Diploma thesis

Immunohistological analysis of members of the TGF-beta superfamily in inflammatory skin diseases

Submitted by

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Graz, 2015 *Bálint Kovács eh.*

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Zusammenfassung

Langerhans-Zellen, eine Subpopulation dendritischer Zellen in der Epidermis, fungieren als Türsteher der Haut. Eindringende Antigene werden von ihnen aufgenommen, degradiert und in den Lymphknoten präsentiert, wodurch eine adaptive Immunantwort produziert wird. Die Differenzierung der Langerhans-Zellen wird durch bestimmte Mitglieder der TGF-β-Superfamilie geregelt. Diese heterogene Gruppe von etwa dreißig strukturell ähnlichen Proteinen signalisieren durch Rezeptor-vermittelte Phosphorylierung von R-SMADs. Das Endprodukt dieser Signalkaskade, eine trimere pSMAD Einheit, wird in den Zellkern verlagert, wo es als ein Transkriptionsfaktor agiert.

Wir führten dreifache immunhistologische Färbungen in gesunden und entzündeten Hautproben durch. Selektive Mitglieder der TGF-β-Superfamilie und ihre Antagonisten wurden in *Psoriasis vulgaris* und *Chronisch Diskoiden Lupus Erythematodes* Läsionen genauer charakterisiert.

Überraschenderweise zeigten unsere Ergebnisse eine allgemeine Hochregulierung von TGF-β Familienmitgliedern in entzündlichen Hauterkrankungen mit einigen besonderen Eigenheiten bezüglich der Veränderung von Langerhans-Zellen. Die Intensität von TGF-β1, BMP4, BMP7, BMP9 und des Antagonisten NOGGIN waren in pathologische Proben erhöht. Ein auffälliges Ergebnis war die explizite Trennlinie zwischen proBMP7 und aktivem BMP7 an der Grenze der basalen Schicht zum Stratum spinosum in der gesunden Epidermis. Die intrazellulären Elemente des Signalkaskade, die R-SMADs, wurden nicht wesentlich durch die Fehlregulation der TGF-β-Superfamilie beeinflusst.

Das Ziel dieser Arbeit war es, die Rolle der TGF-β-Superfamilie in epidermalen Langerhans-Zellen in Gesundheit sowie Entzündung zu beschreiben. Dadurch konnten wir mögliche neue Therapieansätze für spezifischere und gezieltere Behandlungen dieser dermatologischen Erkrankungen aufzeigen.

Abstract

Langerhans cells (LCs), a distinct subpopulation of dendritic cells in the epidermis operate as gatekeepers of the skin. Intruding antigens are ingested, degraded and presented in the draining lymph nodes, thereby activating an adaptive immune response. The differentiation of LCs is regulated by certain members of the transforming growth factor β (TGF-β) superfamily. This heterogeneous group of structurally similar proteins signal through receptor-mediated phosphorylation of R-SMADs. The end product, a trimeric pSMAD, is translocated into the nucleus, where it acts as a transcription factor.

We performed immunohistological analysis of healthy and inflamed human skin from *Psoriasis vulgaris* or *Discoid lupus erythematosus* lesions to characterize selected members of the TGF-β superfamily and their antagonists.

Surprisingly, our findings have shown a general upregulation of TGF-β family members with some peculiar mannerisms concerning LCs in inflammatory skin diseases. TGF-β1, BMP4, BMP7, BMP9 and the antagonist NOGGIN were increased in pathological samples. One striking result was the explicit separation between proBMP7 and active BMP7 at the limits of the basal layer in the healthy epidermis. The second messengers, R-SMADs were not influenced by the dysregulation of the TGF-β superfamily.

The aim of this study was to characterize the role of the TGF-β superfamily in epidermal Langerhans cells in health and inflammation. New paths could be depicted with more specific and precisely targeted treatments for affected patients.

Contents

Glossary and abbreviations

Acanthosis: diffuse epidermal hyperplasia

ACTH: Adrenocorticotropic hormone

APC: antigen presenting cell

Auspitz's sign: Small bleeding points where the thin suprapapillary epithelium is torn of

BMP: bone morphogenetic protein

BMPER: BMP-binding endothelial cell precursor-derived regulator

CD(number)…cluster of differentiation with designed number of molecule

CDK: cyclin dependent kinase

CGRP: calcitonin gene-related peptide

DC: dendritic cell

DLE: *Discoid lupus erythematosus*

H&E: Haematoxylin and Eosin

Hyperplasia: the enlargement of an organ or tissue caused by an increasing cell division

INF-γ: Interferon gamma

LAP: latency associated protein

LC: Langerhans cell

LN: lymph node

LPS: lipopolysaccharide

LTBP: Latent TGF-β Binding Protein

MAPK: mitogen activate protein kinases

MHCII: major histocompatibility complex two

α-MSH: alpha-melanin stimulating hormone

NOGGIN: slang English word for head (also name of molecule)

PFA: paraformaldehyde

PPM1A: Protein Phosphatase, Mg^{2+}/Mn^{2+} Dependent, 1A

pSMAD: phosphorylated small body size, and mothers against decapentaplegic

Ring of Woronoff: Vasoconstriction around active plaques of psoriasis

Smurf1: SMAD specific E3 ubiquitin protein ligase 1

SnoN: Ski-related novel protein N

TARC: thymus and activation-regulated chemokine

TGF-β1: transforming growth factor beta one

Th1: T lymphocyte helper 1

Th2: T lymphocyte helper 2

T-lymphocytes: T-cells: thymus-lymphocytes

TMPEAI: N-[2-(2-Trimethylammoniumethyleneoxy-7-methoxy)ethyl]propionamide iodide

TNF- α: tumor necrosis factor alpha

TSR: TGF-β superfamily receptor type I (also called Alk1)

VEGF: Vascular epithelial growth factor

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1 Introduction

1.1 Human skin

The functional unity of skin (Integumentum commune) is constructed of cutis and subcutis. It covers approximately two square meters in toto and fulfills various objectives such as protection from physical or chemical harm, thermoregulatory, water regulatory, barrier of the body interior and exterior and sensation of haptic and thermic stimuli. The skin can be broken down into the cutis and the subcutis, describing the fatty layers and the interposed connective tissue separated by the basal lamina from the dermis and the epidermis which two compose together the cutis. The dermis is responsible for the mechanical resistance of our skin and histologically it can be classified in two portions: the papillary dermis and the reticular dermis. Our field of interest lays in the epidermis, therefore we are not going to elaborate on the other fractions (Lüllmann-Rauch 2012; Elston 2010; Burns et al. 2010; Heronimus 2014).

1.1.1 Epidermis

The human epidermis is a stratified squamous epithelium. It has an average width between 50 and 100µm (the palms and feet are ten times thicker) and possesses no vascularization. The cells are adequately supplied with anabolic nutrients through diffusion of the dermis.

The epidermis is constructed of four sublevels of dissimilar thickness (Fig. 1). From the most inner to the most exterior they are named stratum basale, stratum spinosum, stratum granulosum and stratum corneum. On the skin of palms and feet a fifth layer interposed can be distinguished between stratum corneum and spinosum, the stratum lucidum.

The basal lamina is the separation point between dermis and epidermis. It is mainly composed of collagen IV and anchored to the dermis by collagen VII and to epidermis by hemidesmosomes. The stratum basale (also stratum germinativum) is only one prismatic cell layer thick and composed of epidermal stem cells and progenitor cells. Mitosis explicitly takes place here and the cell pool for the upper layers is replenished from here.

The stratum spinosum consists of 2 to 5 layers of polygonal cells bound together by desmosomes. (Together with stratum basale also considered as Malpighian layer)

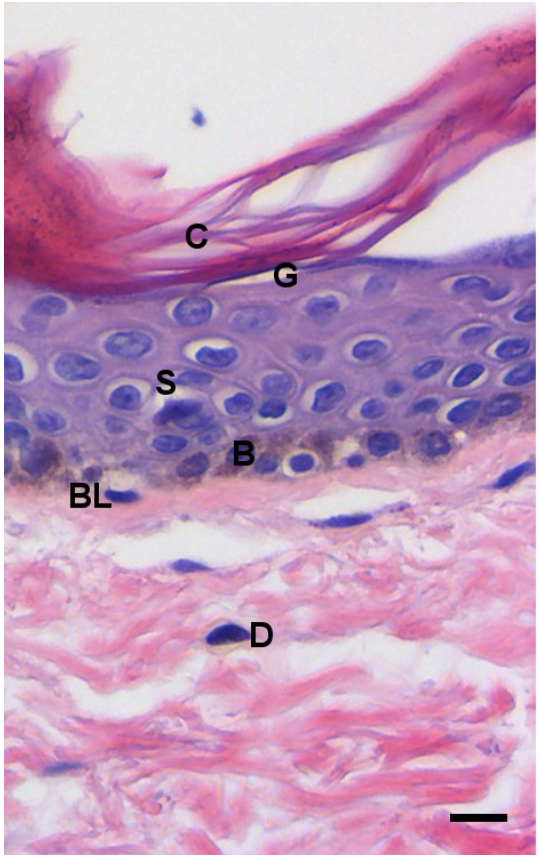

Figure 1. Overview of human skin layers
Original magnification x40, Haematoxylin & Eosin; Paraffin embedded tissue; Bar represents 10 µm; 4 µm longitudinal section; Dermis (D), Basal Lamina (BL), Stratum basale (B), Stratum spinosum (S), Stratum granulosum (G), Stratum corneum (C)

The stratum granulosum, approximately three layers thick, contains microscopically visible small aggregates of cytokeratin filaments and proteins indicating the stadium of degradation in the more outer layers. The stratum corneum or horny layer is built up of approximately 25 layers of dead cells linked together by corneodesmosomes. In this stadium the cells no longer have organelles or a core and they are lost through desquamation.

The life cycle of a keratinocyte from the basal lamina till desquamation requires roughly one month whereupon half of the time is spent in the stratum corneum (Elston 2010; Heronimus 2014; Burns et al. 2010; Lüllmann-Rauch 2012).

1.1.2 Different cell types in the epidermis

Keratinocytes are the predominant cell type in the epidermis with the main objective to maintain its barrier function.

Although 90 percent of the cells are keratinocytes we will also shortly consider the two other cell types present in the Epidermis and then focus on Langerhans cells.

Melanocytes are situated in the basal layer and their function is the production of melanin thereby protecting the DNA from damages through UV-radiation. The pigment melanin, pivotal for the coloring of the skin is produced in melanosomes, which are then secreted into the keratinocytes through local dendrites. The production process is regulated through paracrine stimulation with the hormones ACTH and α-MSH. One melanocyte builds a functional unit with approximately 36 keratinocytes and is connected to its environmental cells via E-Cadherin comparable to LCs.

Merkel cells are cells of debated origin located in the basal layer and responsible for the sensation of mechanical pressure. They are linked to an axon on their basal side and transmit thereby their level of stimulation (Burns et al. 2010; Heronimus 2014; Lüllmann-Rauch 2012; Elston 2010).

1.2 Langerhans cell

Langerhans cells [LC] where discovered in 1868 by Paul Langerhans, a medical student descendant from a family dynasty of physicians, at the University of Jena at the age of 21. While performing a gold chloride staining on sections of the human skin he spotted and described them as cells with a dendritic body shape and concluded thereby prematurely that they were part of the epidermal nervous system (Langerhans 1868). This was later refuted and LCs were correctly established as essential part of the dermal immune system.

Recently the question about the origin of the LCs was discussed highly controversially. LCs constitute one of the four main subsets of the dendritic cell line: myeloid, plasmacytoid, CD14+ and LCs (Collin et al. 2013). Thus, LCs can be derived from peripheral stem cells as well as from an in situ tissue precursor cell (Fig. 2) (Chopin & Nutt 2014; Haniffa et al. 2014). The developmental pathway of LCs can be induced by some members of the TGF-β

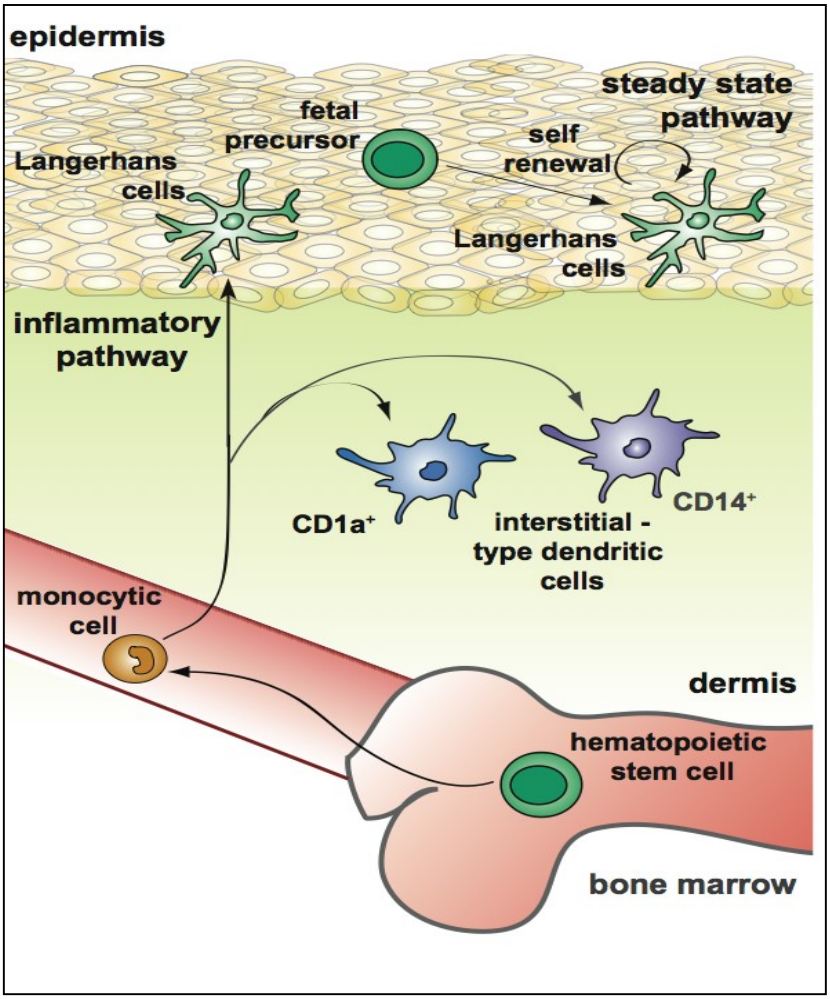

Figure 2. Development of LCs
LCs can differentiate either from monocytes or from fetal precursor LCs which reside in the skin. Figure provided by (Borkowski et al. 1996)

superfamily, notably TGF-β1 (Kel et al. 2010) and BMP7 (Yasmin et al. 2013) have been shown to play an important role in this process. In steady state LCs are kept in a tight network by being replenished from local precursor pols, whereas during inflammation they migrate into the lymph nodes more abundantly and are replaced from differentiating monocytes (Fig. 3) (Chopin & Nutt 2014). Additionally Activin A is known to be an inducer of human LC differentiation from human monocytes (Musso et al. 2008).

Located in the epidermis they have a close interaction with their embedding keratinocytes which provide through paracrine secretion of stimulating survival factors a favorable milieu. The cell itself has a round nucleus and numerous dendrites which form a three dimensional meshwork supporting thereby the objective of an intact immune surveillance system (Fig. 3). Inside the cell body we can find Birbeck granules, one of the defining structural elements besides characteristical proteins e.g. CD207 (Langerin), CD1a and E-Cadherin (Geissmann et al. 1998) and others.

