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The Extracellular Matrix Protein 1 (ECM1) in Skin Biology: An Update for the Pleiotropic Action

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Abstract: Extracellular matrix protein 1 (ECM1) is a secreted glycoprotein that plays a pivotal role in the structural and homeostatic biology of the skin, particularly in angiogenesis, reconstitution of basement membrane, proliferation and differentiation of epidermal keratinocytes and dermal fibroblasts, and malignant transformation. This rationale is substantiated by loss-of-function mutations in the ECM1 gene in an autosomal recessive genodermatosis lipoid proteinosis and circulating IgG autoantibodies to this molecule in a humoral autoimmune condition lichen sclerosus, both of which are counterpart disease conditions sharing comparable skin pathology. In the recent decade, considerable progress has been made in determining the *in vivo* function of ECM1 in animal model studies. Furthermore, underlying insights arose for the genetic predisposition in inflammatory bowel disease ulcerative colitis, acquisition of immune tolerance and allergic responses *via* particular T cell subsets such as CD4+CD25+ regulatory T cells and Th2 cells, regeneration of certain organs, and clinical advantages for diagnostic and prognostic significance in various cancers. Following our latest review in 2009, we now update the most recent evidences for the pleiotropic action of ECM1 in skin research, and also highlight the novel pathogenic relevance of this molecule in a variety of human disorders.

Keywords: Extracellular matrix, protein 1 (ECM1), pleiotropic action.

INTRODUCTION

An increasingly large number of gene defects and autoantibodies has been linked to specific heritable and autoimmune disorders with skin manifestations. In fact, pathogenic mutations are now known to present in over 500 different genes in a manner that these genetic variations help to explain the characteristic phenotypes of certain heritable diseases with skin involvement [1-3]. The genodermatosis mutation database has been available and apparently revealed both candidate genes and predictable skin manifestations [4]. Of these, investigation for pair of genetic abnormalities and autoimmunity targeting the same molecule in skin may often explore the underlying insight of the molecular-based pathogenesis. One of the exciting candidates for such an association is the extracellular matrix protein 1 (ECM1).

ECM1 is an 85-kDa secreted glycoprotein that was first identified in the conditioned medium from the murine osteogenic stromal cell line MN7 [5,6]. It was termed extracellular matrix protein 1 because of the isolation from a variety of connective tissue proteins, such as type I collagen, osteopontin, osteonectin, cathepsin B and L, and bone sialo protein. Thereafter, the human ECM1 homolog was characterized [7,8]; the gene is located on chromosome 1q21.2 and encodes four splice variants, ECM1a-d. ECM1a (1.8 kb, 540 aa) comprises 10 exons, whereas ECM1b (1.4 kb, 415 aa) lacks exon 7 and ECM1c (1.85 kb, 559 aa) contains an additional exon 5a within intron 5, which shares high homology with exon 6 of murine ECM1 [9]. These three major variants show wide-spread and different expression patterns in human tissues [7,9,10]. ECM1a is expressed in the vast majority of organs, including skin, liver, intestine, lung, ovary, prostate, testis, skeletal muscle, pancreas, and kidney, albeit greater expression levels in placenta and heart. In contrast, ECM1b has an extremely restricted expression in tonsils and epidermal keratinocytes, and tissue-specific expression of ECM1c has yet to be fully identified except for skin [11]. ECM1d comprises a splicing variant, with an out of frame insertion of 71 nucleotides at the 5' end of exon 2, resulting in a truncated protein of 57 amino acids [12], whose biological significance and *in vivo* expression is still unknown. In skin, ECM1a was expressed in the epidermal basal layer, dermal blood vessels, the outer root sheath of hair follicles, sebaceous lobules, and sweat gland epithelia, whereas ECM1b was in the suprabasal layers of epidermis [10,11,13]. Thus, *in vivo* association between each ECM1 splicing variant and skin biology has gradually been defined.

The ECM1 protein contains a signal peptide of 19 amino acids, followed by four domains: a cysteine-free N-terminal segment, two tandem repeats and a C-terminal fragment. The two tandem repeat domains and the C-terminal domain contain a specific cysteine arrangement, C-(X_{12-38})-CC-(X_{7-10})-C [6,7]. More recently, a computational model for the three-dimensional structure of ECM1a was determined in

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order to identify putative region(s) for binding with candidate partners in human skin [14]. Functional studies have revealed the biological significance of ECM1 in angiogenesis, endochondral bone/cartilage development, and malignancies. A series of these evidences has followed the concept of the early observation for the multifactorial action of ECM1 in various organs and tissues. Most of these biological activities represent the positive regulation for cell proliferation, migration, and differentiation, finally resulting in tissue formation, but inversely show negative effects on chondrocyte hypertrophy, matrix mineralization, and endochondral bone formation [13-17]. On the other hand, the pathogenic impairment of ECM1 function has recently been demonstrated in human genetic and autoimmune disorders, lipoid proteinosis (LiP) and lichen sclerosus (LS), respectively [18,19], providing further understanding of *in vivo* ECM1 biophysiology. These discoveries led to the progress in several trials for recapitulating both disease conditions by gene knockout and antibody transfer experiments for ECM1 [20,21]. Main stream of these evidences has been addressed in the 2009-review article [22]. In this review, we focus on the most updated evidences of the ECM1 function in skin research, and also highlight the novel pathogenic potency of this molecule in the genetic background of inflammatory bowel diseases, T cell-dependent allergic responses, cancer biology, and skin ageing.

GENETIC AND AUTOIMMUNE IMPAIRMENT OF ECM1 FUNCTION

Lipoid Proteinosis and ECM1

Clinics and Skin Pathology

LiP (OMIM 247100), also known as Urbach-Wiethe disease or hyalinosis cutis et mucosae [23,24], is an autosomal recessive mucocutaneous disorder caused by loss-of-function mutation of ECM1 gene [19]. The disease is characterized by hoarse voice, generalized thickening and scarring of the skin and mucosae (Fig. **1**) [23-25]. Hyperkeratosis and pronounced infiltration are obvious in regions exposed to mechanical stimuli or trauma, such as eyelids, dorsa of hands and feet, elbows, knees, axillae, and buttocks, clinically resembling with verruciform skin. Other underrecognized skin features include photosensitivity [26,27], thus being manifested as Koebner's and photo-Koebner's phenomena. Normally, the LiP skin do not undergo a delayed wound healing or keroids during the clinical course, implicating that ECM1 deficiency does not participate in the wound healing process, or otherwise can be at least compensated. Extracutaneous manifestations include opthalmological features [28], epilepsy (~25% of all cases), and various neuropsychiatric symptoms, such as dystonia, or impairment of memory, perception and emotional control, perhaps in association with cerebral calcification [25,29-34]. Among a variety of these manifestations, the most reliable clinical hallmarks are hoarse voice from an early childhood and a limited protrusion of the tongue [27]. Therapies are mostly unhelpful. Recent progress for the treatment option includes oral retinoid acitretin that was challenged to three patients, but the clinical efficacy is limited; of the three patients, one showed an improvement of hoarseness and indurative skin lesion [35], and the other two had some regression and softening of the skin induration [36].

