


Figure 9: Matriptase expression in normal and IFAP skin using immunohistochemistry Matriptase (i, k, m) was found to be expressed in the cytoplasm of epidermal basal cells in normal skin (i), whereas in cytoplasm of upper granular layer cells in IFAP (k, m) as compared to DAPI stain (j, l, n). The localization of Matriptase was visualized using anti-rabbit antibody (green color). Nuclei of the cells were visualized using DAPI dye (blue color). Images were taken by a fluorescence microscope and captured at x20. Images m and n were captured at x40.

4. Keratin 1 expression in normal and IFAP skin

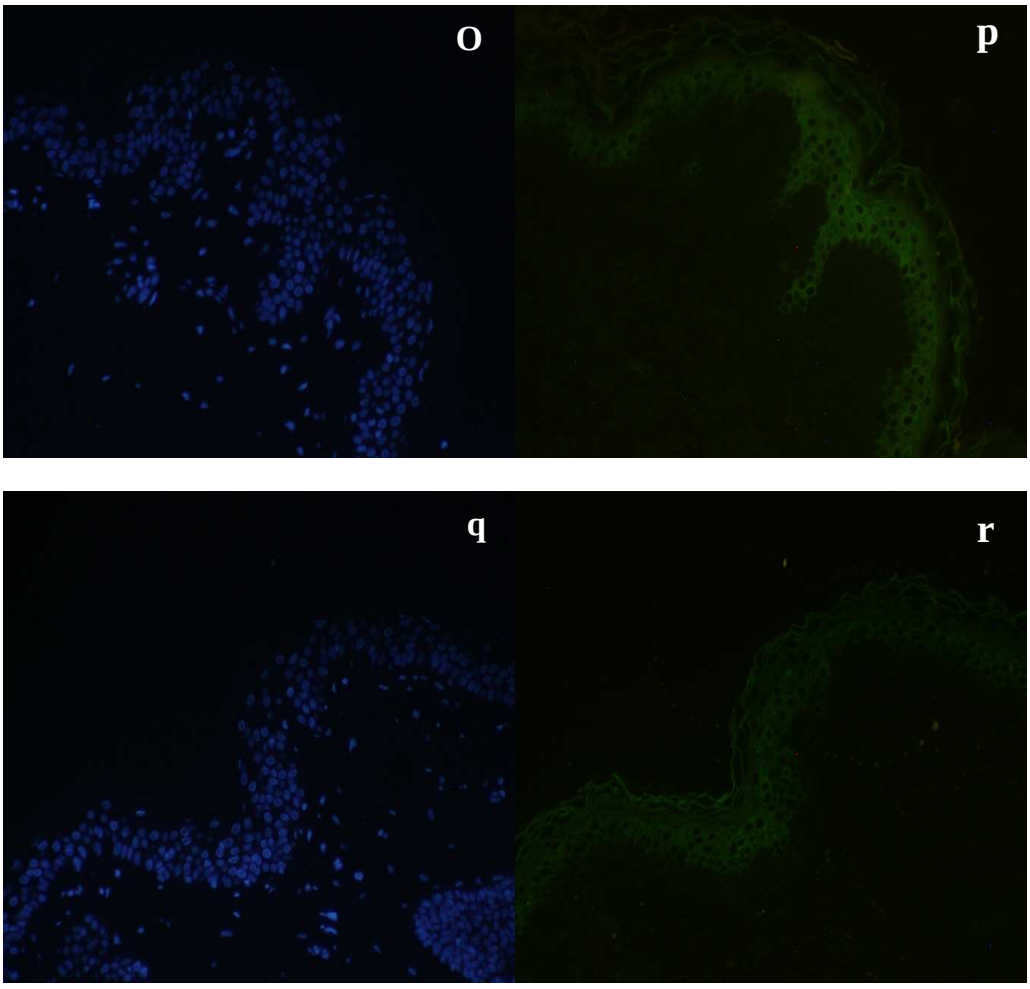


Figure 10: keratin 1 expression in normal and IFAP skin using immunohistochemistry keratin 1 (p, r) was found to be expressed in the cytoplasm of epidermal suprabasal cells in normal skin (p) and IFAP (r) as compared to DAPI stain (O, q). The localization of keratin 1 was visualized using anti-mouse antibody (green color). Nuclei of the cells were visualized using DAPI dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