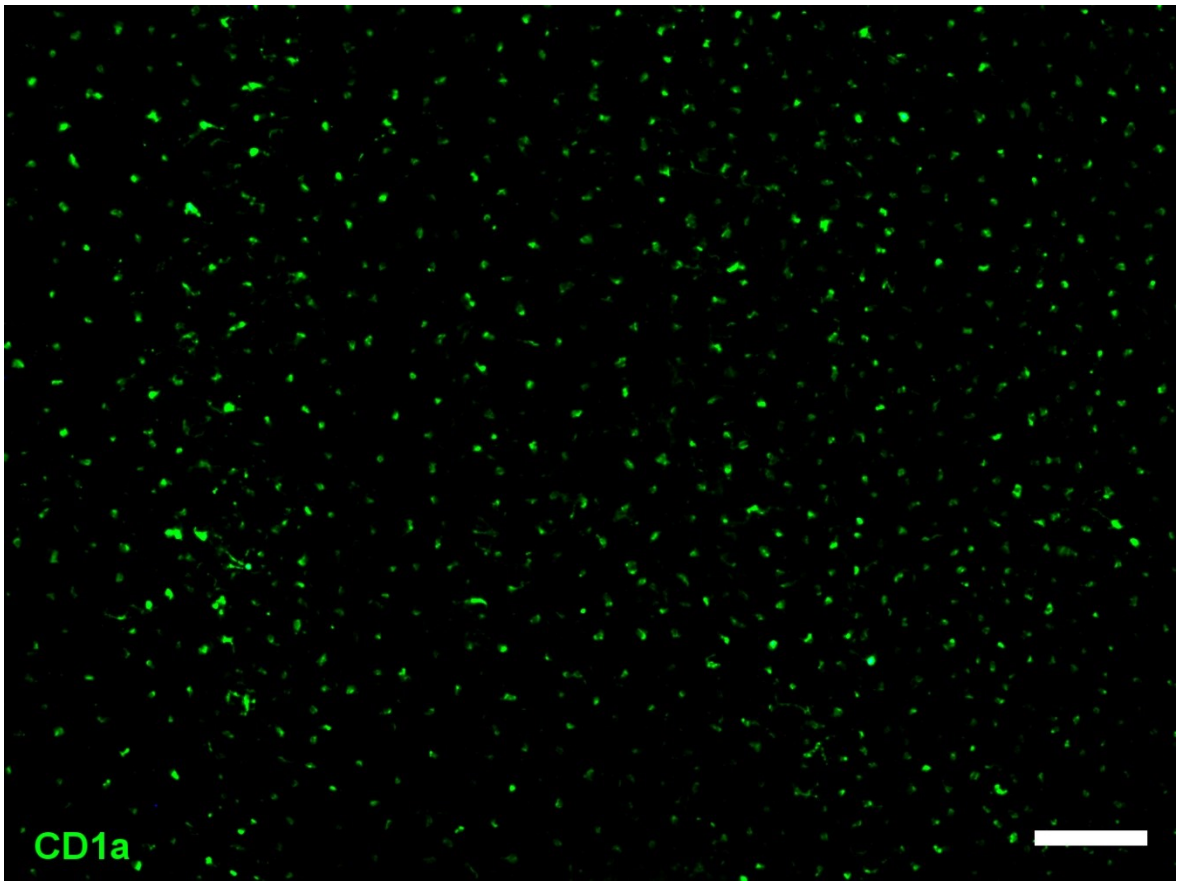

Figure 3. Langerhans network in epidermal sheets
Immunofluorescent staining; Original magnification x4; Bar represents 100 µm; CD1a (green); picture provided by Izabela Borek;

Since LCs are a subset of Dendritic Cells [DC] and therefore by definition Antigen Presenting Cells [APC], they monitor the epidermal microenvironment and capture possible antigens. Following pathogen capture, LCs have to decide whether an antigen is potentially dangerous or innocuous and thereby adjust their reaction in an immunogenic or respectively tolerogenic pattern. After activation, they migrate into the sub epidermal lymph nodes and present processed epitopes of the absorbed pathogen through MHCII molecules [major histocompatibility complex two] to naïve Thymus-Lymphocytes [T-Lymphocytes]. Through this priming, T-Lymphocytes get activated and can induce an adequate immune response to the impending intruders.

Concerning dysfunctional LCs various pathologies are known. Among the most important is Langerhans Histiocytosis (Histiocytosis X).

1.2.1 Langerin CD207

Langerin is a C-type (Ca^{2+} dependent) Lectin responsible for the generation of Birbeck granules. It is dispensable for LC development. Although langerin was thought to be expressed only by epidermal LC, a subset of $CD8a^{+}$ DCs found throughout the secondary lymphoid tissues (LN, spleen and thymus) also express it in murine samples. (Kaplan et al. 2008) It has a single carbohydrate recognition domain especially for mannose-like structures. Pathogens bound by langerin include Mycobacterium leprae, HIV, herpes simplex virus 2, measles virus, fungi including Malassezia furfur and Candida species (Feinberg et al. 2013). It is considered as a strong inducer of membrane superimposition thus creating the Birbeck granules (Valladeau et al. 2000).

1.2.2 Birbeck granules

They are cell organelles constituted mainly out of langerin molecules. In appearance Birbeck granules have a tennis racket shape and two superimposed membranes separated by leaflets with periodic “zipperlike” striations. The molecular signal cascade leading to their formation is unknown, and their role in Langerhans cell physiology is still much debated due to limited research in this area (Valladeau et al. 2000).

1.2.3 CD1a

CD1 proteins are transcribed from one locus on chromosome 1 which contains only five slightly different variants (CD1A, CD1B, CD1C, CD1D, and CD1E). They show a low rate of genetic polymorphism and are not linked to any particular disease. In comparison, the mouse genome contains only one orthologue of the human CD1D thus limiting the utility of mouse models for studying functions of LCs concerning CD1 biology (Seshadri et al. 2014). The physiological function of CD1a is the presentation of lipid antigens to naïve T-cells. They are expressed on a few related cell lines, like LCs, thymocytes and some specific DC subsets. (Seshadri et al. 2013)

1.3 Transforming Growth Factor-β superfamily

TGF-β type proteins are important in various complex cell signaling structures and impact functioning processes in almost every organ in the human body during the developing embryonal phase, as well as during the steady state in adult homeostasis. TGF-β superfamily members presence is restricted to vertebrates. Dysregulation of family members quantity or quality lies at the base of many miscellaneous diseases (Xu et al. 2012).

The superfamily embodies a wide range of similar proteins, which act through signal cascades with a limited number of receptors. They have multiple well established and researched functions including: Regulation of organogenesis and tissues, the heart, lungs, eyes and palate are implied. Instruction of the adaptive immune system with a special focus on the antigen presenting cells and coordination of wound healing.

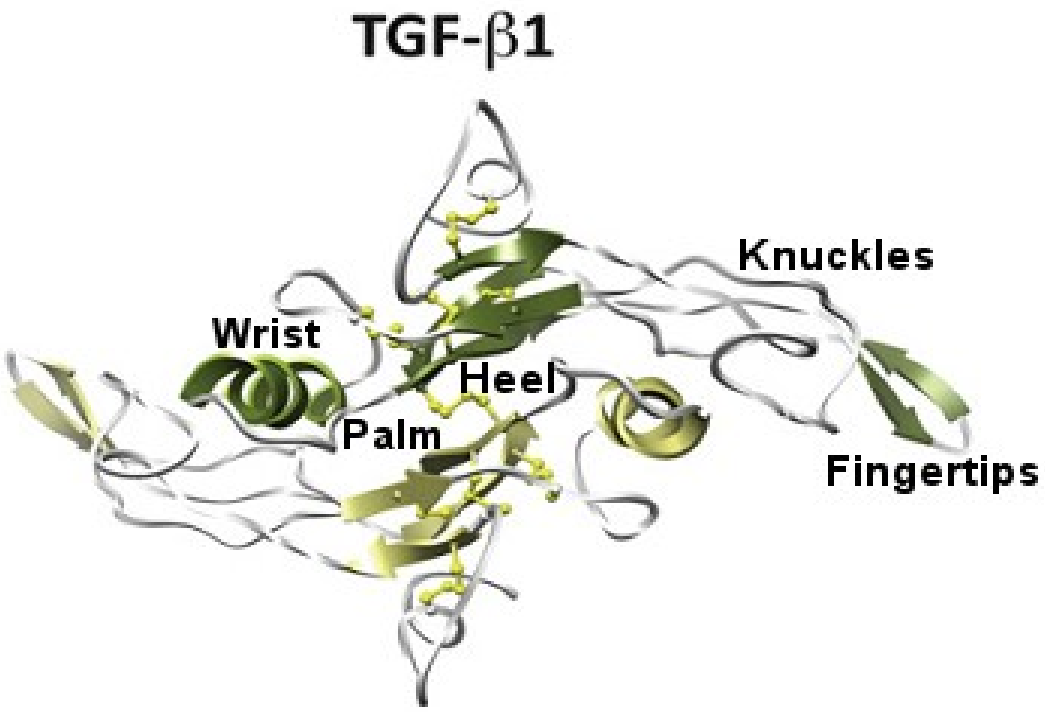

Figure 4. Structure of TGF-β1
Molecular structure of TGF-β1 dimer; monomers respectively in (olive drab) & (pear); β-sheets (arrows) and α-helices (loops); resemblance to a left hand can be noted with wrist, palm, knuckles and fingertips visible, hand can be mirrored through the cysteine knots (yellow); (Hinck 2012)

The TGF-β superfamily is classified in different subfamilies according to their sequence similarities in human and other species, including but not exclusively TGF-βs, BMPs, GDFs and Activins. The molecule of the most investigated family member, TGF-β1, is presented as a structural prototype for the TGF-β superfamily (Fig. 4). Their homodimer shapes are structurally described as a left hand with wrist, palm, knuckles and fingertips.

A genealogical tree regarding sequence similarity between the different family members can be established (Fig. 5). The TGF-β superfamily can be subdivided into two main branches. Firstly the ones activating gene transcription through pSMAD2/3 including TGF-βs, activins, GDF-8, -9, -11, nodal and BMP-3 and secondly the ones signaling through pSMAD1/5/8: BMP-2, -4, -5, -6, -7, -8, -9, -10 and GDF-1, -3, -5, -6, -7. However this repartition is not perfect because some of the molecules rest unattended.

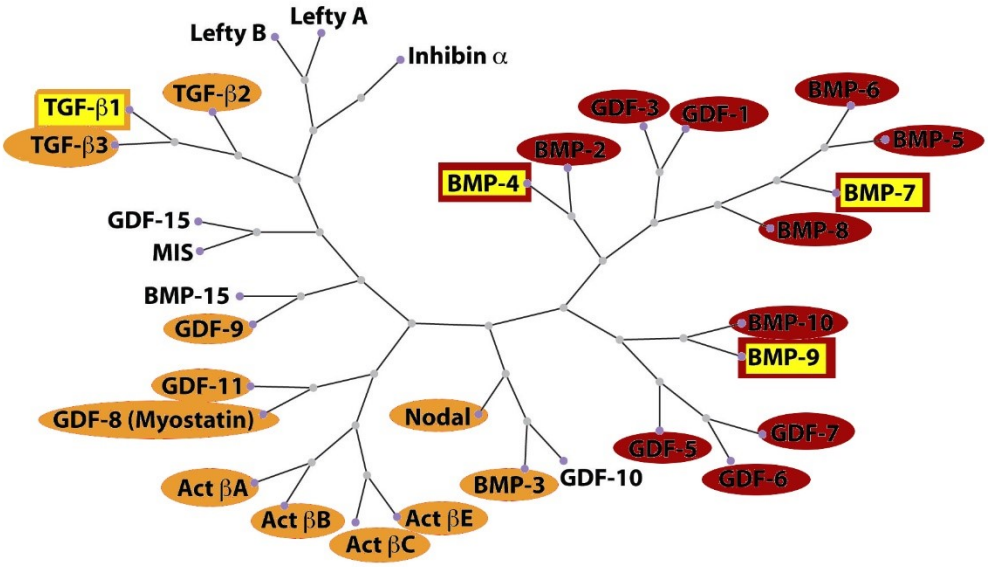

Figure 5. Phylogenetic division of TGF-β superfamily
Canonical signaling through pSMAD2/3 (brown); Signaling through pSMAD1/5/8 (red); Target TGF-β family members (yellow); Modified figure from (Hinck 2012)

Moreover receptor availability is very restricted due to the fact that for more than 30 TGF-β family members only seven type I and five type II receptors have to suffice (Mueller & Nickel 2012). Further distinction can be made by the fact that TGF-βs have a restrictive manner of receptor binding, mainly adhering to one type of receptor complex (TβR-I:TβRII) whereas other TGF-β superfamily members, such as BMPs and GDFs are very promiscuous and can bind up to three different type I receptors and three different type II receptors (Hinck 2012).

Heteromeric combinations of type I, type II receptors and their activation by dimers of TGF-β family members reach a very high level of versatility that can be made responsible for a highly specific and divers cell signaling pattern (Xu et al. 2012).

Protocol of receptor docking also distinguishes in these two subcategories, whereby TGF-β and activins assembles their receptors in an ordered manner by starting with type II receptors and then recruiting their type I receptor. Moreover Tβ-RI and Tβ-RII display a characteristic receptor-receptor contact before SMAD phosphorylation. Although the receptors are structurally very similar the TGF-β type binding is predominantly a combination of hydrophobic, hydrogen-bonding and electrostatic interactions.

On the contrary BMPs and GDFs show a much more heterogeneous approach on their receptors respectively and no contact between receptors during the activation. Their driving interaction force is hydrophobic (Hinck 2012).

1.3.1 Canonical signaling pathway

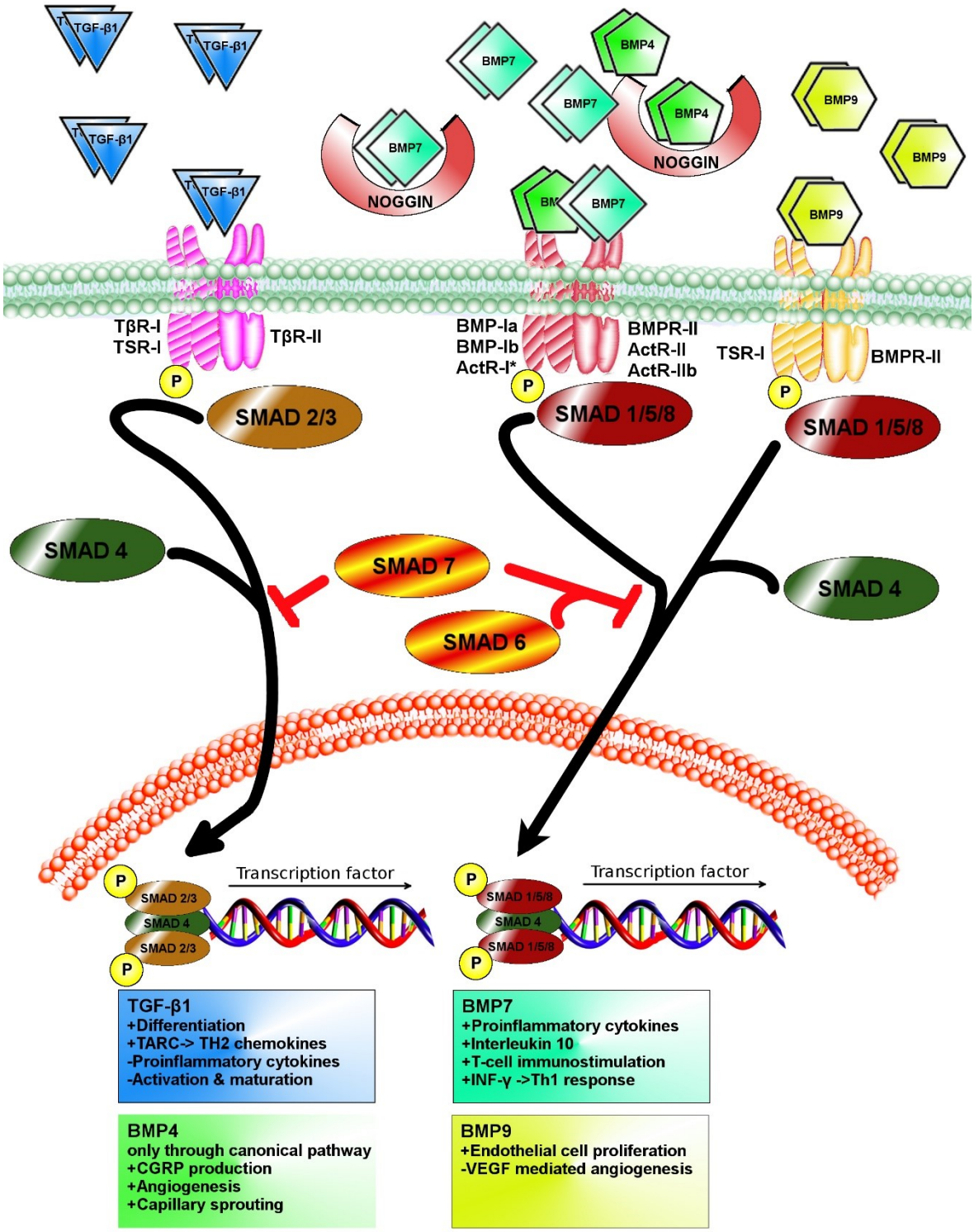

Figure 6. Signaling pathway of selected TGF-β superfamily members
Type one receptors (belted); type two receptors (bland); only for BMP7 (*) Signaling from extracellular till effects of transcription is depicted. Figure was designed on the basis of this work.