Update of the ECM1 Gene Mutation Database

Following the latest review of 41 mutations in the ECM1 gene in LiP individuals and families in 2007 [27], the addition of 21 pathogenic mutations to the database has been reported. Of these, only 4 were novel and different mutations [37-41], with the exception that only one case shares the c.1019delA heterozygous mutation published previously in a Kuwait patient [18] and the C269Y homozygous mutation was recurred between patients in the Middle East countries (Table **1**) [39,40]. All the remaining were recurrent mutations reported elsewhere. Thus, the combined mutation database now contains up to 45 different mutations of the ECM1 gene [27, this review]. Overall mutation characters vary considerably; for example, nonsense/missense, frame shift, different sizes of deletion and insertion, or splice site mutations. These mutations span throughout all the 10 exons, except for exon 5a, but more than half of all mutations (25/45, 56%) occur within exons 6 or 7 (including adjacent splice sites). These findings favor the hypothesis that: i) epitope(s) within exons 6 and 7 represent the plausible target for the establishment of LiP phenotype, ii) ECM1a and c (isoforms containing exons 6 and 7) are of much more biological significance than ECM1b (an alternative splicing isoform lacking exon 7).

Clinically, hoarse voice and protrude tongue were found in all cases with the novel 4 mutations (Table **1**), highlighting the clinical hallmark manifestations in LiP. Looking back to the updated ECM1 gene mutations and clinicopathology of the affected individuals, however, there seems no apparent genotype-phenotype correlation, even in familial and sibling cases with the same mutations, as being comparable with the latest review [27]. Therefore, the variable clinical manifestations of LiP are more likely to be a consequence of the different acquired settings in each case.

Table 1. Characters of the Updated ECM1 Gene Mutation in Lipoid Proteinosis

Mutation(s)	Site of Mutation(s)	Geographical Origin	Hoarseness/Protrude Tongue	Reference
c.240delTC/c.1019delA[1]	Exon 4/exon 7	Arabian	+ / +	[37]
p.Q206X/p.Q206X	Exon 6	Pakistani	+ / +	[38]
p.C269Y/p.C269Y[2]	Exon 7	Saudi Arabian, Iranian	+ / +	[39,40]
p.C477R/p.C477R	Exon 9	Chinese	+ / +	[41]

[1]The heterozygous mutation c.1019delA has been published previously in a Kuwait patient [18].
[2]The homozygous mutation C269Y was recurred between patients in the Middle East countries [39,40].

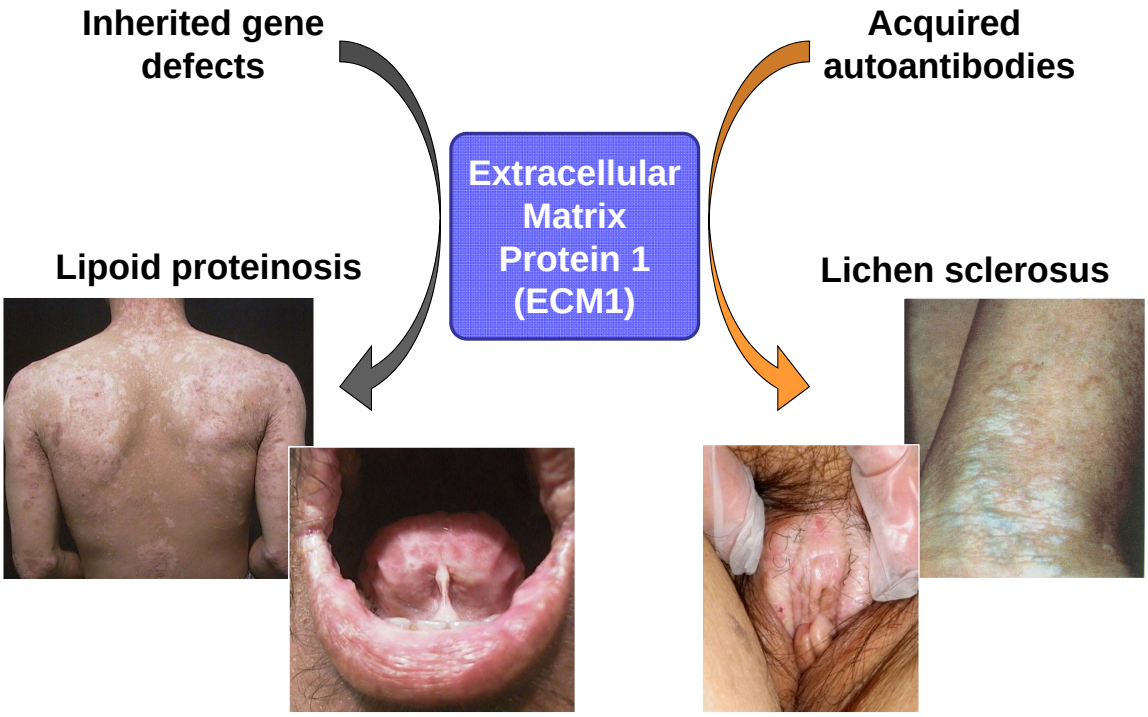


Fig. (1). A counterpart disease concept for genodermatosis LiP and autoimmune condition LS, both of which target ECM1. LiP and LS are considered a disease entity for genetical and immunological impairment of ECM1 function, respectively [18,19]. Both diseases have considerable overlapping of the skin clinicopathology; trauma-induced inflammation (Koebner's phenomenon) and microscopic findings such as epidermal atrophy with hyperkeratosis, disruption and duplication of the basement membrane, and hyaline (glassy) changes and telangiectasia in the upper dermis [25,27,42,43,64]. The use of all clinical photographs was permitted by the corresponding patients according to the approval of the institutional board of Matsuda General Hospital.