5. Loricrin expression in normal and IFAP skin

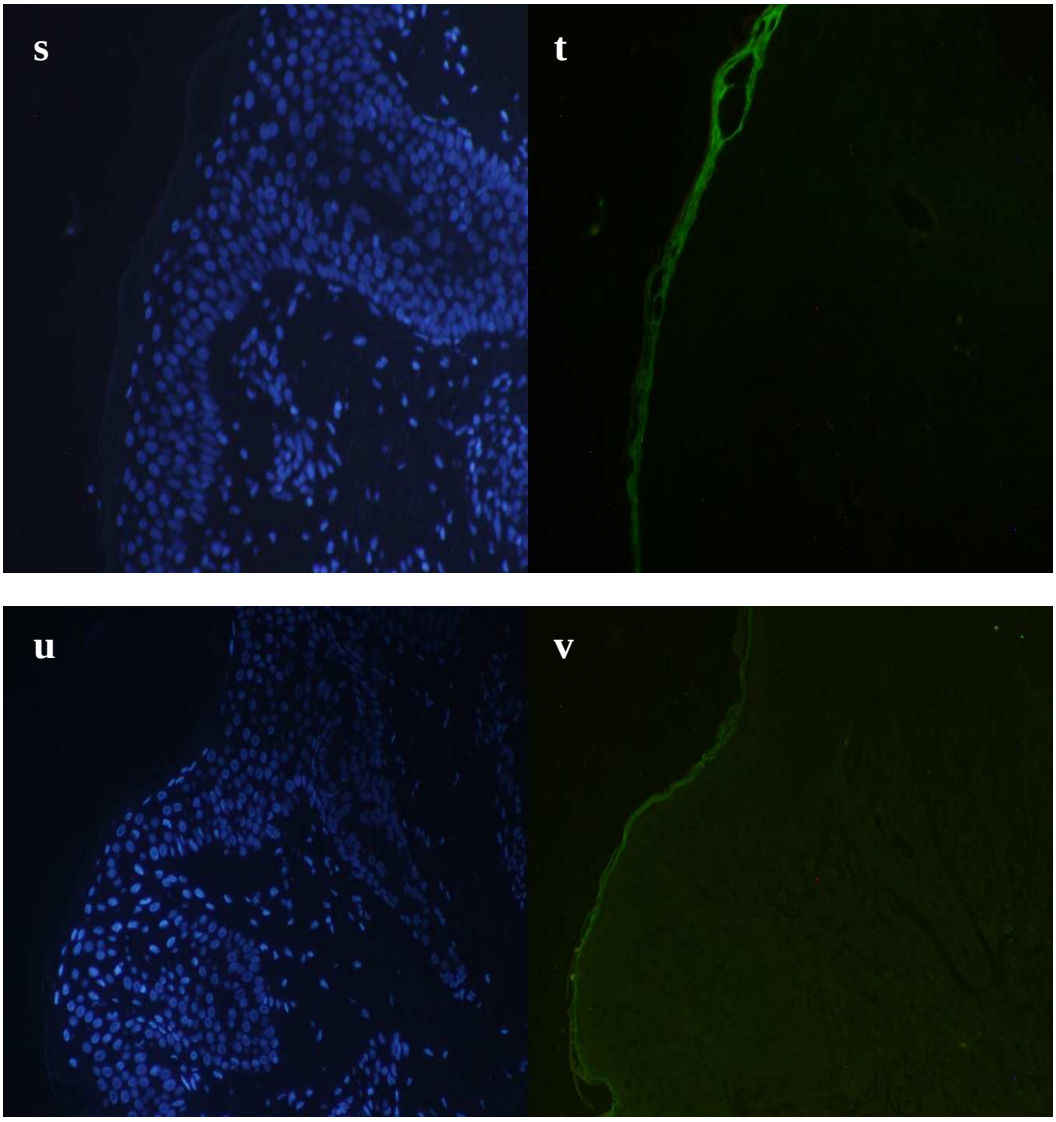


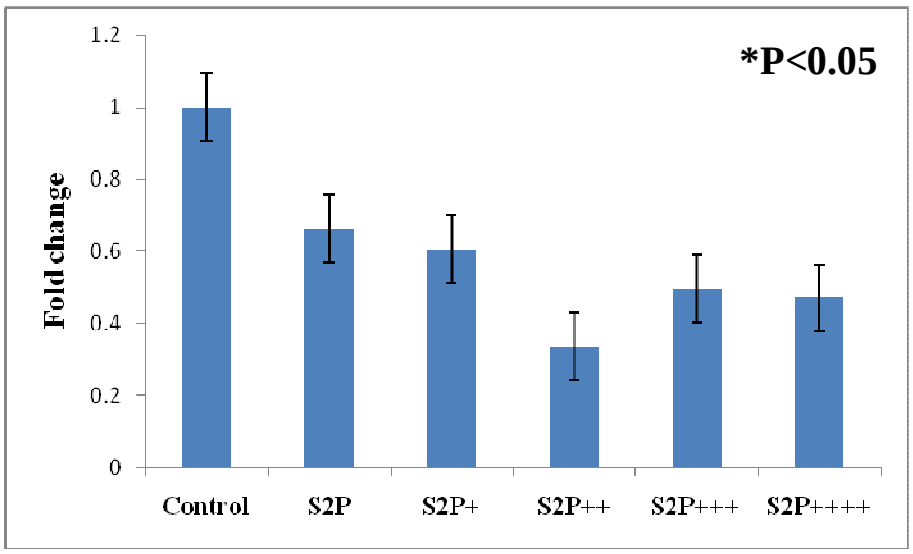
Figure 11: loricrin expression in normal and IFAP skin using immunohistochemistry loricrin (t, v) was found to be expressed in the cornified envelop in normal skin (t) and IFAP (v) as compared to DAPI stain (s, u). The localization of loricrin was visualized using anti-rabbit antibody (green color). Nuclei of the cells were visualized using DAPI dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

In normal skin, Tp63 was found to be expressed in the nuclei of epidermal basal cells, whereas MBTPS2 and matriptase were expressed in the cytoplasm of epidermal upper granular layer and basal cells respectively. However, in IFAP skin the expression of Tp63 was found to be more spread throughout the epidermis, MBTPS2 had a decreased expression in the upper granular layer and matriptase expression changed from epidermal basal layer to the upper granular layer. As for, keratin 1 and loricrin, keratin 1 were found to be expressed in the suprabasal layers and loricin in cornified envelop of normal and IFAP skin.

B. Regulation of Gene Transcription

In order to assess whether MBTPS2 has an impact on the regulatory function of Tp63 and NOTCH, transactivation assays were done. HEK293 cells were transiently cotransfected with 3μg of the promoter (Tp63 or NOTCH/luc) per two wells and increasing concentrations of Wild type MBTPS2.