An overview of the TGF-β superfamily members which were selected for characterization for this study (Fig 6).

1.3.2 Transforming growth factor β 1

TGF-β1 is expressed in great numbers in different cell lines, e.g. it controls differentiation and proliferation of hematopoietic and immune cells. Usually it is responsible for inhibition of growth in most cell types by blocking the cell cycle in G1 (Mueller & Nickel 2012) but distinct reactions in each specific cell type are possible and occasionally even opposite responses can be triggered depending on the additional growth factors involved.

After translation into a proprotein, the molecule is proteolytically cleaved into a non-covalently linked mature TGF-β1 and latency-associated protein [LAP] (Zi et al. 2012). In the case of TGF-β1 the prodomain is constructed comparable to a straitjacket where it blocks the mature molecules receptor epitopes. It is not responsible for the correct folding pattern, whereas it confers latency and activity can only be achieved through its removal. Furthermore the straitjacket is essential to TGF-β1 transporting because it covers large hydrophobic spots and makes it therefore adaptable for diffusion (Mueller & Nickel 2012).

Following the secretion, this TGF-β1-LAP complex is bound by Latent TGF-β Binding Protein [LTBP], one of the molecules which regulates bioavailability of TGF-β1. This triple complex can be cleaved proteolitically and its result, the bioactive TGF-β1 is mostly bound by various non-receptor cell surface proteins (Decorin, Biglycan and Betaglycan), which support the bioavailability regulative system by enrichment of the plasma membrane. When TGF-β1 is liberated in its ligand form, a 25 kilo Dalton dimer covalently bound by disulfide bonds, the protein stimulates its receptor in a heterotetrameric complex consisting of two TβR-Is and TβR-IIs each. The binding process initializes autophosphorylation of the TβR-II, whereupon through conformational changes its receptor partner TβR-I is transphosphorylated and the kinase activity is switched on. Both TGF-β receptors are characterized as dual specificity kinases. Besides their phosphorylation of serine and threonine, they are also able to use tyrosine as a substrate (Xu et al. 2012). TβR-I in the signaling cascade of TGF-β1 can alternatively only be replaced by TSR. (Mueller & Nickel 2012)

Signal termination of TGF-β1 is achieved through clathrin-mediated or lipid raft-dependent receptor internalization by endocytosis and lysosomal degradation. In addition literature still discusses controversially possible recycling when caveolin-1-mediated endocytosis occurs (Xu et al. 2012).

TGF-β1 signaling can be antagonized at different levels. SMAD7 acts as a pseudo substrate for the TGF-β1 receptor complex and competitively inhibits R-SMADs. It is also involved in repression of the TGF-β1 target genes at nuclear level. Other notable antagonist which we lack to treat in this work are TMPEAI and SnoN (Zi et al. 2012)

TGF-β1 induces LC differentiation from $CD34^+$ hematopoietic progenitor cells. Thereby it uses the non-canonical pathway through BMPR-Ia (ALK3). Its expression pattern in the human epidermis is suprabasal and it is responsible for the inhibition of proinflammatory cytokine production in LCs via TβR-1 (ALK5). TGF-β1 also induces the production of thymus and activation-regulated chemokine [TARC], a molecule deviating immune response to Th2 (Yasmin et al. 2013).

1.3.3 Bone Morphogenetic Protein

BMPs constitute a branch of the TGF-β superfamily. They occur in various tissues and show evidence of different functions, whereas their activities are mainly localized at sites of epithelial-mesenchymal interactions. The proteins were named after their ability of de novo bone formation in a classic subcutaneous implantation assay. Although not all BMPs are able to fulfill this requirement (Ducy & Karsenty 2000).

BMPs are synthesized as large precursor proteins of about 400-500 amino acids. This molecules are cleaved by a subtilisin-like convertase, resulting in a smaller protein of about 100 to 140 amino acids including a cystine knot in its center (three intramolecular disulfide bridges). The homodimers are bound together by an intermolecular disulfide bridge capacitating their biologic activity. At times BMPs can also be secreted in their preform, however this molecules show no specific activity (Carreira et al. 2014).

BMP receptor binding can activate two different molecular pathways, the canonical signaling, defined by the phosphorylation of R-SMAD1/5/8s and the non-canonical signaling through triggering the enzyme mitogen activated protein kinase [MAPK]. In this work we will mainly focus on effects achieved through the canonical pathway.

During inflammatory responses BMPs induce the maturation of DCs and stimulate specific cytokine secretion such as IL-8 and tumor necrosis factor α [TNF-α] improving this way the T-cell activating capacity of DCs and their survival capability. Moreover they block macrophage proliferation and protect vascular integrity (Moura et al. 2013).

BMP signaling can be antagonized by different proteins which form complexes with the homodimer and thereby prevent its binding to the active site, such as Noggin, Twisted gastrulation, Gremlin and Follistatin. (Carreira et al. 2014)

1.3.3.1 BMP4

BMP4 shows osteochondrogenic potential and stimulates production of VEGF by osteoblasts (Carreira et al. 2014). BMP4 activates the BMP-SMAD pathway but is not signaling via the non-canonical pathway. Its effects on cell homeostasis include modulation of cell adhesion, extracellular matrix remodeling, motility, metabolism, signaling and transcription. BMP4 is a strong inductor of CGRP [Calcitonin gene related peptide] expression and can switch thereby the neuropeptide phenotype. The protein promotes angiogenesis and extracellular signal regulated kinases dependent capillary sprouting. It has been shown that CGRP Axons are situated anatomically near LCs where they block Th1 responses and preferably enhance Th2 responses (Madva & Granstein 2013). It is a known inhibitor of LPS-induced maturation of DCs (Pluchino et al. 2009).

Various BMP4-antagonist can be defined: BMP-binding endothelial cell precursor-derived regulator [BMPER] directly interacts with BMP4 and SMAD5 and inhibits also vasculogenesis (Moura et al. 2013) . NOGGIN and follistatin are pseudoreceptors for BMP4 and are also able to keep it from actively docking (Robson et al. 2008) (McMahon et al. 1998) (Singh et al. 2012). BMP4 has already been stained in the human epidermis and it has been shown that it can inhibit the skin pigmentation by blocking the melanin synthesis and its transfer to keratinocytes. Interestingly these capacities can be antagonized by BMP6 which increases skin pigmentation. (Singh et al. 2012)

Moreover BMP4 in mice is involved in limb patterning and sceletogenesis. Mice which lack BMP4 show a phenotype with mesodermal defects in early gastrulation. (Mueller & Nickel 2012)

1.3.3.2 BMP7

BMP7 has been discovered and named as an osteogenic factor, active in mature osteoblasts and also present in dental epithelium. Since its finding in the 1980 more physiological functions have been described. BMP7 is critical for kidney development and seems to be involved in sceletogenesis ophtalmo-, cardio- and neurogenesis (Mueller & Nickel 2012).

It can stimulate periodontal wound healing in furcation defects (Carreira et al. 2014) and it has proven value as anti-inflammatory agent in rat bowel disease (Moura et al. 2013).

BMP7 induces LC differentiation via the canonical BMP signaling pathway. BMP7-induced LCs exhibit higher amounts of proinflammatory cytokines, IL10, T-cell stimulation and prefer Th1 responses due to INF-γ production compared to LCs generated in response to TGF-β1. (Yasmin et al. 2013).

Recombinant BMP7 was approved by the U. S. Food and Drug Administration for medical use in 2001. It is applied in fracture healing, reunion issues and bone regeneration (Carreira et al. 2014).

1.3.3.3 BMP9

BMP9 promotes certain subtypes of endothelial cell proliferation, however simultaneously it also inhibits basic fibroblast growth factor-induced endothelial cell proliferation and VEGF mediated angiogenesis (Moura et al. 2013). Its interacting receptors are TSR-I and BMPR-II (Mueller & Nickel 2012).

Factual information about BMP9s functional role in DCs or LCs is not existing. The protein characterization in the epidermis can be considered as a pioneer work (Botchkarev 2003).

1.3.3.4 NOGGIN

NOGGIN is a specific antagonist of distinct BMPs amongst others BMP4 and BMP7 (Carreira et al. 2014). NOGGIN binds them as a pseudo receptor on their active epitopes, simulating the structure of the BMP-receptors (Mueller & Nickel 2012).

It is considered as a major developmental stem cell regulator and has shown the capacity to block the inhibition function of BMP4 on DCs, in result stimulating the maturation of DCs and their function as APCs in lymph nodes (Pluchino et al. 2009).

1.3.4 SMAD (Mothers Against Decapentaplegic homologue)

Eight different SMAD proteins are found in human organisms. They are numbered consecutively, whereat 1, 2, 3, 5, 8 are receptor-regulated (R-SMADs), 4 is the only cooperative SMAD (Co-SMAD) and 6, 7 are inhibitory proteins (I-SMADs). Once an R-SMAD is activated by its TGF-β superfamily receptor through phosphorylation it forms a trimer with another activated R-SMAD and a SMAD4. This activation can also occur through non-canonical pathways such as MAPK and CDK. This trimeric complex is translocated into the nucleus, where R-SMADs act as transcription factors (Mueller & Nickel 2012). SMAD4 deficient cell lines are also able to process TGF-β superfamily signaling, albeit with lesser intensity, thus SMAD4 is not an essential factor for signal transduction but an enhancer or corepressor depending on the cell type.

I-SMADs interrupt SMAD signaling by binding to the activated type I receptor and thereby blocking the active site for R-SMADs. SMAD6 is especially affixed to BMP-Rs through pSMAD1/5, whereas SMAD7 shows no preferences. TGF-β inhibits overwhelming signaling through a direct feedback line by inducing the promotor of SMAD7 through SMAD3/4 (Xu et al. 2012).

1.3.4.1 SMAD 2/3

SMAD2 and SMAD3 are second messengers for multiple TGF-β family members.

SMAD molecules and pSMAD complexes are shuttled constantly between cell plasma and nucleus staying functional. One trimeric SMAD complex regulates the expression of approximately 100-300 genes. Most models assume identic dynamics of pSMAD2 and pSMAD3, whereas their biological functions differ quite clearly. (Zi et al. 2012) In this study we have chosen only to stain one of both, pSMAD3.

Besides the rapid phosphorylation activation through TGF-β1 family members there is also a slower through GSK3 on different linker sites. This second phosphorylation is a typical marking for proteasomal degradation through polyubiquitylation and consequently signal termination defining thereby the half-life of activated SMADs and signal amplitude. Signal termination is also achieved through desphosphorylation of R-SMADs by PPM1A, a specific phosphatase. Moreover PPM1A promotes nuclear export of pSMAD2/3 (Xu et al. 2012).

1.3.4.2 SMAD 1/5/8

Analogue to SMAD2/3 SMAD1/5/8 are also second messenger proteins. One way of increasing their effectivity is by CDK8/9 which phosphorylates the linker region of SMAD1, thus amplifying the translation procedure but also marking it for degradation through ubiquitylation by Smurf1. (Xu et al. 2012)

1.4 Inflammatory skin diseases

A vast number of different inflammatory skin diseases exist. Therefore our choice for Lupus cutaneous and *Psoriasis vulgaris* was guided by availability of sections and level of specific knowledge respectively understanding of pathophysiological processes. Professor Peter Wolf from the institute of dermatology, Medical University Graz, was so kind to provide us with skin sections from *Psoriasis vulgaris* and Lupus cutaneous.

1.4.1 Discoid lupus erythematosus

DLE, also called *Cutaneous lupus erythematosus* or *Chronic discoid lupus erythematosus* is an inflammatory skin disease belonging to the subgroup of connective tissue diseases. It is a benign skin disorder characterized by red scaly patches in different stages with atrophy and pigmentary changes. It can be diagnosed through typical hematological, serological and histological deviations. These evidence points DLE out as of autoimmune etiology (Burns et al. 2010).

1.4.1.1 Epidemiology and Etiology

DLE has a typical distribution pattern where women are affected twice as often as men and the age of onset peaks in the fourth decade (Damm & Soennichsen 1964). The incidence of DLE in the population is approximately 5 in 1000.

In four percent of the cases DLE shows affection of other family members. A strong association with certain HLA types has been found. Among others HLA-B7, -B8, -Cw7, -DR2, -DR3 and -DQw1 are reported (Millard et al. 2001).

Furthermore various environmental factors play important roles in disease exacerbation. Traumas, including X-rays, diathermy and chemical burns occur in 11%, stress induces the onset of exacerbation in 12% (Wolfe et al. 1997) and temporarily exacerbation is situated pre-menstrual (13%) and during the summer season in half of the cases (Burns et al. 2010). Aggravation of symptoms in sunlight exposure was found in 68%, which could also be reproduced with UVB and UVA radiation (Bijl & Kallenberg 2006). Antibodies against reovirus RNA can be found in 42% percent of DLE patients (Grunwald et al. 1982).

1.4.1.2 Pathogenic mechanisms

DLE is a complex immunological disease whereas different immunoglobulins can be found at the dermal-epidermal junction in skin lesions. IgG, IgA, IgM and complements can exclusively be found in affected skin, however Properdin, a protein which indicates complement activation and the complements C3 and C4 can also be found in healthy skin (Prystowsky & Gilliam 1975). Besides Properdin is also elevated in serum. These molecules can be presented via immunohistological stainings (Schrager & Rothfield 1977).

The lymphocytic infiltration is dominated by $CD4^+$, $CD8^+$ and HLA-DR^+ cells. Some memory cells could also be distinguished (Volc-Platzer et al. 1993). These skin homing cytotoxic lymphocytes produce a special cocktail of cytokines, consisting of IL-5, IL-10 and predominantly interferons of type 1 which induces auto reactivity. Unfortunately for our understanding of these basic mechanisms the target antigen has not been identified so far (Wenzel et al. 2005).