Lichen Sclerosus and ECM1

Clinics

The breakthrough discovery of ECM1 gene mutations in LiP led to open another window for humoral autoimmunity to this molecule in LS [19]. LS is an acquired chronic inflammatory disorder that affects skin and mucous membranes, with highly occurrence in anogenital area [42]. In a large cohort retrospective study and review of previous reports, extragenital LS alone may occur in approximately 15% of all cases investigated [43,44]. As a constant observation including most recent cross-sectional studies, the disease prevalence ranges from 0.1-0.3%, with a male to female ratio of 1:10 [43,45,46]. Clinical features include erosions, pale patches, papulo-plaques, and porcelain-like atrophic scarring with intractable itching and soreness (Fig. **1**), thus causing dysuria, sexual dysfunction, and physical morbidity. There is also an increased risk of squamous cell malignancy in longstanding lesions [43,47]. In female LS, twin age peaks (prepubertal/postmenopausal) suggest a hormonal origin in the pathogenic background, but hormone therapy was mostly unsatisfactorily [47].

Scenario for Autoimmunity to ECM1 in LS

The progress for screening of disease-specific autoantibodies in LS is based on the considerable prominence for possible genetic susceptibility and humoral autoimmune basis to the disease [48-50]. Reports have shown variable intra-familial cases with LS [51,52], an association with particular HLA class II antigens DQ7-9 [53,54], and an increased incidence of various autoimmune diseases and autoantibodies, such as morphea, thyroiditis, pernicious anaemia, type I diabetes mellitus, alopecia areata, vitiligo, bullous pemphigoid and mucous membrane pemphigoid [46,55-61]. A hundred years ago, evidence implicating a humoral autoimmune response in LS has been demonstrated in a case with probable LS, being induced by injection of serum from an affected individual into non-lesional skin [62]. More significantly, LS has considerable overlapping of the skin clinicopathology in an ECM1-deficient genodermatosis LiP; for example, trauma-induced inflammation (Koebner's phenomenon) [27,63] and microscopic findings such as epidermal/epithelial atrophy with hyperkeratosis, disruption and thickness of basement membrane, and hyaline (glassy) changes and teleangiectasia in the upper dermis [27,42,43,64].

Immunoblotting and an antigen-specific ELISA have identified serum IgG antibodies to ECM1 in 74-80% of female patients with genital LS, with 94% specificity in discriminating LS from other autoimmune diseases and healthy control [21]. Interestingly, higher titers of anti-ECM1 antibodies correlated with more longstanding and refractory diseases, and cases complicated by squamous cell carcinoma. Also, a reliable number of male patients with penile LS (n=80) tend to have higher titers of serum anti-

ECM1 antibodies than age-matched normal males [65]. For an immunological background, male LS patients have a relatively lower prevalence of associated autoimmune and autoantibody disorders in comparison with female LS patients [66,67]. Thereafter, several case reports have revealed the presence of serum anti-ECM1 antibodies in LS with different clinical features, particularly extragenital lesions [68,69]. These evidence series provide further weight for a humoral autoimmunity to ECM1, without regard to genders and disease phenotype, being a pathogenic axis in LS.

ECM1 PROTEIN STRUCTURE AND ITS BINDING PARTNERS

A computationally predicted three-dimensional structure of the ECM1a protein revealed further detailed insight for a disease-specific impairment of ECM1 function in LiP and LS. ECM1a protein can be divided into 4 distinct domains (Fig. **2B**); the first domain consists mainly of α-helices (αD1), while the three remaining domains, namely Serum Albumin SubDomain-Like domains 2-4 (SASDL), were topologically comparable with the subdomain of the third serum albumin domain [14]. SASDL2 and SASDL3 are capable of binding with most of the extracellular matrix proteins identified so far, e.g., laminin 332, fibulin 1C/D and 3, and matrix metalloproteinase-9 (MMP-9), phospholipid scramblase 1 (PLSCR1), while the C-terminus of ECM1 interacts specifically with perlecan and cartilage oligomeric matrix protein (COMP) (Fig. **2A**) [11,14,70-74]. These ECM1-protein interaction partners have been identified by yeast two hybrid (Y2H) library screening or ELISA-based methods (e.g. fibronectin, type IV collagen), while ECM1-carbohydrate interactions were established through *in vitro* binding experiments (hyaluronic acid, chondroitin sulphate A, heparin) (Fig. **2A**). Most of these binders co-localize immunohistologically with ECM1 in human skin.

COMP is a 524-kDa pentameric, disulfide-bonded glycoprotein that represents a prominent non-collagenous component of cartilage extracellular matrix. It is expressed in tendon, bone (osteoblasts only), and synovium [75,76]. Mutations in the human COMP gene cause the development of pseudo-chondroplasia and multiple epiphyseal dysplasia, autosomal-dominant forms of short limb dwarfism characterized by short stature, normal facies, epiphyseal abnormalities, and early onset osteoarthritis [77], thus implicating the pivotal roles in osteo-chondrogenesis and matrix mineralization. Perlecan is a major heparan sulfate proteoglycan that is involved in the binding and cross-linking with basement membrane and interstitial dermal components, such as laminins, fibronectin, type IV collagen, fibulin-2, and heparin [78,79]. PLSCR1 is an endofacial plasma membrane protein believed to carry out calcium-dependent nonspecific and bidirectional movement ("scrambling") of phospholipids across the plasma membrane, contributing to cell proliferation/differentiation and apoptosis *via* direct interaction with epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF-7, vascular endothelial growth factor (VEGF), and caspase [80-83]. Moreover, ECM1 can bind to both collagen IV and laminin-332 (formerly designated as *laminin-5*, which consists of α3, β3, and γ2 sub-chains) and also enhances their binding [14,70].

All of these binders are regular constituents of the basement membrane, its surrounding interstitials, and appendages in the skin, which possess a large regulatory potency for enhancing and reducing functions, depending on the cellular or tissue context (Fig. **3a**). On this basis, ECM1 can be a multifunctional binding core and/or a scaffolding protein, acting as a "biological glue" by its promiscuous interaction with a variety of extracellular and structural proteins, thereby contributing to the maintenance of skin integrity and homeostasis [70, 84-86]. Hence, disruption of the ECM1 function may cause the failure of multi-communication among the surrounding skin structural and interstitial molecules, resulting in the torsion of lamina lucida/densa [64] and lamellate or punctuated structures below basement membrane, accompanied by excess deposition of collagens IV and laminin-332, perlecan, extracellular matrixes around the thickening blood vessel walls [85,86], and disorganization of type VII collagen [64], a major constituent of anchoring fibril for skin basement membrane, as seen in LiP and LS skin pathology (Fig. **3b**).