1. ***TP63 and MBTPS2***



2. ***NOTCH and MBTPS2***

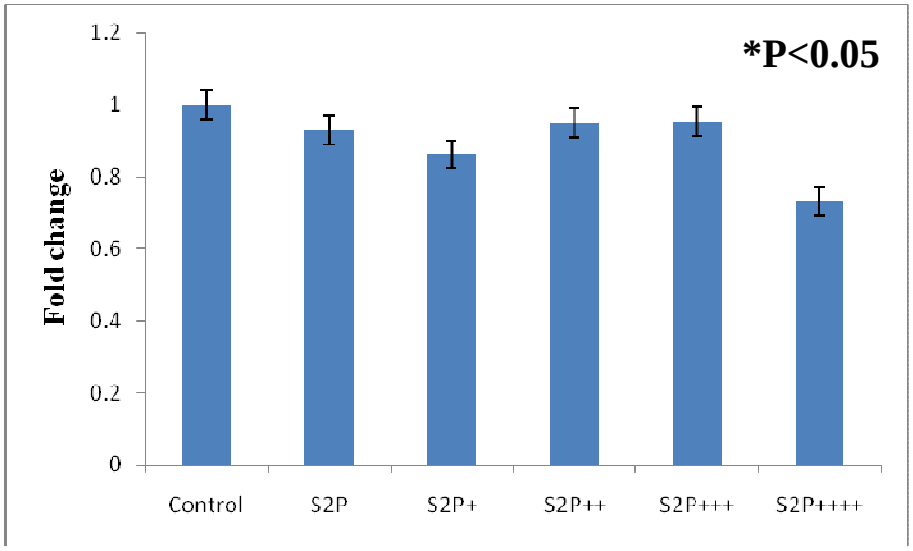


Figure 12: Transcriptional Activity of MBTPS2 on Tp63 and NOTCH promoter. 1- Transcriptional activity of MBTPS2 (25ng, 50ng, 100ng, 250ng and 500ng) with p63 (3µg/2 wells). 2- Transcriptional activity of MBTPS2 (25ng, 50ng, 100ng, 250ng and 500ng) with NOTCH (3µg/2 wells). Relative luciferase activities are represented as fold

change. The data are the means of 3 independent experiments done in duplicates ± standard error. Significance ($p<0.05$) was assessed using the Students T-test.

Luciferase assay was done to assess the effect of MBTPS2 on the transcriptional regulation of p63 and NOTCH. The results showed that MBTPS2 is a relatively good suppressor of the Tp63 promoter with a maximum fold decrease of 0.6. Interestingly, MBTPS2 had no effect on NOTCH promoter.

CHAPTER IV

DISCUSSION

Skin development is a tightly regulated process involving different steps starting with basal cells proliferation followed by outwards differentiation to form the stratum corneum. Abnormalities in any of these processes lead to impaired skin development associated with variety of conditions among which is ichthyosis and ichthyosiform disorders, a rare group of genetic disorders characterized by impaired epidermal differentiation or excessive protease activity at the level of the granular layer. To date, no definitive therapy is available to such disorder, and treatment is targeted at managing the signs and symptoms. Studies have identified mutations in several genes implicated in skin development as being responsible for different forms of ichthyosis. MBTPS2 is a candidate gene whose role in epidermal proliferation and differentiation has not been well investigated, yet mutations in this gene have been associated with severe phenotypic features combining proliferation and differentiation abnormalities in IFAP syndrome and its spectrum of disorders.

In this study, we evaluated the role of MBTPS2 in epidermal proliferation and differentiation and its possible interaction with major players in skin development including Tp63, NOTCH and its downstream mediators and matriptase.

The process of differentiation in the skin relies on the normal production of lipids, mainly cholesterol (Feingold et al., 2014). MBTPS2 is a zinc metalloprotease essential for cholesterol homeostasis and endoplasmic reticulum stress response (Rawson, 2013) and mutations in this gene lead to disturbed differentiation of epidermal

structures evoking the triad of IFAP phenotype (Wang et al., 2014; Mégarbané et al., 2011) with features of both over proliferation and impaired differentiation suggesting a role of MBTPS2 in these processes.