1.4.1.3 Histopathology and dermatological features

The histological features of DLE cannot be easily distinguished from other forms of Lupus erythematosus. For diagnosis two out of three criteria must be observed under the microscope (Fig. 7; Right).

- Liquefaction degeneration of the basal cell layer of the epidermis
- Degenerative changes in the connective tissue, consisting of hyalinization, edema and fibrinoid change, most marked immediately below the epidermis
- A patchy dermal lymphocytic infiltrate with a few plasma cells and histiocytes, particularly around the appendages, which may be athropic

Besides the milestones of diagnostics for DLE, other less relevant features can also be discovered in the histological picture like epidermal atrophy with relative hyperkeratosis and plugging of the follicular mouths, associated with pilosebaceous atrophy, thickening of the basement membrane of the epidermis and sometimes of small vessels and premature elastotic degeneration of collagen in light-exposed areas (Montgomery & Prunieras 1956; Jerdan et al. 1990).

Clinical symptoms of DLE include Raynaud's phenomenon, chilblains, joint pains and poor peripheral circulation (Rothfield et al. 1963).

Two major forms of DLE can be distinguished. In localized DLE only the head and the neck are affected however in disseminated DLE also other areas of the body can be impaired.

Localized DLE shows alopecia and well defined erythematous patches of diameters up to 15 centimeters (Fig. 7; Left). The lesions have adherent scales and undersurface they show macroscopically visible horny plugs which can block the pilosebaceous glands. These are also called tin-tacks. The surface of the affected skin may look dirty brownish yellow and rough because of the plugs. Non-itching hyperkeratotic papulonodular lesion may also be found on the upper limb. Symptom regression is usually achieved through cribriform scarring, particularly on the face (Burns et al. 2010). Other types of lesions which occur in localized DLE are tumid lesions and annular atrophic plaques (Christianson & Mitchell 1969).

Disseminated DLE is widespread across the trunk and the limbs. It usually appears in cigarette smokers, is associated with severe psychological upset and tends to be very irresponsive to treatment (Parish 1967). Symptoms could be described as papulosquamous or reticulate telangiectasia. Impaired skin can be found either as purplish plaques on hands, palms, toes, the front and the back of the knees or as persistent blotchy telangiectasia on face, neck ears, hands, breasts, heels and on the lateral feet (Crocker 1893).

Scarring occurs under pigmentary disturbances and the typical remaining picture can be described as patches of leukoderma scattered with hyper pigmented spots with calcification enclosures (Kabir & Malkinson 1969).

Nails, mucous membranes and eyes may also be affected but we will not further elaborate (Heronimus 2014; Burns et al. 2010).

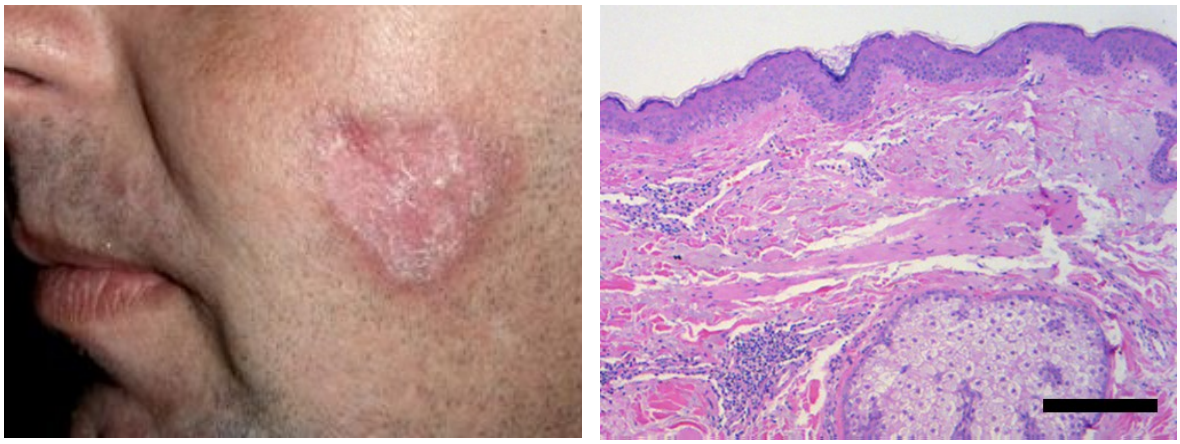

Figure 7. Characteristical DLE lesion on facial localization. DLE punch biopsy.
Disc-shaped erythematous lesion with diameter of 3 cm (Left) (Eastham 2014). Paraffin embedding; Haematoxylin & Eosin; Original magnification x10; Bar represents 100 µm; 3.5 µm longitudinal sections; Dermis with strong lymphocytic infiltration and Acanthosis (Right).

1.4.1.4 Evidence based therapy to date

Patient management can be subclassified into three major parts, general precautions, topical therapy and systemic therapy.

General measures include avoiding mental stress, work overload and fatigue, adjusting sun exposure to an absolute minimum, the use of camouflage creams and smoking cessation (Callen 2004).

Local therapy is the first line therapy and it achieves good responses as single therapy. The objectives are to control and clear skin lesions. First choice drugs are corticoids. 0.025% fluocinolone and 0.1% betamethasone 17-valerate have been proved of value in numerous publications (Barikbin et al. 2009). Intralesional corticosteroid injections may be considered as a recent alternative (Callen 1985). Recombinant Interferon-alpha is another cytokine which has been evaluated intralesionally with success (Martínez et al. 1992).

Other non-active agent dependent focal treatments are cryotherapy, surgical excision, overpainting with trichloracetic and local laser therapy (Burns et al. 2010).

Severely attained patients will also need oral therapy. First line oral treatment should be started with antimalarials such as hydroxychloroquine or chloroquine sulphate 200mg/d. These active agents achieve positive responses in up to 75% of all patients in the first six weeks (Aylward 1993). Possible second line treatments are oral corticosteroids (methylprednisolone, prednisolone), mepacrine or oral thalidomide are also promising and effective but they have a more severe adverse effect profile than antimalarials (Burns et al. 2010).

1.4.2 Psoriasis vulgaris

Psoriasis is a chronic, proliferative, inflammatory skin condition, with well-defined characteristic lesions on various and typical localizations on the body. Its pathophysiology is not fully understood, whereas genetic and environmental elements seem to play an important role. (Burns et al. 2010)

1.4.2.1 Epidemiology and Etiology

The Caucasian race has a prevalence between 1,5% and 3%, however there are significant distribution differences (Burns et al. 2010) compared to other races, which vary considerately. The age of onset is typically scattered in two major peaks, with the early one between 16 and 22 years and a late one between 57 and 60 years (Henseler & Christophers 1985). Due to the fact that sunlight enhances the symptoms of psoriasis, most of the cases are diagnosed in winter and spring months (Bell et al. 1991). Both sexes are equally affected.

Psoriasis has a strong genetic association with HLA-Cw6 and in twin studies it has also been shown that the condition is hereditary. 64% of monozygotic twins compared to 15% for dizygotic twins, which correspond to a 91% of estimated heritability (Brandrup et al. 1982). If both parents are affected there is a risk of 41% for the child to be also affected, whereas with a single parent with psoriasis the risk is only 14% (Henseler & Christophers 1985).

Besides the genetic susceptibility we can also find specific environmental risk factors which can exacerbate *Psoriasis vulgaris*. Among others trauma (Koebener and inverse Koebner phenomenon), infections (post streptococcal and HIV) and drugs should be mentioned. There is strong evidence for certain drug types like lithium salts, antimalarials, beta-adrenergic blocking agents, non-steroidal anti-inflammatory drugs, tetracyclines, angiotensin converting enzyme inhibitors and the withdrawal of corticosteroids (Basavaraj et al. 2010). Furthermore consistent clinical data supports psychogenic factors as one of the major environmental influences on psoriasis and its course (Fortune et al. 1998). It is one of the most diagnosed chronic skin conditions, therefore molecular understanding of its basic processes could help to provide a better health care for individual patients.

1.4.2.2 Pathogenic mechanisms

It has been shown that there is an increase in endothelium of microvasculature in the upper vascular plexus. Epidermal keratinocytes, which are the primary source of angiogenic activity produce a wide array of soluble mediators including vascular endothelial growth factor (VEGF). VEGF is over expressed in psoriatic epidermis(Detmar et al. 1994). Two of the most important molecules which are induced by lesioned skin are E-selectin and intercellular adhesion molecule-1, thus yielding a favorable environment for leukocyte homing. In consequence dermal capillaries are induced through inflammatory processes which involve especially leukocyte homing to produce histamine, neuropeptides, interleukin-1, interleukin-8 and tumor necrosis factor-alpha(Griffiths & Barker 2007)(Heidenreich et al. 2009).

Immunological studies suggest that T-lymphocytes play a crucial role in inflammatory development of psoriatic plaques. Evidence includes: early influx of T-cells into expanding lesions(Baker et al. 1984), strong association with the particular MHC-class (HLA-Cw6)(Brandrup et al. 1982), positive effect of anti T-cell therapy (Kirby & Griffiths 2002)and increased antigen presentation in lesions(Baadsgaard et al. 1989).

Defensins and cathelicidins are found abundantly in the stratum corneum, so that the activation of the innate immune system can be attributed to this factors and its dysregulation provides the means for a yet unknown antigen to establish T-cell expansion and activation in the psoriatic skin (Nickoloff 1999). Several facts are consistent with this theory: limited clonality of infiltrating T-cells, persistence of T-cell clones in plaques over a long time period and identical T-cell clones in tonsils and skin of patients with Streptococcus-associated psoriasis (Diluvio et al. 2006). The principal pathogenic T-cell subset involved in this mechanism are T17 cells, which are generated by stimulation through the cytokine IL-23 occurring in large numbers in psoriatic plaques (Lee et al. 2004).

1.4.2.3 Histopathology and dermatological features

Three principal histological features can be distinguished in psoriatic skin (Fig. 8; Right):

- Epidermal hyperproliferation with loss of differentiation
- Dilatation and proliferations of dermal blood vessels
- Accumulation of inflammatory cell, particularly neutrophils and T lymphocytes

The perceived increase of proliferating cell compartments takes place in the basal and suprabasal levels of the epidermis and is independent from shortened cell-cycle time, whereas the active cycling cell number is sevenfold elevated (van de Kerkhof 2003).

One of the most striking clinical features are the Koebner phenomenon and its counterpart the reverse Koebner phenomenon. Whereat due to a local traumatic reaction psoriatic plaques appear at the site of previously uninvolved skin and show the same shape as the traumatic injury. The Koebner phenomenon appears in average in seven to fourteen days and it is present in more than two third of psoriatic patients (Farber & Nall 1974). Reverse Koebner describes when plaques disappear upon the lesioned stimulation of skin injuries on the psoriatic concerned loci and only about 25% affected patients show this type of reaction (Eyre & Krueger 1982).

The morphology of psoriatic lesions are very characteristic and could be described as salmon pink, pyknic and surmounted by a silvery white scaling, which varies in thickness (Fig. 8; Left). The lesions are slightly elevated from the skin level, possess well defined borders, usually show Auspitz's sign and are mostly uniform concerning their general appearance. The psoriatic discs vary in size from one to several centimeters diameter, form irregular patterns and may converge. The plaques may be surrounded by a lighter peripheral zone the, ring of Woronoff and they may show pruritus in certain cases. (Griffiths et al. 2007; Almqvist & Wiksell 1967)

Psoriasis has more specialized phenotypes such as guttate psoriasis, inverse psoriasis, psoriatic arthritis etc. which we have voluntarily chosen to omit in this chapter since our work concerned only lesions from *Psoriasis vulgaris*.

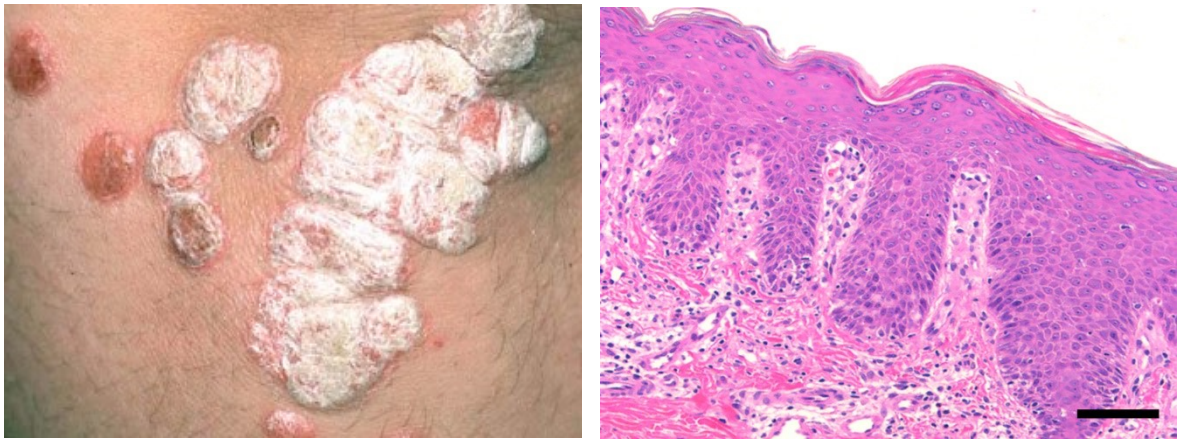

Figure 8. Characteristical psoriatic lesion on knee. Punch biopsy of psoriasis.
Salmon pink well circumscribed plaque with 5-6 cm diameter and silvery white scaling (Left) (Griffiths et al. 2007). Paraffin embedding; Haematoxylin & Eosin; Original magnification x10; Bar represents 100 µm; 3.5 µm longitudinal sections; Epidermal hyperproliferation with deep krypts and lymphocytic infiltrates are visible (Right).

1.4.2.4 Evidence based therapy to date

Psoriasis vulgaris is widespread dermatological pathology with yet unresolved genesis. Therefore physicians throughway the last decades approached its treatment with various concepts. We will treat only well-established pharmaceutical agents and omit novel drugs, albeit many more active agents which are currently in phase 3 trials should be mentioned.

First line treatment should consist of tar preparations, Dithranol, topical corticosteroids, vitamin D3 analogues and UV-therapy if possible. Vitamin A analogues were omitted by choice.

Coal tar is an enormous mixture of thousands of different substances whereas only 400 of them are known and these comprise only 55% of tar by weight. The quality and quantity of this mixture varies in every center, therefore a quantification of results obtained with tar treatment is simply impossible (Gruber et al. 1970). It has been shown, that tar has a positive effect on psoriasis, whereas neither its optimal quantity nor duration could be fixed due to the above mentioned fluctuations (Comaish 1981).

Dithranol and thereby its active agent chrysarobin, a tree bark extract are applied only to psoriasis affected parts of the skin (Runne & Kunze 1985). The initial concentration once per day is 0.05% whereupon it can be increased cautiously up to 4% according to response and tolerance. Dithranol is exceedingly irritant, lacks systemic toxicity and it leaves brownish staining on the loci of its appliance, which disappear in short time period (Vella Briffa et al. 1978).

Topical Corticosteroids in appropriate concentrations are still treatment of choice for certain parts of the body as face, neck, flexures and genitalia. It is also used as second line treatment if tar, dithranol or Vitamin D anologues fail to show effect (David & Lowe 1989). Active agents are clobetasol propionate, betamethasone dipropionate, halcinonide and fluticasone propionate. Adverse effects can be a suppression of plasma cortisol levels, tolerance and other typical cushingoid symptoms. Consequently topical corticoids should be applied in low dose and frequency (Katz et al. 1987).