DIFFERENCES IN MOLECULAR-BASED PATHO-MECHANISM BETWEEN LIP AND LS

Conceptionally, genodermatosis LiP and acquired autoimmune condition LS can be recognized a counterpart disease condition targeting ECM1 (Fig. **1**) [18,19]. Considering ECM1 protein structure and its corresponding interaction partners, however, the pathomechanism of ECM1 function impairment in both diseases would be different. Combined with the ECM1 gene mutation database [27, this review], the hot spot region for ECM1 gene mutations in LiP patients (exons 6 and 7) lies within the complete SASDL 2 and part of SASDL 3 (aa177-aa361, Fig. **2B**, **C**) [14,70]. Notably, most of these mutations are non-sense or out-of-frame changes, causing deletion of SASDL2 and its downstream region. In contrast, epitope mapping studies revealed that most of detectable IgG antibodies to ECM1 in LS patients' sera react with both NH2- (32-203aa) and COOH-terminal ends (349-480aa) of ECM1 epitopes, just avoiding the SASDLs 2 and 3 (Fig. **2B**) [21]. This illustrates a heterogeneous IgG reactivity to "spared" antigenic epitopes within ECM1 protein. Together with basic research data, one may speculate that anti-ECM1 antibodies in LS mainly affect the functional binding of ECM1 with type IV collagen, COMP, and perlecan, whereas LiP mutations affect laminin 332, PLSCR1, fibulines-1C/D and -3, MMP-9, COMP, and perlecan interactions (Fig. **2A**) [11,14,42,70-74]. The differences in the affected binding partners between both diseases may cause other subtle microscopic changes; the granular material composed of type VII collagen, a major component of anchoring fibrils in the sublamina densa, was deposited in the dermis of LS skin, but almost restricted to the dermal periappendage regions in LiP skin [64]. The ECM1-dependent reaction chain may drive further advanced damages in the interaction between ECM1 and other ECM components, ultimately causing the clinicopathology in LiP and LS. Further fine epitope mapping within ECM1 now warrants to identify the pathogenic specificity and/or concealed region(s) for binding of *in vivo* ECM1 with candidate partners in human skin. Likewise, whether the development of multiple antigenic targets within ECM1 has any pathogenic significance for LS or whether it merely

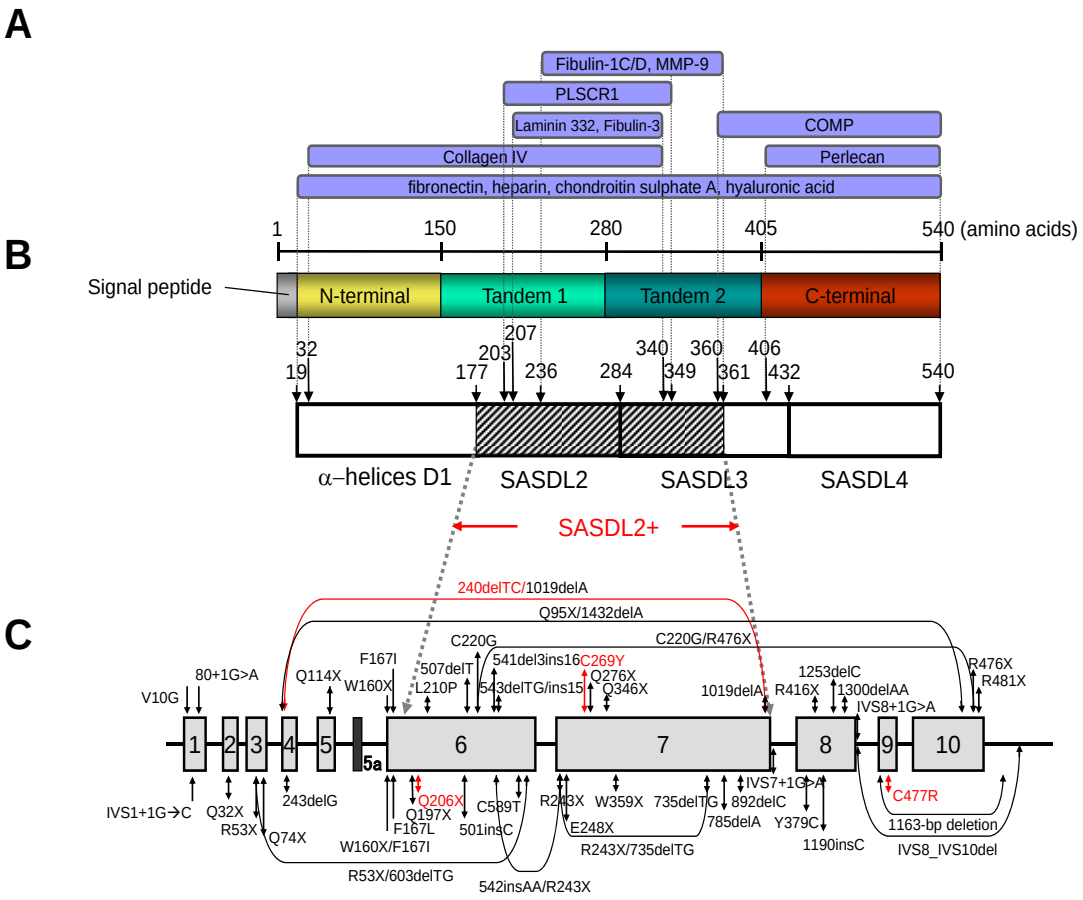


Fig. (2). Summary of the gene and domain organization of the human ECM1 and its binding partners. (**A**) Site-specific binding of ECM1a protein to different structural and ECM molecules in the skin. A series of *in vitro* and *in vivo* binding studies have revealed the biological interaction of ECM1 with the following proteins and polysaccharides at different binding sites; perlecan (406-540 aa of ECM1a) [11], fibulin-1C/1D and MMP-9 (236-361 aa) [70,71], type IV collagen (32-340 aa) [70], laminin 332 and fibulin-3 (207-340 aa) [14,70], PLSCR1 (203-349 aa) [14,73], and COMP (360-540 aa) [74]. The binding of the full-length ECM1a with fibronectin, heparin, chondroitin sulphate A, and hyaluronic acid has also been demonstrated [70], but their exact binding sites within ECM1a has yet to be characterized. ECM1a is capable of enhancing the binding of type IV collagen and laminin 332 [70]. (**B**) A predicted secondary structure and its corresponding domains of ECM1a protein. The full-length ECM1a contains a 19-amino acid residue signal peptide and the following four distinct domains; an N-terminal cysteine-free domain, two tandem repeat domains, and a C-terminal domain. The latter three domains contain the characteristic cysteine arrangement CC-(X7-10)-C patterns [6,7], typically observed in the albumin protein family members. This pattern generates double-loop structures that are involved in the protein/protein interactions. The first domain exists of α-helices (αD1), and the remaining three domains are comparable topologically with the subdomain of the third serum albumin domain, named SASDL2, 3 and 4 [14]. The amino acid numbers corresponding with a mature ECM1a precursor protein were indicated. The SASDL2+ region was used to screen a foreskin cDNA library by Y2H and instrumental in the identification of fibulin-3, laminin 332, and PLSCR1 as putative ECM1 binders [14,73]. (**C**) Schematic representation of ECM1 gene structure and mutations in LiP patients. ECM1 gene consists of 10 exons (*boxes*) and alternative introns (*horizontal lines*). All the pathogenic ECM1 mutations thus far reported were depicted; previously reported mutations were indicated in *black* [27], whereas four novel mutations were in *red* [37-41]. The homozygous mutations were indicated as *double arrows*. Note that more than a half of all nonsense and flame-shift mutations are located within exon 6 and alternatively spliced exon 7.

represents a secondary phenomenon occurring within the setting of more severe and chronic disease need to be clarified [21].