Tumor protein p63 (TP63) has an important role in skin development (Chikh et al., 2007) and is a main factor involved in proliferation. We investigated a possible role linking the function of Tp63 and MBTPS2. Immunohistochemical evaluation of MBTPS2 and Tp63 in normal and IFAP skin tissue sections showed cytoplasmic staining of MBTPS2 in cells of epidermal granular layer and nuclear staining of Tp63 in cells of epidermal basal layer. As expected, the expression of MBTPS2 in IFAP decreased as compared to normal skin, whereas the expression of Tp63 was more spread and involving the spinous cell layer in IFAP compared to normal skin. These results correlate with features of proliferation observed in IFAP disease and suggest that MBTPS2 is a regulator of Tp63. To further validate these results, we checked for the transcriptional regulation of Tp63 promoter by MBTPS2. Results showed that wild type MBTPS2 is a relatively good suppressor of the Tp63 promoter, which explains the wider expression of Tp63 in IFAP disease where MBTPS2 is mutated. We have recently identified a novel IFAP causing missense mutation F475S (c.1621U>C) in MBTPS2, which we plan to introduce into wild type MBTPS2 cDNA using site directed mutagenesis to confirm the role of MBTPS2 in regulating Tp63. In addition, Co-immunoprecipitation (Co-IP) would be a good future experiment for detecting possible interaction at the protein level.

To check the role of MBTPS2 in skin differentiation, we evaluated the expression of factors involved in this process. NOTCH is a cell surface receptor that has a role in promoting keratinocytes commitment to differentiation (Rangarajan et al. 2001,

Sasaki et al., 2002). Studies have shown that Notch suppresses Tp63 in the upper epidermal layers (Sasaki et al., 2002; Dotto GP., 2009) and regulates Keratin 1 and loricrin formation, which are markers of differentiation. Given its role in differentiation, we decided to check for possible transcriptional regulation of NOTCH promoter by MBTPS2. Interestingly, results showed no effect of MBTPS2 on NOTCH expression. Therefore, we decided to check for the expression of Keratin 1 and loricrin whose formation is regulated by NOTCH to check whether MBTPS2 is mediating its effect on them through other pathway. Immunohistochemical staining for keratin 1 and loricrin on normal and IFAP skin tissue sections showed no change in expression in keratin 1 and loricrin between normal and diseased skin suggesting that the differentiation problem in IFAP disease is not mediated through NOTCH.

Matripase is another protease involved in skin development. Studies have shown that matriptase is highly expressed in epidermal basal and spinous layers and is likely to be involved in the regulation of keratinocytes proliferation and mainly early phases of differentiation (Ya-Wen Chen et al., 2014). Sales et al. evaluated the role of matriptase in Netherton syndrome (a suyndrome with overlapping features with IFAP) and concluded that its expression changes to the suprabasal epidermis and it prematurely activates epidermal pro-kallikreins leading to premature desquamation at the granular transitional cell layer boundary. Thus, it appears that the timing of matriptase expression is essential in the appropriate steps in epidermal differentiation. Mutations in matriptase have been associated with two congenital skin disorders, autosomal recessive ichthyosis with hypotrichosis (ARIH) and ichthyosis follicular atrophoderma, hypotrichosis, and hypohidrosis (IFAH), which have clinical features similar to IFAP disease (Ya-Wen Chen et al., 2014). Consequently, in this study

matriptase expression was investigated for possible role in IFAP disease. We compared matriptase expression in IFAP disease with its expression in normal skin and saw that matriptase expression changed from basal layers in normal skin to granular layer in diseased skin, similar to Netherton syndrome, where transition from proliferation to differentiation usually takes place. This change in space and time of expression will most likely disrupt skin physiology and thus interfere with proper epidermal proliferation and differentiation. We will be performing assays to determine the possible transcriptional regulation and interaction between MBTPS2 and matriptase.

We have shown that NOTCH does not decrease in response to MBTPS2 and since it regulates keratin 1 and loricrin formation, the expression of these factors seemed normal in IFAP patients unlike matriptase; therefore, we suggest that the differentiation problem in IFAP disease is mainly through matriptase. Further experiments have to be performed to validate this hypothesis.

In conclusion, this is the first report describing and characterizing a potential role for MBTPS2 gene in the context of skin proliferation and differentiation. This report depicts the novel *in vitro* regulation of Tp63 by MBTPS2 and possible role for matriptase in IFAP disease. Despite being a non-perfect representative of the *in vivo* system, the *in vitro* system will help delineate the regulatory pathways leading to IFAP and cite novel molecular markers for therapy. Nevertheless, *in vivo* studies remain essential to validate our hypothetical model.

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