In current therapies four different Vitamin D Analogues are applied: 1,25-dihydroxivitamin D3 (calcitriol), calcipotriol, 1,24-dihydroxivitamin D3 (tacalcitol) (Van de Kerkhof et al. 1996) and 1-alpha, 25-dihydroxy-22-oxacalcitriol (maxacalcitol) (N.barker et al. 1999). Detailed analysis has shown that their mechanism of action is derived from two different levels. Firstly they influence proliferation and differentiation of epidermal keratinocytes via the vitamin D receptors (Langner et al. 1996). Secondly they manipulate the immunological features of psoriatic plaques towards a Th2 shift of the cytokine profile of the primarily Th1 cells (Kang et al. 1998). The main side effects of vitamin D analogues are local irritation and alterations of the systemic calcium homeostasis with hypercalciuria and hypercalcaemia (Bourke et al. 1997).

Narrow-band UV-therapy at 311nm turned out to be the most effective light therapy for *Psoriasis vulgaris*. It is superior to conventional broad-band 290-320 nm, producing longer remission, lower incidence of burning and lower risk of UV carcinogenesis (Man et al. 2005)(Picot et al. 1992). Its major side effect consists of asymptomatic or painful blistering at the sites of psoriatic lesions during a therapy course. It usually resolves with continued narrow-band treatment (George & Ferguson 1992). Elaboration on Psoralen/PUVA was voluntarily omitted.

Despite the fact that topical therapy demonstrates quick remissions and generally acclaimed positive results it should always be accompanied by systemic therapy because thereby higher levels of synergy between two reagents could be attained (Heronimus 2014). Most notable active agents would be Metotrexate, Hydroxycarbamide, Acitretin and Ciclosporin (Burns et al. 2010).

1.5 Mouse versus human data

Due to the fact that recovering prospective experimental data from human LCs is a sheer impossible endeavor (gene knockout experiments or DNA-modification), scientist often walk alternative paths. In the case of LCs and ISDs it consist mainly in mouse models and in vitro investigation. From murine prospective experiments we could derive excessive information about the behavior of LCs and DCs in various circumstances. From most recent murine studies we learnt that murine LCs suppress exaggerated inflammation reactions of the skin through suppression of IL-6 when confronted with innocuous commensal antigens.(Scholz et al. 2015) An independent group found similar results whereat murine LCs produce IL-10 suggestive of an immunosuppressive role and especially an anti-inflammatory role during active psoriasis could be considered (Glitzner et al. 2014). (Yao et al. 2015) attributed murine LCs as very effective foreign antigen presenting capacity and therefore the distinct function of eliciting germinal center B cells and an adequate humoral immune response.

This new findings were achieved through the sacrifice of numerous mice and it was anticipated that results could be transmit one-to-one from murine models to human conditions thereby enabling the discovery of new suitable, specific and effective molecular therapies. Unfortunately human LCs turned out to be less comparable to murine LCs concerning their transcriptional program and thereby their effective protein outcome. Concerning their genetic profile they resemble more another subtype of murine DCs, namely $XCR1^{+}CD8A^{+}$ $CD103^{+}$ cells than murine LCs. This new findings implicate that in the past by focusing on a few exterior visible hallmarks like $CD207^{+}$ or other proteins instead of the transcriptional products researchers conducting comparative interracial studies could have been distracted from evaluating the physiology of the functional "LCs" in other species. (Artyomov et al. 2015)

The question which remains to be asked is about the validity or obsolescence of murine and therefore also other animal models for human phenotypes.

The success of alternatives like in vitro models is also limited due to its obvious debilities such as lack of systemic interactions and artificial environment with synthetic stimuli. Consequently our choice of performing retrospective immunohistological staining seems to be more than just a reasonable decision, even by taking in account that only a retrospective approach was feasible.

1.6 Hypothesis and Questions

We replicated the immunohistological data of (Yasmin et al. 2013) concerning the BMP7 and the TGF-β1 in adult skin and therefore we addressed multiple questions in the context of our hypothesis that BMP7 is a strong proliferative cytokine for LCs in the basal layers whereas TGF-β1 acts as a conserving signal in the suprabasal layers of the skin in steady state.

Specific aims

Aim 1: To establish a standard staining protocol inclusively a well-designed antibody panel for the quantification of TGF-β superfamily members and their cellular signaling in miscellaneous skin samples.

Aim 2: Evaluate whether the pSMAD2/3 and pSMAD1/5/8 which are downstream signaling molecules of the canonical TGF-β1 and BMP7 pathways respectively, are expressed in coherent histological patterns in the skin.

Aim 3: Evaluate whether inflamed skin shows a perturbed SMAD and/or LC marker expression.

Aim 4: Provide a comprehensive overview of the analyzed cytokines regarding LCs in inflammatory skin diseases.

2 Results

The staining procedure was done according to the protocol described in the methods chapter. Initially, we started for each sample by performing a Haematoxylin & Eosin [H&E] staining, so that we were able to evaluate the existence or absence of pathological features in ISD samples and physiological features in healthy samples. Based on this observations we restricted our initial pool of healthy specimens.

2.1 Different expression patterns of TGF-β1 in healthy and inflamed skin

We reproduced histological data from (Yasmin et al. 2013) and found that TGF-β1 is expressed in the epidermis in a special pattern with increasing concentration from the basal layer to the suprabasal layers in healthy postnatal skin (Fig. 9). We performed an intensity analysis on digital microscopy pictures (not shown) and a verified thereby the described gradient.

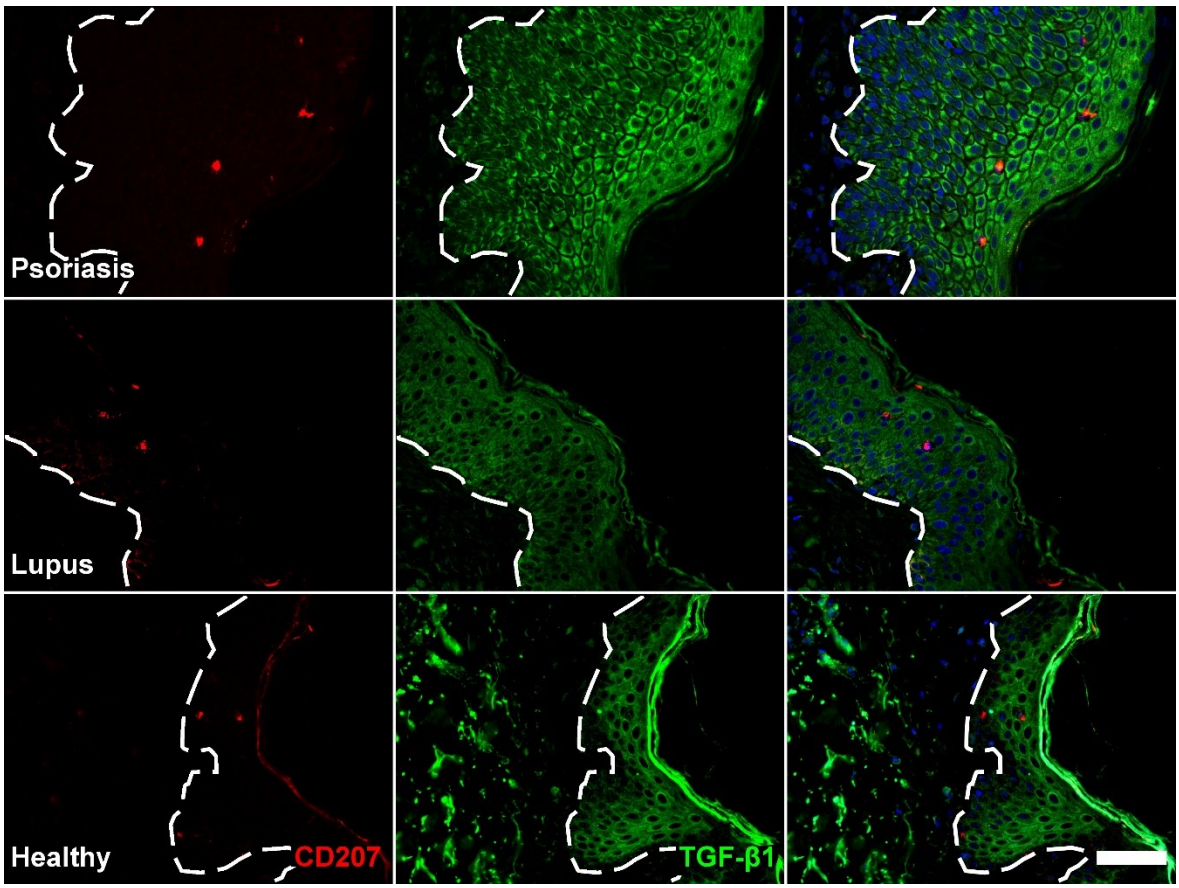

Figure 9. TGF-β1 immunohistology of healthy and inflamed human skin
Immunofluorescence double-labeling was performed on paraffin sections (3.5 µm) of adult human skin. Samples were analyzed for CD207 (red) and TGF-β1 expression (green). Nuclei were visualized with DAPI (blue). The dashed lines represent the dermal–epidermal junction. Data are representative of 6 independent experiments. Scale bar: 100 µm.

However, pathological samples display a loss of the typical gradient and show an augmented intensity of TGF-β1 in all epidermal layers. In some specimens the gain of intensity is hard to distinguish due to the fact that the epidermis shows a major acanthosis with a manifest hyperplasia of the germinal layers and therefore less degraded nuclei in the outer layers than in the healthy skin, which are bigger in size and appear therefore bluer.

2.2 Inverse activation of BMP7 pro and active form

Stainings were done with two different highly specific antibodies against BMP7, one targeted the preform and the other bound to the active compound of BMP7. We observed an absolutely non-overlapping expression of these two forms of the same protein in the healthy specimens (Fig. 10). The active form can exquisitely be found in the epidermal basal layer in contrast to the latent protein before activating cleavage, which is found in the suprabasel layers. Interpretation will be given in the discussion session.

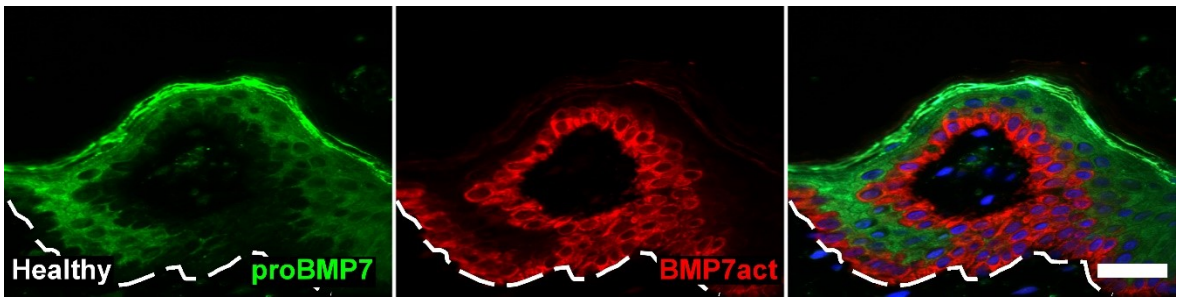

Figure 10. Expression pattern of BMP7 pro-peptide and cleaved active form in healthy human skin
Immunofluorescence double-labeling was performed on paraffin sections (4 µm) of adult human skin. Samples were analyzed for BMP7act (red) and proBMP7 expression (green). Nuclei were visualized with DAPI (blue). The dashed lines represent the dermal–epidermal junction. Data are representative of 6 independent experiments. Scale bar: 25um.

The inflamed skin indicates a loss of the distinct separation barrier between these two forms and showed a broader distribution of the active form (Fig. 11A). We could further observe an all over-expression pattern of the pre- and the active form in the psoriasis samples (Fig. 11), although the lupus specimens kept their typical appearance (not shown). The distribution of the labeled cytokines have been much more diffuse compared to the healthy samples.

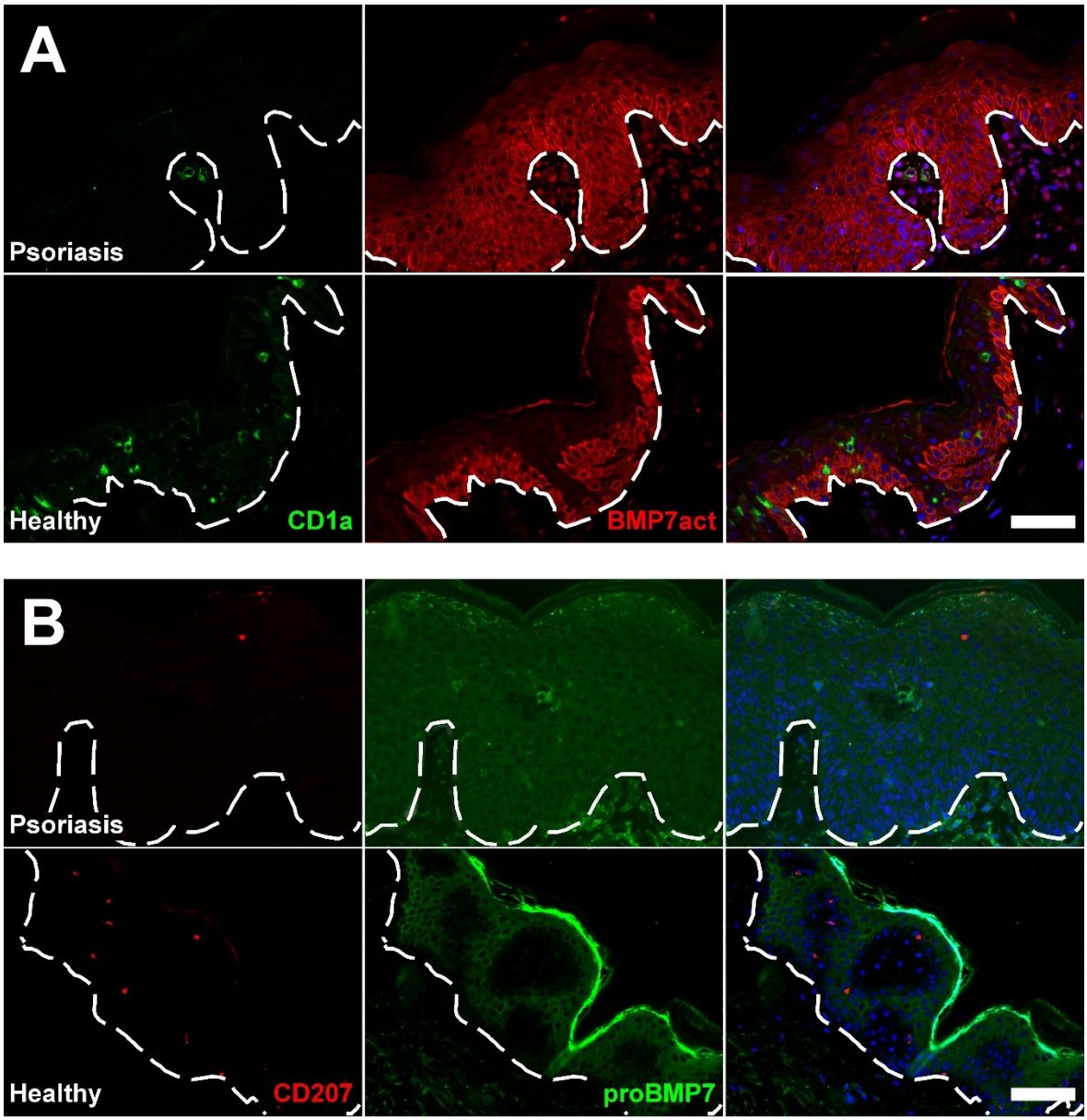

Figure 11. Loss of characteristical staining pattern of BMP7 in psoriasis
A) Inflamed psoriatic skin is characterized by strong upregulation of BMP7act expression.
B) Distributional changes in the expression pattern of proBMP7 in psoriasis. Immunofluorescence double-labeling was performed on paraffin sections (3.5 µm) of adult human skin. Samples were analyzed for A: CD1a (green) and BMP7act (red); B: CD207 (red) and proBMP7 expression (green). Nuclei were visualized with DAPI (blue). The dashed lines represent the dermal-epidermal junction. Data are representative of 3 (psoriasis) and 6 (healthy skin) independent experiments. Scale bars: 50 µm.