INFLAMMATORY BOWEL DISEASES AND ECM1

Substantial advances using epidemiological twin and linkage analysis have been achieved in defining the genetic architecture of inflammatory bowel diseases (IBDs). To date, roughly 60 definite susceptibility genes and loci have been identified in IBDs [87]. Ulcerative colitis (UC; MIM 191390) is one of two major IBDs, characterized by chronic relapsing and remitting inflammatory conditions affecting the colon, invariably with rectum. Normally, inflammation is restricted to the mucosal epithelia, thus establishing a current working hypothesis that the disease pathogenesis is a defective mucosal barrier and dysregulated inflammatory response to the intestinal epithelium [88].

Recently, hypothesis-free genome-wide scan for non-synonymous single nucleotide polymorphisms (SNPs) has yielded the strong association with UC at two different SNPs (rs3737240 and rs13294) in the ECM1 gene [89]. This locus, spanning 290-kb, also contains MRPS21, PRPF3, and TARS2, those of which are mitochondrial and ribonucleoprotein family members. Interestingly, the ECM1 genetic variants have recently been re-screened with the same cohort for one the other major IBD, e.g. Crohn's disease (CD; MIM26600), but there were no significant association with reliable statistical power [90], implying that ECM1 gene confers a susceptibility locus specific for UC. The association of ECM1 with UC was confirmed in the Dutch population [91]. In contrast, *ECM1* rs 13294 was not associated with either CD or UC in Eastern European patients [92]. These evidences may raise the question why LiP and LS, genetic and autoimmune impairment conditions of ECM1 function, respectively, do not consistently exhibit any of intestinal manifestations, and if any, whether it is simply underrecognized or an occult manifestation. Limited case reports for intestinal involvement in LiP are only available [93].

Nevertheless, ECM1 is a plausible pathogenic candidate for UC; it is ubiquitously expressed throughout the intestine [7], and represents structural integrity and gatekeeping action of transmembrane permeability in the mucosal epithelia. It is therefore possible that ECM1 may be part of the key molecular cascade for the structural/immunological breakdown of mucosal barrier function, thereby allowing an increased antigenic load to trigger a disease-specific intestinal autoimmunity in UC [94,95]. Furthermore, rs 3737240 (exon 6) and rs 13294 (exon 8) encode the amino acid substitutions of T130M and S290G. Thr130 resides within the binding domain for type IV collagen [70] and is conserved in primates, whereas Ser290 is not. Therefore, it is tempting to speculate that rs37372240 would be rather a pathogenic SNP and may affect the interaction of ECM1 with collagens (e.g. type IV collagen) resulting in excessive collagen production, which is deposited under the membrane giving the histological appearance of a subepithelial collagen band, typically for collagenous colitis [96].

Animal Models for Unraveling *In Vivo* ECM1 Function

Ecm1 Knockout Animals

Among a number of animal models recapitulating features of inherited or acquired autoimmune disorders, mice can be a preferable platform to understand the detailed and/or concealed pathomechanistic insight of the disease. However, mice homozygous for the ECM1 null-mutations, reflecting the pathogenic nature of LiP, are not viable and *Ecm1*-deficient embryos die during pre-implantation development (< day 3.5 postcoitus, E3.5) following a conventional knockout targeting strategy for *Ecm1*. At that stage, the provisional conclusion that ECM1 is indispensable for the early embryonic development has been made [97].

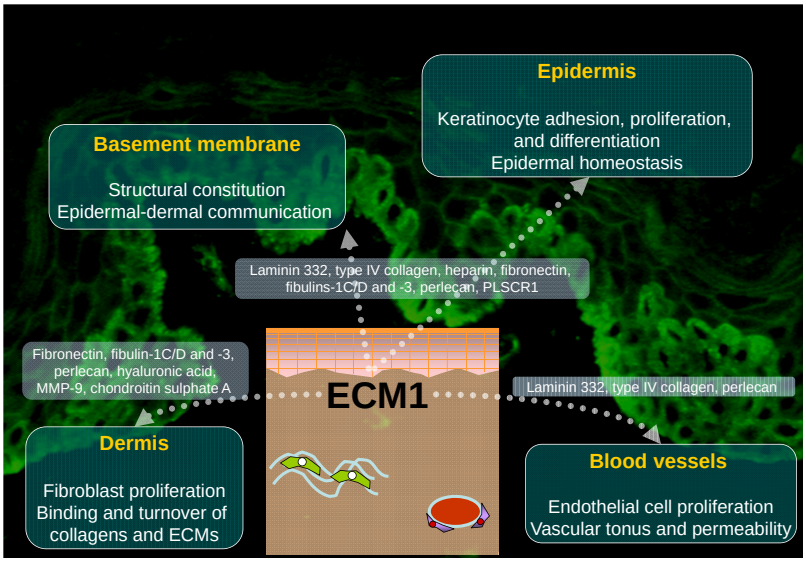
As an alternative approach, a freshwater fish zebrafish (Dario rerio) has recently been utilized to establish a novel animal model for LiP [98]. There are several characteristic advantages that favor choosing this small freshwater fish [99]; it has a rapid maturation and development of organs, as well as skin compartments. Fully developed adult zebrafish skin composes of stratified (multi-layered) epidermis and the underlying collagenous stroma, separated by hemidesmosomal structures in basement membrane zone, those seen in human skin. Interestingly, *ecm1*-knockout zebrafish normally survive and phenotypically reveals altered contour of the skin surface with micropapules and loss of microridges [98]. Microscopically, the mutant zebrafish skin reveals apparent reduplication of basement membrane and pinocytotic vesicle formation in the periphery of dermal fibroblasts, findings seen in LiP skin. Although the identified zebrafish ECM1 protein has ~45% homology to the equivalent human protein [100,101], the results from rescue transgene experiments using human ECM1a cDNA for the knockdown phenotype suggest accuracy of the ECM1 orthologue in zebrafish [98]. However, the study limitation using zebrafish in skin research includes lack of epidermal differentiation molecules, such as filaggrin, involcurin, trichohyaline genes (the latest zebrafish genome in NCBI database), for which the zebrafish skin do not undergo terminal differentiation, and lower genetic similarity to human (~56%) [100,101]. These observations may be a common inevitable difficulty in establishing the overall skin features of LiP in in-water animals.