BMP7act showed a similar pattern with TGF-β1 whereby the overlapping area was reduced to a minimal amount likewise to proBMP7, TGF-β1 was not accordingly intense (not shown).

In healthy skin no overlapping at all means some enzyme is present in high numbers in basal layers where it cleaves preform to active form, in pathological skin no clear gradient was observed and an all over-expression from the stratum basale through the stratum corneum could be described.

2.3 BMP4 and BMP9 deviations

BMP4 in physiological state has a constitutional gradient which starts with weak intensity in the basal layers (Stratum basale), grows more intense in the middle suprabasel layers (Stratum spinosum) and loses brightness in the highest still nucleated layers (Stratum granulosum), occasionally with stained spots in the nuclei (Fig. 12).

In pathological specimens we identified an all-over expression pattern of the BMP4 and no gradient could be defined. Equally a few cases showed a potential induction of the BMP4 quantity (Fig. 12).

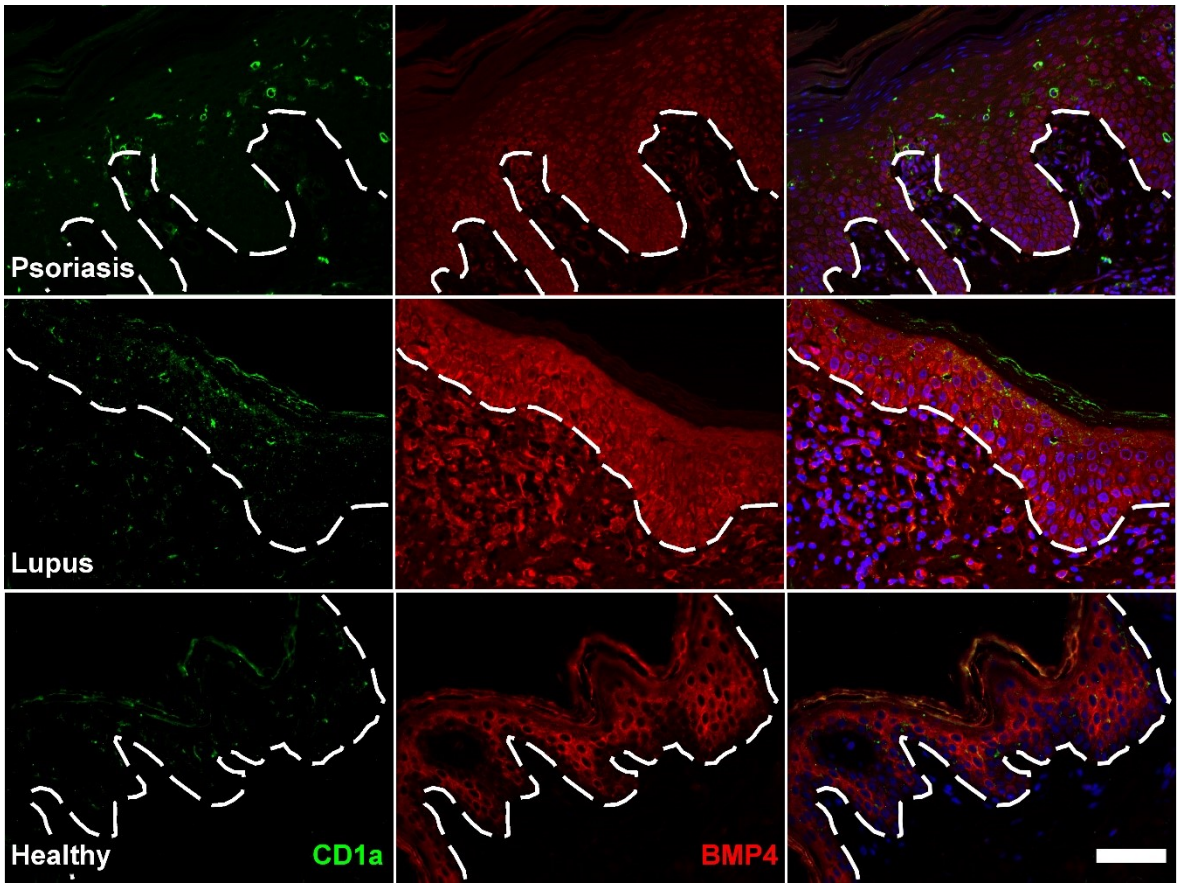

Figure 12. Loss of typical BMP4 low-high-low gradient in ISD
Immunofluorescence double-labeling was performed on paraffin sections (3.5 µm) of adult human skin. Samples were analyzed for CD1a (green) and BMP4 expression (red). Nuclei were visualized with DAPI (blue). The dashed lines represent the dermal–epidermal junction. Data are representative of 6 independent experiments. Scale bar: 50 µm.

Compared to other BMP intensities, BMP9 is particularly weakly expressed, nevertheless it is present in our findings, contrary to former publications in the field (Fig. 13) (Botchkarev 2003). Moreover it seems to be particularly specific for the epidermis as there is no staining at all visible in the dermis. Pathological donors show an increase in BMP9 quantity. This trait can especially be seen in psoriatic skin.

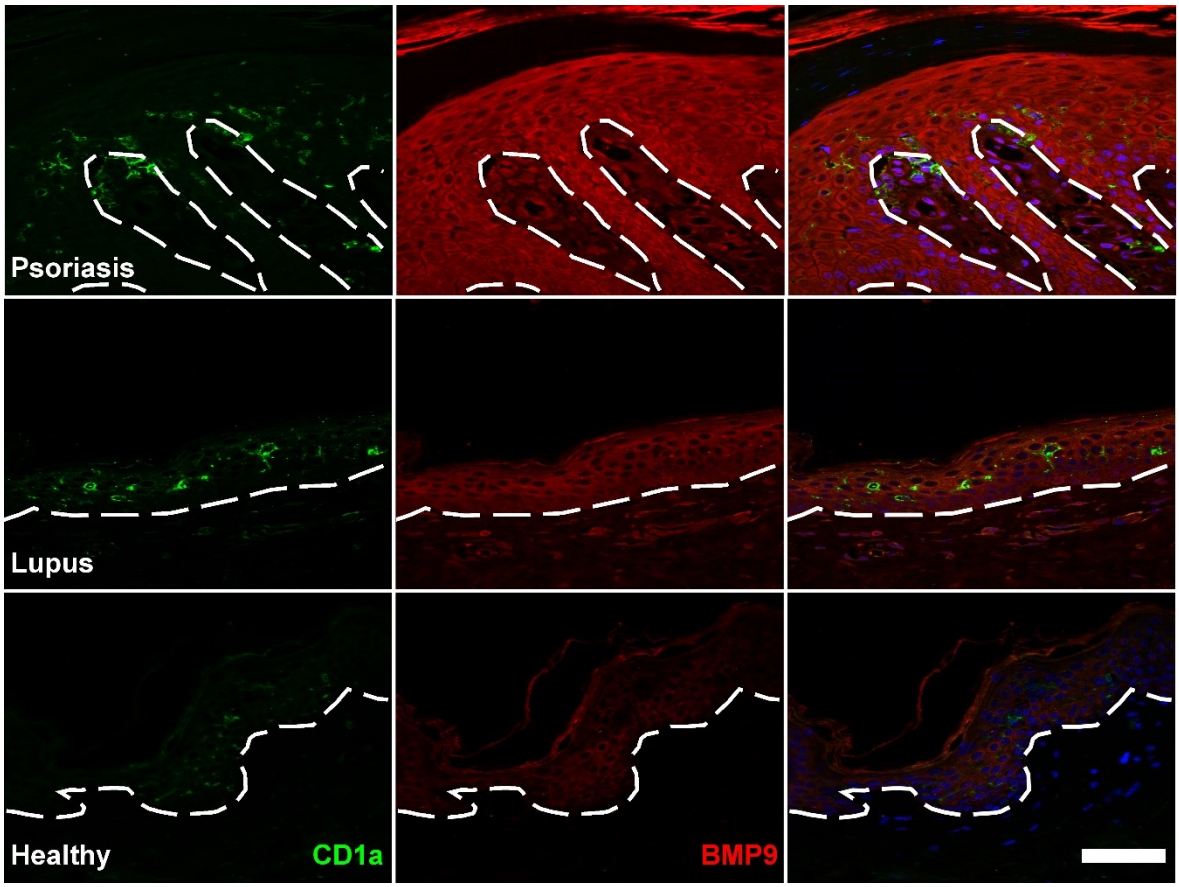

Figure 13. BMP9 expression is upregulated in epidermis from psoriatic lesions.
Immunofluorescence double-labeling was performed on paraffin sections (3.5 µm) of adult human skin. Samples were analyzed for CD1a (green) and BMP9 expression (red). Nuclei were visualized with DAPI (blue). The dashed lines represent the dermal–epidermal junction. Data are representative of 6 independent experiments. Scale bar: 50 µm.

2.4 Strong psoriatic induction of NOGGIN

The physiological shape of NOGGIN in the healthy skin can be described as a heterogeneous distribution over all layers of the epidermis with hotspot-like intensity elevations in certain areas (Fig. 14).

In psoriasis we can see a strong increase in the expression of NOGGIN, whereas cutaneous lupus shows no factual alteration of its pattern. The described blotchy stains could also be found in Lupus samples. We performed standardized microscopy pictures to compare intensity levels in the altered sections and found that psoriasis induces the expression of NOGGIN.

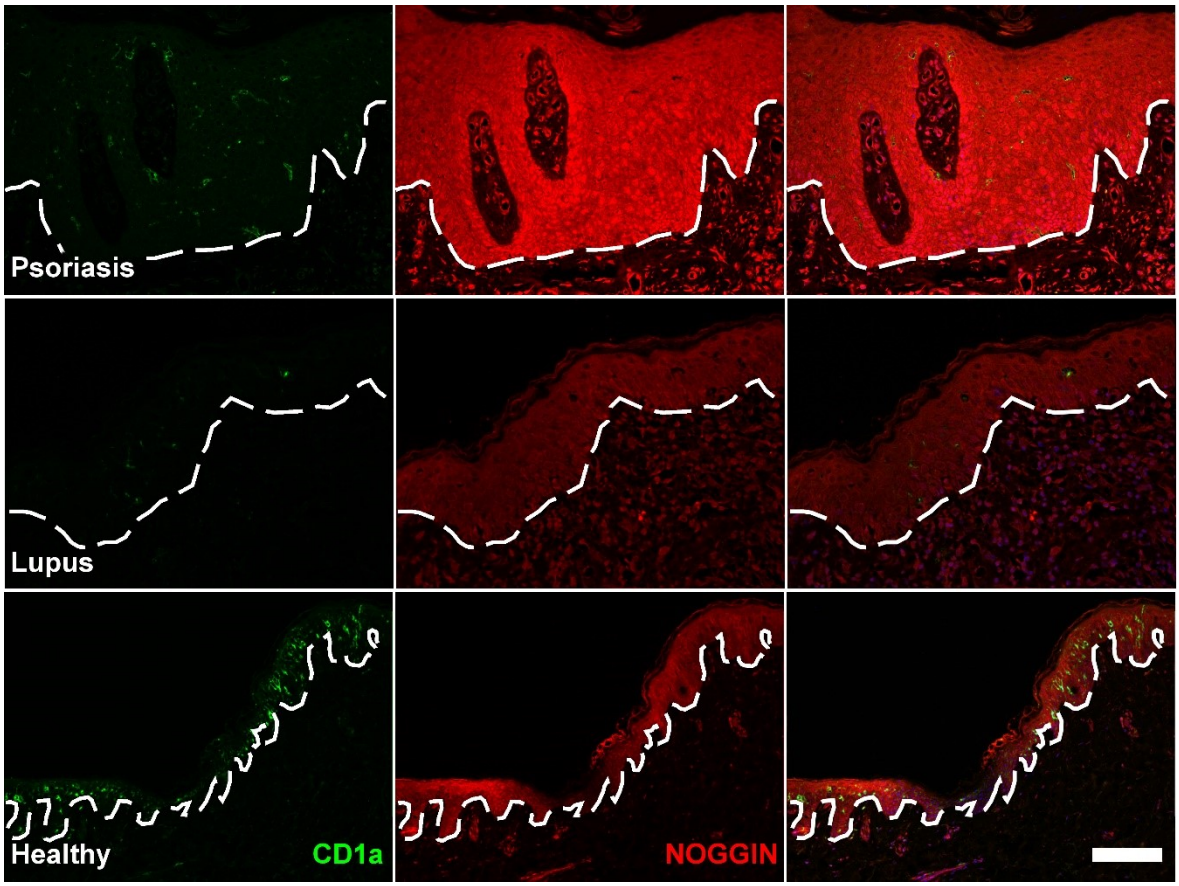

Figure 14. Psoriatic induction of NOGGIN
Immunofluorescence double-labeling was performed on paraffin sections (3.5 µm) of adult human skin. Samples were analyzed for CD1a (green) and NOGGIN expression (red). Nuclei were visualized with DAPI (blue). The dashed lines represent the dermal–epidermal junction. Data are representative of 6 independent experiments. Scale bar: 100 µm.

2.5 Unchanged pSMAD2/3 and pSMAD1/5/8

Phosphorylated SMAD stainings are very recognizable because the cells are only dyed in their nucleuses, so the affected cells show a blueish reddish core. Per contrast the cell bodies show no color at all. Keratinocytes are consecutively active for both SMAD signal pathways so we could observe their positivity as a reference point (Shimizu et al. 1998).

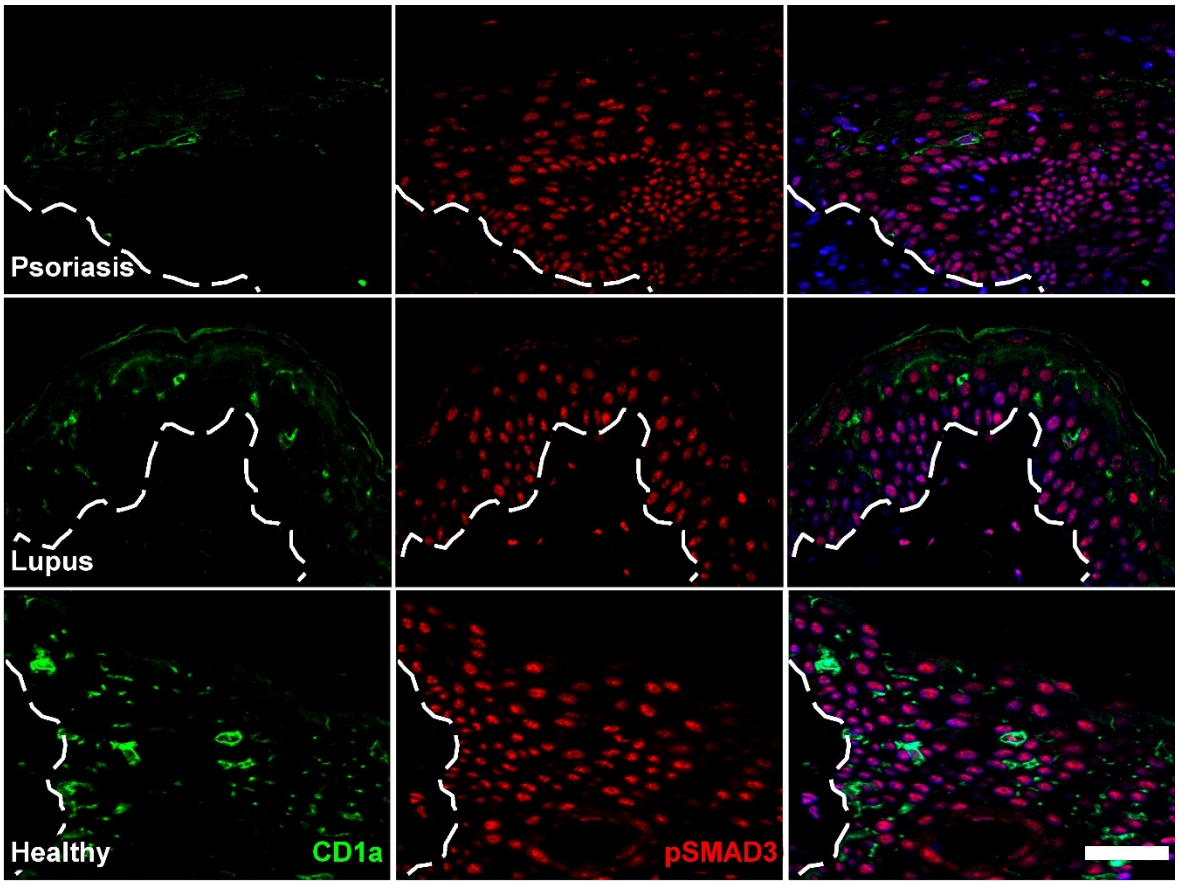

Figure 15. pSMAD3 in healthy and inflammatory skin
Paraffin embedded tissue; Immunofluorescent staining; Original magnification x20; Bar represents 50 µm; 3.5 µm longitudinal sections; Data is representative of three (Top, Middle) and six (Bottom) independent donors; CD1a (green); pSMAD3 (red); DAPI (blue); No consistent deviation of pSMAD 3 could be observed

Unexpectedly no significant uniform changing trend could be observed between ISD and healthy samples (Fig. 15). The SMAD stainings, although done with great effort and precision remained inconclusive on immunohistological level due to inconsistency in their staining pattern. Identified and counted Langerhans cells were also mainly positive for both pSMADs with very few exceptions. pSMAD1/5/8 stainings are not presented.

2.6 LC phenotype variation in ISDs

Generally lower numbers of LCs could be observed in pathological skin samples. Interestingly we also found one exception whereby in one of the psoriatic tissues a reactive Langerhans cell proliferation with far higher quantities than in the control samples could be observed.

Both proteins could be observed in LCs, however localization of CD207 was restricted to the cell body in opposite to CD1a which was also staining the dendrites to a greater extent.

Another immunohistological feature was the extensive phenotype loss of CD207 in comparison to CD1a, which was conserved unaffected. The last striking observation was the migration of LCs into higher layers within the skin. In physiological state LCs are mainly located in the basal layer, contrary to ISDs where a shift of the already scarcer LCs towards the more exterior layers can be noted.

2.7 Schematic summary of results

The last representation is a general approach of our most important results in overview (Fig. 16). This representation should serve as a one glance representation of the distribution pattern of selected TGF-β family members in the human skin.

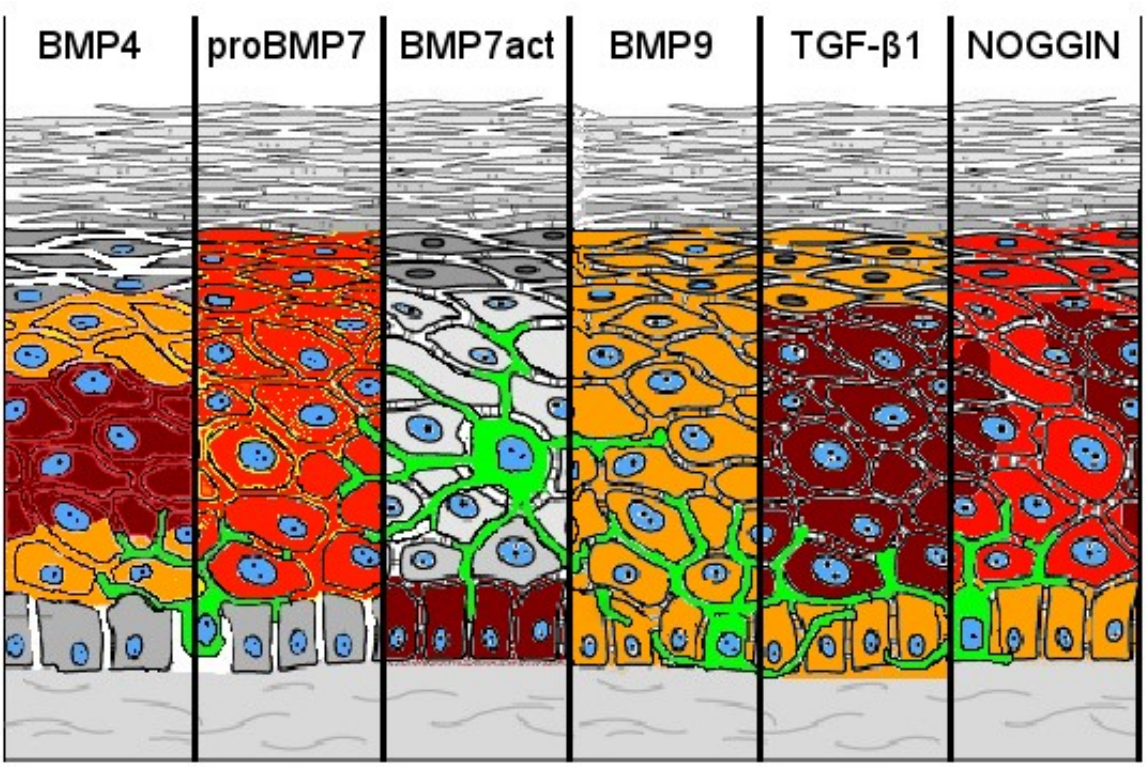

Figure 16. Expression pattern of selected TGF-β superfamily members in steady-state human epidermis
The different skin layers are shown (stratified human epidermis) schematically with intensities corresponding to the concentrations analyzed by microscopy. LCs (green), Intensities (white to dark red) Data is representative for 6 independent donors;

3 Discussion

Despite the increasing information and understanding about the function of Langerhans cells and their pivotal role in skin conditions, particularly inflammatory skin diseases more experimental set ups need to be realized before we could claim realistically to apprehend basic pathophysiological and biochemical processes behind the symptoms.

The immunohistological overview we performed was a first step towards the comprehension of only one of countless factors involved in ISD. The TGF-β superfamily accomplishes an irremissible role in regulating the immune system of the epidermis. Our stainings helped to gain little insights into the still blurry picture of the fine tuning of certain TGF-β family members in health and disease.

3.1 Interpretation of results

The challenge in immunohistological stainings consists in finding the flawless explanation for certain phenomenon of differentially expressed proteins. Moreover an excellent ability of visual observation is necessaire to perceive common staining trends and discriminate them against distracting fluctuations on the highly variable samples.

3.1.1 Bio-active BMP7 restricted to basal keratinocyte layer in steady-state but not during inflammation

The most striking result of our series was the clear separation barrier between active and proBMP7. We deduced that the converter from the pro to the active form, namely a substilin-like convertase, has to be restricted to the basal layers in healthy skin samples. Interestingly this equilibrium is destroyed in ISD and also the clear border between the two isoforms of BMP7 disappears. As for the known functions of BMP7, the induction of proinflammatory cytokines, IL-10, the capacity of T-cell immunostimulation and especially the IFN-γ production inciting preferably a Th1 response (Yasmin et al. 2013) are largely compatible with former publications of psoriasis pathogenesis in molecular detail where induction of INF-γ (Campanati et al. 2014) and preferential Th1 response (Griffiths & Barker 2007) are pointed out as a key feature. Therefore we suggest BMP7 as a gap linking explanation in the pathogenesis of psoriasis where it is upregulated in order to induce this specific cytokine production profile and thereby shape the skin condition. This would suggest a possible new treatment line in *Psoriasis vulgaris* with BMP7 antagonizing agents. One of the questions which still remains to be answered is the cause of BMP7 dysregulation or may it be the source of the skin condition itself.

3.1.2 Upregulation of BMP4 and BMP9 in inflammation

We found that BMP4 and BMP9 were equally upregulated in inflammation and lost their specific staining pattern from the healthy epidermis.

Typical pathohistological common features of ISD like angioneogenesis and thickening of the epidermis are currently unsatisfactory justified by explanations on molecular level. BMP4 is known to induce angiogenesis and capillary sprouting in certain cell lines. Moreover BMP4 is a prominent inducer of CGRP in neural cells which lay their axons adjacent to LCs (Madva & Granstein 2013). BMP4s all-over expression pattern could be considered hypothetically as the reason for augmented angiogenesis in ISD. Other directs effects of CGRP on the epidermis can also be seen, such as vasodilatation, recruitment of inflammatory cells. The last important action of CGRP is a deviation of the immune response of Th1 reactions to preferably Th2 responses.

However the induction of BMP9 is difficult to explain, particularly for the reason that strong evidence suggest that one of his functions is an inhibition of neoangiogenesis by the decrease of VEGF production. On the contrary in ISD (shown in *Psoriasis vulgaris*) we find an upregulation of VEGF production through which the neovasculogenesis is mediated. A possible explanation would be that BMP4s effect outweighs BMP9s. For further elaboration of this argument quantitative experiments should be performed measuring the amount of the two proteins and their relative impact on the epidermis.

3.1.3 Increase of NOGGIN expression pattern during inflammation

Deducing from our observations we can say that, the BMP family members which were in our focus have generally been augmented in ISD. Contemplating the elements of the TGF-β superfamily as generally being upregulated by ISD we could hypothesize that the increase of NOGGIN implies an unspecific response moderating the absolute deregulation of the BMP family (one part of the TGF-β family) comparable to a protective security mechanism preventing over-signaling. Other partial antagonist of the TGFβ superfamily like follistatin or Snon could also be increased in a similar pattern thereby blocking different branches of the TGF-β family and completing their inhibitory and regulative task.

3.1.4 Unchanged SMAD expression in health and inflammation

Although our results on SMAD stainings showed no satisfactory common trend towards one or the other R-SMADs a few questions remain to be asked. Firstly, how is the transcriptional function of R-SMADs exactly transmit when it acts as a second messenger for proteins which sometimes have the exact opposite protein product (Mueller & Nickel 2012)?

Two possible justification can be envisaged. The transcription is regulated through its co-stimulation by other proteins and only the accurate activating mix produces the specific response. In conclusion we would then have to admit that in vitro stimulating experiments with recombinant solo protein stimulation shows a completely distort picture of the physiological reality.

Alternatively we can consider that every TGF-β superfamily member acting through the canonical SMAD pathways also activates parallelly other co-transcriptional factors which in their mix are so unique that the protein output produced by the complex sum of transcription factors also becomes unique and thereby the signaling proteins function. In this case it would be an imperative to discover and identify all non-canonical pathways for the TGF-β superfamily members in order to fully understand their harmonies.

3.1.5 General LC phenomena and TGF-β1 overexpression during inflammation

LCs play a role as first line defense or gatekeepers of the immune system in the skin. When they encounter pathogens in the skin they decide whether to let them pass through unaffected by tolerating them or to identify and mark them as enemies. One of their receptors responsible for material uptake and degradation is the CD207 molecule.

The intracellular distribution bias between CD1a and CD207 can be enlightened when we take in account their distinct functions. For CD207 (mannose like pathogens) is an information collecting tool, whereas CD1a is an information presenting tool (lipid antigens). The cell bodies of LCs are usually found in the basal layers, contrary to the dendrites which are spread all over the epidermis. This distribution could be aimed at not being too eager to react to antigen stimulation. So the level of presentation is only reached when the pathogen manages to penetrate into deeper areas of the epidermis.

In ISD LCs in general are diminished by reason of migration into the lymph nodes and activation of the adaptive immune system. We have also shown that the few remaining LCs change their distributional pattern from strictly suprabasal to more holistic in the epidermis thereby adapting to the newly created acanthosis. This migration into the higher layers could be directed by the observed TGF-β1 overexpression (and/or the BMP7 upregulation) which seems to be needed as a continuing signal for LC maintenance after their development from $CD34^+$ cells.

From our presented findings we deduce that the disappearance of CD207 in ISD identifies the protein as a peacetime protein. It is expressed when the skin is not under inflammation so that it can monitor its surroundings and if required present special antigens after migration into the lymph nodes to T-cells. Contrary when under attack during inflammatory processes, CD207 in the LCs which are still situated as outposts in the epidermis is probably internalized, degraded and downregulated in order not to reinforce unnecessary immune tolerance anymore. The loss of CD207 implies a loss of the characteristic Birbeck granules.

It was a long debated question whether LCs replicate at all through mitosis, till Hashimoto (Miyauchi & Hashimoto 1987) showed in 1987 through a skin depleting experiment that LCs recover mainly through mitosis. We could establish further direct prove through the immunohistological picture of one LC in the middle of the telophase of mitosis (Fig. 17).

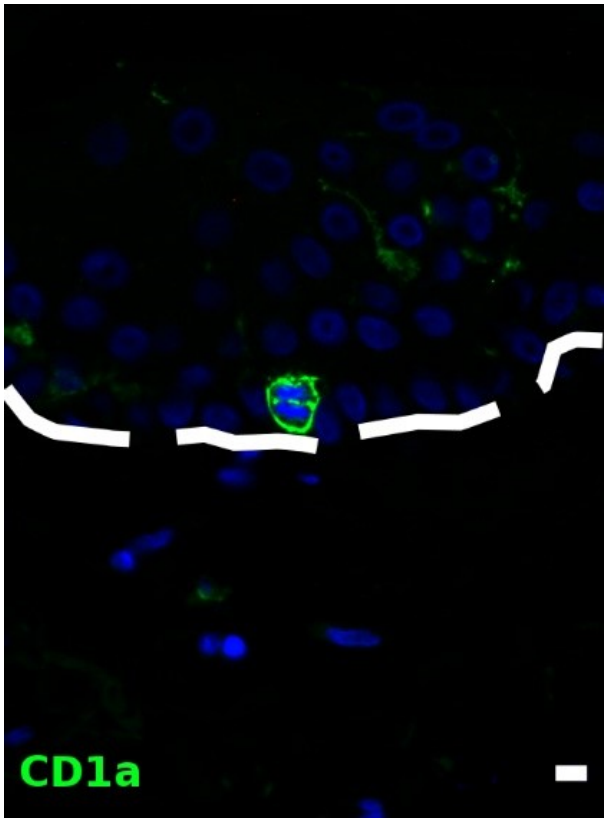

Figure 17. Langerhans cell in mitosis
Immunofluorescence labeling was performed on paraffin sections (4 µm) of adult human skin. Samples were analyzed for CD1a. Nuclei were visualized with DAPI (blue). The dashed line represents the dermal–epidermal junction. Scale bar: 5 µm.
Typical cell picture of mitotical telophase with two nuclei separating along the cell equator line

3.2 Challenges on corresponding work field

In the end we would like to add a few minor remarks impacting the experimental part of this work.

One of the major defaults in the classification was to consider DLE and Psoriasis as one entity solemnly by the fact that they are inflammatory skin diseases. Eventually, judging by the discrepancies perceived in our immune histological stainings the differences between those two skin conditions seem to outweigh by far their communalities.