In 2010, Li *et al*. have first demonstrated the ECM1 knockdown in mice by deleting exons 2-11 of the mouse *Ecm1* gene [20]. Since the mice die around 6-8 week ages with uncharacterized autoinflammatory condition, they next generated chimeric mice transplanted with bone marrow cells from *Ecm1*-deficient mice. The most striking observation in the chimeric mice for *Ecm1* was significant attenuation of ovalbumin-induced airway allergic response, a histology showing fewer infiltration of eosinophils, lymphocytes, and macrophages in the bronchoalveolar lavage. Compared with wild-type mice, the activated CD4+Th2 cells of *Ecm1* chimeric mice, but not other T cells including Th1, Th9, Th17, CD8+T cells, and inducible regulatory T cells (Tregs), were specifically retained in the peripheral lymphoid organs, and did not migrate into the inflammatory sites. Functional assays for several T cell lineages from *Ecm1* knockout mice exhibited no substantial differences in the proliferation activity, cytokine/chemokine profiles, and polarization of their differentiation, suggesting the direct action of ECM1 in Th2 cell emigration from lymph nodes into the blood [20]. Thus, Li and coworkers discovered a novel role for ECM1 in controlling Th2 cell trafficking during an allergic immune response.

ECM1 Overexpression in Mice Skin

Considerable progress to address the *in vivo* ECM1 function has been evolved during the last half decade. Preliminary implication draws functional importance of ECM1 in epidermal differentiation, because the human ECM1 gene locates within a region on chromosome 1q21.2 centromeric to a gene cluster of the epidermal differentiation complex [7,8,13,102]. However, *in vitro* experiments using ECM1a/c-deficient (mutations outside exon 7) and ECM1b-deficient (mutations within exon 7) cultured keratinocytes from LiP patients showed no significant differences in the expression of early and late epidermal differentiation markers, keratin 10 and involucrin, respectively, compared

(**a**)



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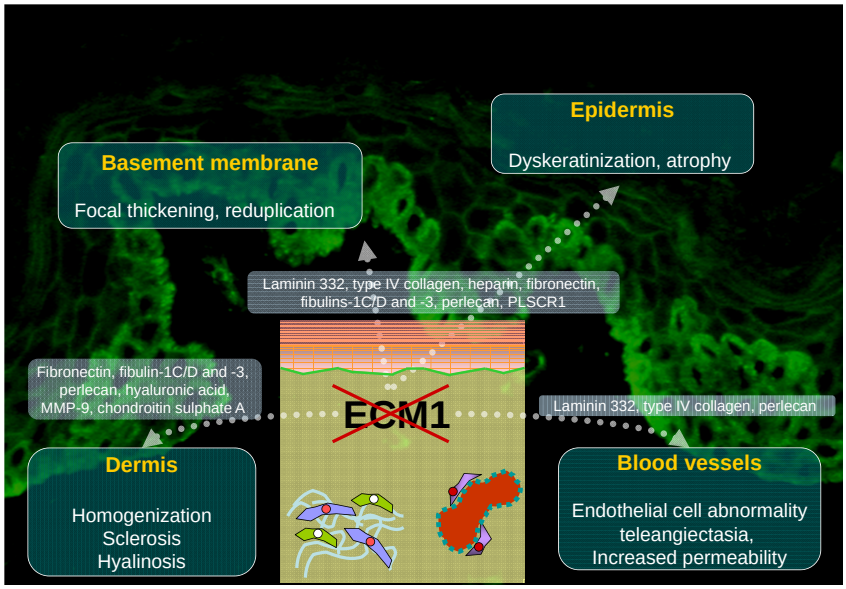

Fig. (3). ***In vivo*** **ECM1 function and its multifocal assembly in the skin structural and ECM molecules.** (**A**) In human skin, ECM1a is expressed in the epidermal basal layer, dermal blood vessels, the outer root sheath of hair follicles, sebaceous lobules, and sweat gland epithelia, whereas ECM1b was in the suprabasal layers of epidermis [10,11,13]. ECM1 binds to a variety of structural and ECM molecules (*gray boxes*), forming the stratified epidermis, hemidesmosome, and dermal components in the skin. Most of these molecules are secreted by keratinocytes and fibroblasts, as well as endothelial cells. Laminin 332, fibulin-1, and type IV collagen specifically localize in the basement membrane and dermal blood vessel walls. Major interstitial dermal proteins and polysaccharides contain fibronectin, chondroitin sulphate A, and hyaluronic acid, which bind to ECM1 with different affinities [70]. MMP-9, a proteolytic enzyme for type IV collagen, is capable of binding to ECM1 directly [71], although this specific binding down-regulates the enzymatic activity [152]. ECM1 can act as a ‘‘biological glue’’ in the whole basic framework and physical flexibility in human skin. Background pictures were the immuno-labeling with rabbit anti-ECM1 antibody [19] in human skin section; positive signals (*green*) were seen in the cytoplasm of the lower epidermal keratinocytes and dermal blood vessel walls, as well as along with basement membrane zone. PLSC1, phospholipid sclamblase 1; MMP-9, matrix metalloproteinase-9. Impairment of ECM1 function, which caused by genetic ablation (LiP) or autoimmunity (LS), may collapse the multiple assembly with the surrounding skin structural and interstitial molecules, resulting in the clinicopathology characteristics for LiP and LS; hyperkeratosis and atrophy of the epidermis, disruption and duplication of the basement membrane, excess deposition of type IV collagen, perlecan, laminins, and extracellular matrixes around the thickening blood vessel walls with telangiectasia and hyaline changes [42,43,64,85,86].

with normal human keratinocytes [103]. This observation was further verified with immunolabelling of LiP skin. More surprisingly, mice overexpressing ECM1a at the basal (keratin 14 promoter-driven) and suprabasal layers (involucrin promoter-driven) have demonstrated no morphological and histological changes in the skin, comparable with those of wild type mice [103]. These data indicates that *in vivo* ECM1 function can be dispensable or otherwise at least compensable for early and terminal differentiation of keratinocytes in a steady-state condition. This notion provides the clinical perspective that warty keratotic appearance of the LiP lesional skin is more unlikely to be a direct consequence of deficient ECM1 function, but may result from chronic exposure with extrinsic factors, such as mechanical stress, temperature, dry, or UV [10,26,103].

Passive Transfer of ECM1 Antibodies in Mice

To elucidate the pathogenic importance of serum anti-ECM1 autoantibody in LS, a passive transfer experiment of affinity-purified IgG from LS patients has been challenged to the mice ear skin [21]. This approach reproduced limited features of the clinicopathology seen in LS; the injected skin sites showed marked erythematous swelling with telagiectasie. The pathology of the injected skin sites displayed moderate inflammatory cell infiltration, dilated blood vessels and interstitial edema in the dermis, all of which are compatible with the early skin pathology seen in LS. These changes are anti-ECM1 antibody specific, because i) control human IgG injection did not show any skin changes, ii) the skin sites injected with affinity-purified LS IgG revealed IgG deposition in the lower epidermis (intracellular) and surrounding dilated dermal blood vessels, being similar to those found in ECM1 immuno-labeling of normal human skin or mice skin immunostained with anti-ECM1 rabbit polyclonal antibody [19,21]. Thus, LS IgG is capable of binding with *in vivo* native ECM1, and also inducing some of LS skin pathology. Despite these specific immunoreactions, the injected skin sites showed no evidence of hyaline and scarring changes, typical skin pathology in established LS [42,43].

A passive transfer experiment failed to recapitulate the complete pathological features of LS skin, but instead, an extrapolation simply from these results provides the underlying pathomechanism of the disease; first, much more prolonged inflammation caused by the anti-ECM1 antibody - coordinate with T cell-mediated immune reaction [104-106] - might be needed for establishing the LS skin. Another persuasive evidence is the increased incidence of peri-stoma LS lesions in patients who received urostomy or colostomy [107]. This clinical observation suggests a possible combined role for constitutive urine or stool contact, local skin occlusion, infection (i.e. Gram-negative microorganisms), and perhaps moisturization, a condition akin to the anogenital skin. However, the functional impairment of *in vivo* ECM1 may not be responsible for the lesional predilection, because ECM1-deficient genodermatosis LiP shows neither a predilection for anogenital skin nor the potential for squamous cell carcinogenesis. Together, a series of current experimental and clinical studies for genital LS may thus propose that a combination of intrinsic and extrinsic factors to the local skin is essential for the development of the characteristic LS skin. From the clinical perspective, further animal studies injecting anti-ECM1 antibody to the anogenital area needs to be appraisal.

ECM1 Expression in Different Cell Sources

1. Cell Lines and Normal Cells

In human, various types of normal and malignant cell lineages express at least any of the three ECM1 variants, ECM1a-c. As listed below, *in vitro* and histochemical studies have revealed the expression of ECM1 transcription and/or protein in keratinocytes [7,13], dermal fibroblasts and papilla cells [11,18,108], vascular endothelial cells, squamous carcinoma A431 cells, fibrosarcoma HT1080 cells [11], osteosarcoma Saos2 cells [7], cholangiocarcinoma cells [109], and hepatocellular carcinoma cells [110], bone-metastatized lung adenocarcinoma SPC-A-1BM cells [111], HaCaT keratinocytes [73], mammary epithelial cells, estrogen receptor-positive breast cancer MCF-7 cells, estrogen receptor-negative bone-metastatized breast cancer MDA-MB-231 cells/-435 cells, Hs578T cells, and LCC-15 cells [112-114], embryonic kidney 293 cells [115], chondrogenic ATDC-5 cells [116], and pancreatic cancer SW1990 cells and Capan-2 cells [117].

Again, these evidences suggest that ECM1 has an essential role in the vast majority of cell sources, as well as development and maintenance of malignant potential. As a preliminary data, the amount of ECM1c transcript was estimated to be ~15% of the total ECM1 mRNA in A431 cells and HT1080 cells [11], implying the possibility of an ECM1 isoform-specific regulation in each cell lineage. However, antibodies discriminating these three ECM1 isoforms are currently not available, and also it is not evident that the detectable transcription rates indeed reflect the protein expression levels of the corresponding ECM1 isoforms. Investigation of the isoform-specific expression of ECM1 may be crucial for identifying the detailed characteristics of malignant potential and local behavior in each cancer.

Lymphocyte Biology and ECM1

DNA microarray assays have recently disclosed the expression of ECM1 in mouse hematopoietic cells, particularly in T cells [118,119]. Of note, the ECM1 transcription levels considerably differ in differentiation- and cell lineage-dependent manners; it was much higher in CD4+ helper T cells and CD4+CD25+T cells (Tregs) than CD8+ cytotoxic T cells and CD3-negative naïve T cells, but their expression rates in the equivalent cell sources were unchanged between thymus, spleen, and peripheral lymph nodes. Among CD4+ helper T cell phenotypes, ECM1 expression was almost restricted to the Th2 cells [20], implicating the possible association between ECM1 and allergic reaction. This was supported by the finding that chimeric BALB/c mice transplanted with *Ecm1*-deficient bone marrow cells showed decrease of inflammatory response in experimentally induced airway allergy [20] and depletion of CD25+T cells in wild type C57BL/6 mice dramatically increased the priming and expansion of antigen-specific T-cell precursors *via* gamma-interferon induction in allergic contact skin hypersensitivity [119,120]. In Th2 cells, mRNA expression and protein secretion of ECM1 were detectable at 3 days after antigen-dependent engagement of

T cell receptor. Subsequently ECM1 binds with IL-2 receptor β-subunit (CD122), but neither with CD25 nor CD132, to inhibit the phosphorylation and activation of the downstream signaling molecules, such as STAT5, KLF2, and S1P1 [20], finally resulting in the down regulation of Th2 cell trafficking to the inflammatory sites. On the other hand, both freshly isolated and activated CD4+CD25+Tregs have been confirmed to express higher levels of ECM1 transcript [118]. CD4+CD25+Tregs are well known to regulate innate and adaptive immune responses, an effective tumor immunity to autologous tumor cells, and a potent anti-inflammatory capacity in autoimmune and chronic inflammatory diseases, such as autoimmune encephalitis, diabetes, thyroiditis, IBDs, and contact skin hypersensitivity [120-125]. Naturally occurring Tregs, the other Treg phenotype, which comprise up to 5% of the peripheral CD4+T cell pool, have also shown to express ECM1 [121]. More critically, the ECM1 transcription was significantly increased in naïve T cells by transient transduction of Forkhead box P3 (Foxp3), a transcription factor that acts as a master control molecule for the development and function of CD4+CD25+Tregs in the thymus and periphery [126].