Another issue was the correct choice of antibody. Almost every antibody purchased had to be replaced in the course of the experimental setting due to inappropriate dying or lack of specify. In the end we achieved good quality stainings in the healthy skin but still some of our pathological samples gave very low intensity differences between negative and positive tissue.

The amount of pathological samples analyzed (three independent donors for each condition) was unfortunately not enough to talk about statistical significance much less evidence. Hence our results should be considered with caution and only as preliminary data. Currently we are working on a follow up with more samples from just the *Psoriasis vulgaris* and quadruple stainings. Further data on TGF-β superfamily interaction needs to be established with different biomedical approaches in order to verify these results.

Control samples were taken from independent healthy donors as described in the methods section. A preferable and more state-of-the-art control would have been punch biopsies from the same affected patients but from non-lesioned loci. Unfortunately such samples were not available and could not be provided due to ethnical implications. Furthermore our “healthy” controls were partly taken from punch biopsies out of resection borders from supposed malign neoplasms which then were diagnosed already unaffected. Although no visibly disturbed histological features could be diagnosed nothing can guarantee the cellular integrity and a correct unaffected molecular mode of operation behind the appearance.

A minor flaw in histological analyses were different thicknesses of samples. While the institute of dermatology cut their samples with 3.5 µm we were only able to perform 4 µm microtome slides. Therefore relative comparing was impeded and our self-prepared healthy samples could only be applied as a reference for distribution of the mentioned proteins in healthy skin.

The last weakness of this work is the relative paucity of quantitative data. Unfortunately we had no software to digitally compare intensities in quantitative ways, so absolute numbers were reduced to a minimum by counting affected sample numbers versus healthy ones. On the contrary we presented multiple visual data-sets thereby explicitly emphasizing our arguments and conclusions.

The last issue which hindered this work was the general missing consent in scientific literature, especially considering the promiscuously activating TGF-β family members. Therefore it is very hard to describe exactly which receptor interacts with which protein because contradictory information in nearly every paper is mentioned about this topic.

4 Materials and methods

The present work is basically an immunohistological approach, with an extensive literature research background and a broad imagery analysis. The holistic histological methodology of this highly specialized topic is new on the field and there could not be found any previous comparable overview of these TGF-β superfamily members in the epidermis. Three antibodies were purchased with a grant received from the Medical University of Graz "Förderungsstipendium". The rest was purchased from the budget of Professor Strobls research group.

Material overview	
Machines	
Histology Microscope	Motic BA310
Microscope Camera	Motic: MoticamPro 285B
Fluorescent Microscope	Leica DM4000 M
Microscope Lights	Leica: Kübler Codix
Microscope Camera	Leica DFC300 FX
Microtome	Microm HM 335 E
Water bath	Medite TFB 35
Heating plate	Medite OTS 40
Microtome blades	Feather Microtome Blade R35
Paraffin embedding machine	Medite TDO SAHARA, Microm AP280-1, Microm AP280-2, Microm AP280-3
Cooling room	Viessmann
Scales	Sartorius
Microwave	Silva Homeline
Antibodies:	
Anti-BMP4	Sigma Aldrich SAB4200516
Anti-proBMP7	Abcam ab56023
Anti-BMP7 act	LSBIO IHC-plus™ LS-B4567/39975
Anti-BMP9	Abcam ab35088
Anti-TGF-β1	NovusBio NBP2-22114
Anti-pSMAD3	Abcam ab118825
Anti-pSMAD1/5/8	SantaCruz sc12353

Anti-CD1a	NovusBio NBP2-34313-0.1mL
Anti-CD207	Sigma Life Science HPA011216
Anti-NOGGIN	Abcam ab1605
Anti-Rabbit-Cy3 (goat)	Jackson Immuno Research laboratories Inc. 111-165-144
Anti-Mouse-Dy488 (horse)	Vector laboratories DI2488
Reagents	
Haematoxylin	Merck 1.05174.0500
Eosin	Merck Art. 1345
Xylol	Roth 9713.3
Staining kit	Ultravision LP Detection System: TL-060-HL Thermofisher
Mounting Medium	Fluoroshield F6182-20ML
Antibody diluent	DAKO S3022
Pap pen	DAKO Pen S2002
NaCl	Sigma S3014
KCl	Sigma P9541
Tween20	Sigma-Aldrich P1379 Lot: SZBC2920V
Tris Base	Sigma Life Science T6066; Lot: SLBC1905V
di-Natriumhydrogenphosphat	Roth P030.2
Tools:	
Pipettes	Eppendorf
Microscope slides	ThermoScientific Superfrost Plus J1800AMNZ
Software:	
GIMP	2.8.14
Software for microscopes	
Immunohistology software	Leica Application Suite 3.8.0
Histology software	Motic Images Plus 2.0

Table 1. Overview of disposed material

4.1 Tissue preparation and support

Pathological specimens were obtained from the Department of Dermatology (Medical University of Graz) through a cooperation with Prof Wolf. Tissue samples were acquired from standard punch biopsies (diameter 4 mm) at different body sites directly from the cutaneous lesion. All slides were sectioned with a 3.5 µm width from original paraffin blocks after qualified diagnosis (3 x Lupus cutaneous, 3 Psoriasis cutaneous) by a senior medical doctor. Twenty-four sections of each independent donor were handed over on Super Frost slides with two samples on one microscope slide.

Healthy controls were recruited from two independent sources. First provider was again the Department of Dermatology. We received samples from 5 independent donors. The tissues were acquired from resection margins of various types of cutaneous cancer diagnosed healthy by senior medical staff pathologist. There were no cancerous rests in the tissues, nonetheless there were in some cases significate infiltration and inflammation present in the samples obtained.

Provenance of healthy control samples		
	Type of pathology	***Lesion location***
1.	Basal cell carcinoma	Shank right
2.	Basal cell carcinoma	Dorsum
3.	Basal cell carcinoma	Forehead left
4.	Basal cell carcinoma	Shank right
5.	Melanocytic nevi	Dorsum

Table 2. Provenance of healthy samples from exterior

Secondly we acquired skin flaps (approximately 10 cm x 10cm) from reductive plastic surgery at the Medical University of Vienna two times. We conserved it according to the "Standard tissue fixation protocol with 4% paraformaldehyde (PFA) and paraffin embedding technique for In Situ Hybridization" a SOP used by the institute of pathophysiology and immunology in Graz (see Appendix I). We prepared 14 paraffin blocks with an average sample size of 7 mm x 7 mm. Due to the abundance of our personal healthy stocks we used the afore mentioned blocks for antibody titration and testing. Three different blocks were used for all testing with an overall preference for block number 12. One of the healthy donors from Vienna had to be excluded after paraffin fixation because of a massive destruction of cellular structures, which could be perceived on the Haematoxylin and Eosin and on initial immunofluorescent stainings. Sections were cut with an average width of 5µm.

4.2 Staining

After antibody testing and precise concentration titration for the working reagents the following staining plan was created.

Staining plan				
Primary target	***Secondary target***	***6 Healthy specimens***	***3 Lupus cutaneous***	***3 Psoriasis vulgaris***
TGF b1	CD207	✓	✓	✓
BMP7act	CD1a	✓	✓	✓
proBMP7	Cd207	✓	✓	✓
BMP4	Cd1a	✓	✓	✓
BMP9	Cd1a	✓	✓	✓
NOGGIN	Cd1a	✓	✓	✓
pSMAD2/3	Cd1a	X	✓	✓
pSMAD3	Cd1a	✓	✓	✓
pSMAD1/5/8	Cd1a	✓	✓	✓

Haematoxylin	Eosin	✓	✓	✓
CD1a	CD207	✓	✓	✓
BMP7act	TGF b1	1/6	✓	✓
BMP7act	proBMP7	2/6	X	X

Table 3. Overview of performed stainings

The slides with the anti-pSMAD2/3 were excluded from histological evaluation because stainings showed an inconstant pattern on one and the same section and also great differences between the same specimens on different slides but done at the same staining procedure. Other antibodies which were excluded due to continues inconstant results or nonspecific pattern: anti-Laminin V, anti-HLA-DR.

4.2.1 Immunohistochemistry

H&E stainings were done as an overview and general assessment of all donors. Apart from this outline we performed immunohistochemical stainings for proBMP7 and TGF-β1, whereas this practice was abandoned in later stages of this work by reason that immunofluorescence showed a clearer vision of multiple molecular targets in great proximity and could therefore be assessed more objectively. A thermofischer immunohistochemical staining kit was used.

4.2.2 Immunofluorescence

We established the following staining protocol for the production of histological data. (see in appendix). Antibodies were stored at all times according to manufacturer's instruction manual either in -20°C or in 7°C and in a single stock or multiple smaller stocks in case of none glycerol conserved antibodies. As antibody diluent we performed comparative stainings with PBS, 10% BSA and a DAKO-product after which we eventually settled down with DAKOs antibody diluent. Overnight incubation was done in a cooling room type Viessmann.

Concentrations for the antibodies were determined by exact titration on a healthy specimen and applied as indicated in the following table:

Concentrations of antibodies:			
TGF b1	1:200	pSMAD3	1:500
BMP7act	1:300	pSMAD1/5/8	1:200
proBMP7	1:200	CD1a	1:300
BMP4	1:300	CD207	1:300
BMP9	1:300	anti-rabbit Cy3	1:800
NOGGIN	1:300	anti-mouse Dylight	1:800
pSMAD2/3	1:200	DAPI	1:1500

Table 4. Concentrations of antibodies applied

4.2.3 Quantification of results

Digital pictures were taken with a camera on the microscope and the software: Leica Application Suite 3.8.0, saved in .tif format with all supplementary files and stored on a Seagate 2 terabyte external hard drive. A few pictures were optimized visually for better representation and labelling was always added in later stages. Digital evaluation and visualization was done with GIMP 2.8 freeware. Text was edited on Microsoft Word 2013.

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Appendix I – SOP

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STANDARD OPERATING PROCEDURE (SOP)

Title: A standard tissue fixation protocol with 4% paraformaldehyde (PFA) and paraffin embedding technique for In Situ Hybridization	**SOP #:** MET 01 **Pages:** 1 of 3
Original Issue Date: 29-5-2009 **Effective Date:** 29-5-2009 **Next Review Date:**	
Written By: Carmen Tam-Amersdorfer	
Approved By: Anton Sadjak, Carmen Tam-Amersdorfer	

1 Objective:

Provide a standard tissue fixation protocol with 4% paraformaldehyde (PFA) and paraffin embedding technique for In Situ Hybridization.

2 Location:

Room 92 OG Histology Lab, Institute of Pathophysiology and Immunology.

3 Introduction:

1.1 Background

The goal of fixation is to maintain optimal tissue morphology while preserving the integrity of the nucleic acids (DNA, RNA). Careful sample collection is critical since tissues have an enzyme called RNA-ases that destroy RNA molecules. This activity is more intense after the tissue has been removed from a living body. Therefore, the sooner the specimen is collected and preserved , one can reduce the chance of nucleic acid degradation. As a routine fixation of choice 4% paraformaldehyde provide excellent results between ISH probe permeability and RNA retention. Typical fixation times vary between 14 to 24 hours maximum post fixation at 4°C. Note that over fixation with 4% PFA will significantly reduce ISH quality.

As a health precaution, PFA is a **Carcinogen!** Therefore, one must always work in a chemical fume hood, wear gloves, safety glasses and lab coat.

A standard tissue fixation protocol with 4% paraformaldehyde (PFA) and paraffin embedding technique for In Situ Hybridization	**SOP #:** MET 01 **Pages:** 2 of 3

1.2 Protocol

a. Preparation of 4% PFA in PBS:

1. Fill a 1 liter beaker with 700ml DEPC H^2O. Heat to 60°C using a hot plate with stirring in fume hood.
2. While stirring add 40g PFA (Sigma, Prilled form 95%, Cat. #: 441244-1Kg) to heated water until most particles are in suspension.
3. Add 10X PBS (RNAse free), then correct pH to 7.4 with 10N NaOH drop wise until solution becomes nearly clear, continue stirring.
4. When solution is cleared bring to final volume of 1 liter.
5. Filter the 4% PFA solution into a new beaker with sterile filter paper.
6. Store fixative at 4°C for up to two weeks or in aliquots of 15ml each into 50ml falcon tubes and store in -20°C.

b. Tissue fixation, dehydration and paraffin embedding procedures:

1. Process by HAND and use gloves at all times for RNAse free condition.
2. Fresh tissue is received in 4%PFA (Falcon tube), fix overnight at 4°C .
3. Next day, cut tissue and place in Tissue-Tek embedding cassette (8x8x3mm).

**The duration steps for dehydration and clearing depends on size of tissue. See table for details.

REAGENTS	**Small Tissue**	**Medium Tissue**	**Large Tissue**
70% EtOH/Depc	1hr - o/n	2hr – o/n	4hr – o/n
95% EtoH/Depc	1hr	1hr	2hr
95% EtoH/Depc	1/2hr	1hr	1hr
100% EtoH	1hr	1/2hr	1hr
100% EtoH	1/2hr	1/2hr	1hr
Toluene	1hr	1hr	1hr
Toluene	1/2hr	1/2hr	1hr till clear
Infiltrate Paraffin # 6	1hr	1hr	1hr
Infiltrate Paraffin #9	1hr	2hr	2hr

4. Dehydration is done at room temperature by immersing tissue cassette in beaker with 70% EtoH/Depc H^2O (see table).
5. Decant 70% EtoH/Depc H^2O and add 95% EtoH/Depc H2O and so on (see table for further alcohol dehydration steps and time).

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6. Clear tissue with toluene at room temperature in fume hood (see table for appropriate time).
7. Immerse tissue cassette in infiltration paraffin #6 (Microm Int. GmbH) located in the 60°C oven of Embedding Room KG 10 for desired time (see table).
8. Immerse tissue cassette again in infiltration paraffin #9 (Microm Int. GmbH) for desire time.
9. Transfer the tissue cassette to the Tissue Embedding Console.
10. Fill a metal mold with embedding paraffin liquid and place the tissue in the center of the pre-warmed wax mold.
11. Add more paraffin to fill the chuck and apply a new, clean labelled plastic cassette cover on top of mold.

12. Place the entire mold on cold plate at -8°C for 20 minutes until it's completely solidified.
13. Once solidified, detach the mold from the wax block by using gloved hand to gently separate the adhered wax connecting the metal mold.
14. The block can be store in a box at 4°C until sectioning.

4 Attachments

Attachment 1: Room layout

I have read and understand the standard procedures to be followed with tissue fixation and paraffin embedding technique for In Situ Hybridization .

Name:	Signature:	Date:

Appendix II – Protocol

Staining Protocol for Immunofluorescence Double Staining
1. Deparaffinization
1.1. 10 min Xylol 2x
1.2. 5 min 100% Alcohol 2x
1.3. 5 min 95% Alcohol 2x
1.4. 5 min 70% Alcohol
1.5. 30 s Aqua bidest.
2. Antigen Retrieval: DAKO Sodium citrate 10x dilute 300ml (30ml of DAKO)
2.1. 4 min in microwave on medium 2x
2.2. 2 min in microwave on medium
2.3. Cool at room temperature for 40 min
2.4. Do 1° antibody calculation and dilution during cooling time
3. Rinse in TBST 3x
4. Circle with PAP-Pen
5. Rinse in TBST
6. Apply UV-Protein Block for 7 min
7. Apply 1° antibody
7.1. Incubation overnight in 4°C in wet chamber
8. Rinse in TBST 3x
8.1. Do 2° antibody and DAPI calculation and dilution
9. Apply 2° antibody for 55 min
10. Rinse in TBST 4x
11. Apply DAPI (1:1500) for 20 min
12. Rinse in Aqua bidest 6x
13. Mount samples with Fluoroshield and Cover slip