Immunohistologically, the percentage of FoxP3+lymphocytes was increased in the lesional skin of female genital LS, compared with those of male penile LS and normal control [105]. Although the precise function of infiltrating Tregs in the lesional LS skin remains undefined, the local autoimmune reaction is initiated by decrease or absence of acquired immune tolerance organized by Tregs. This concept is supported by evidence series that the frequency of locally infiltrating Tregs was decreased in autoimmune blistering and sclerotic diseases, such as bullous pemphigoid, scleroderma, and morphea [127,128]. To unravel, the exact pathogenic relationship between Tregs and humoral autoimmunity to ECM1 in LS, passive transfer experiments of anti-ECM1 antibody into the Treg-depleted mice or under conditional Treg-suppression may be of necessary.

ECM1 EXPRESSION IN CELL SIGNALING AND CANCER BIOLOGY

A series of recent evidence has emerged the direct link of abnormal ECM1 expression with aspects of malignant potential. Most of these comprises the overexpression properties of ECM1 in a wide variety of malignant epithelial and internal tumors, including breast carcinoma, esophageal squamous carcinoma, and, gastric, colorectal, pancreatic, hepatic carcinomas [17,109,110,129]. Elevated levels of ECM1 transcript(s) and protein have also been demonstrated in malignant thyroid neoplasms [130], and can clinically represent diagnostic and prognostic significance in individual cases with hepatic and breast cancers [131,132]. This is in line with aberrant ECM1 expression in the cell lines originating from equivalent malignant tissues and organs. ECM1 has been also shown to have an important role in the growth, metastasis and angiogenetic stromal response of laryngeal carcinoma [133]. *In vitro* studies using an established cholangiocarcinoma cell line has shown the increase of ECM1 expression and NF-kB/Akt cascade activity [109]. ECM1 expression is influenced by the Wnt/β-catenin signal transduction pathway, dysregulation of which has been implicated in the tumor pathogenesis in mammary glands, colon and skin [113]. ECM1 and its downstream accessory signals may therefore be involved in the important interaction between tumors and local microenvironment in different tissues. Indeed, ECM1 has recently been shown to be overexpressed in a human tumor metastasis model, further emphasizing a key role for ECM1 in epithelial-stromal interactions [134]. Upon the recent wide-genome screening, however, ECM1 gene loci have yet to be formally associated with secondary developed colorectal cancer in IBDs, as well as any types of primary internal cancers [89]. In addition, LiP patients (an ECM1-deficient human model) have no predilection for skin and internal malignancies. The lack of association between ECM1 and genetic susceptibility for tumor development has yet to be fully characterized.

ECM1 FUNCTION IN SKIN AGEING

Skin ageing is an inevitable progressive deterioration of various physiological functions, which are principally divided into intrinsic (chronological) and extrinsic (photo) ageing, although the latter is mostly superimposed on the intrinsic mode [135]. The intrinsic ageing is slowly progressive occurring in the entire body skin, a morphological feature showing fine shallow wrinkles, lack of elasticity and tensile strength, and hypochromic surface. The skin pathology shows thinner (atrophic) epidermis with degeneration of the underlying connective tissues. These changes are substantiated by reduced expression of extracellular matrix molecules, such as interstitial collagens, elastin, fibulins, and glycosaminoglycans, further accelerated by enhanced release of matrix-degrading metalloproteases and the resultant decrease of ECM and elastin, fibrillin-1 [136-139]. In contrast, the photoageing of skin shows coarse deep wrinkles with yellow-brownish surface, abrupt keratinization and pigmentation, and telangiectasia [135]. Pathologically it shows irregularly thickened (acanthotic) and atrophic epidermis with loss of keratinocyte polarity and dysregulation of melanocyte density, and more significantly, dermal elastolysis caused by repeated degeneration and production of collagen/elastic fibers, and excess deposition of glycosaminoglycans [136,140-142]. Thus, the skin ageing pathology is closely associated with an imbalance of the biological network of ECMs and collagens.

ECM1 expression is downregulated in the intrinsically aged skin protected from chronic UV exposure, but inversely increased by acute (transient) and chronic UV exposure (photoageing) [10]. The bipolar responses of ECM1 are followed by up-/down-regulation cascades of its binding partners, including MMPs, type IV collagen, laminin 332, fibronectin, perlecan, and transforming growth factor (TGF)-β, finally affecting the biological activity of dermal fibroblasts, and recomposition of the structural proteins and surrounding ECM molecules [10,14,70,85,86]. Combining with the clinical observation that sun-exposed LiP skin tends to have severe scarring and photo-aged appearance compared with non-exposed skin [143], ECM1 may play a photoprotective role in human skin. However, the expression levels of TGF-β mRNA and protein did not significantly differ between female genital (non-UV exposed) LS and normal subjects [144,145], suggesting that differences of the disease stage, duration of the disease, and ongoing treatments in each patient might affect the expression of fibrogenic and collagenogenic molecules.

Cigarette smoking is a deleterious extrinsic factor for skin ageing [146]. Not only epidemiological studies using large cohorts or twins [147,148], but also *in vitro* studies using cultured mesenchymal cell sources, dermal fibroblasts and osteoblasts, have demonstrated that cigarette smoke extracts and/or nicotine alter the baseline production of collagens, fibronectin, and elastin, and also increase MMPs, which accelerates the degrading and aberrant deposit of collagens and ECMs [146,149]. Moreover, chronic nicotine exposure decreases the binding activity of transcription factor AP1 with its binding sequences [150], which may cause the down-regulation of AP1-dependent gene expression, including ECM1; human, mouse, and rat ECM1 gene promoter regions contain one or two functional binding sites for AP1 [9]. An imbalance between biosynthesis and breakdown in dermal connective tissue metabolism, as well as direct transcriptional regulation of ECM1 gene, may thus participate in the smoking-induced skin ageing.

CONCLUSION

Considerable progress in animal model studies for determining the *in vivo* ECM1 function and new insights of this molecule into the particular T cell subsets, CD4+CD25+Tregs and Th2 cells, and genetic susceptibility of ulcerative colitis, have been made the last decade. For functional ECM1 impairment, however, genetic ablation and passive transfer of the specific antibody in mice have not fully explained the pathophysiological characteristics in LiP and LS. An extrapolation simply from the clinical observation that LiP patients are viable without a predilection of skin and internal malignancies, ECM1 can be dispensable or otherwise, at least compensable for the development of any types of organs, including skin. Likewise, ECM1 may not be responsible for a higher complication of squamous cell carcinomas in the long-standing LS skin. Nevertheless, multi-angle researches for ECM1 binding partners and associated signaling molecules reinforce the potential importance of ECM1 in the complex skin organization and homeostasis, and more surprisingly, the lung regeneration in post-pneumonectomy mice [151] and its physiological role in ureteric bud branching and nephron development (C. Mendelsohn, unpublished results) Further unraveling the pleiotropic action of ECM1 in the cell and tissue biology needs to be activated.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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