Skin Fibroblasts Are the Only Source of Nidogen During Early Basal Lamina Formation *In Vitro*

Raul Fleischmajer, Alan Schechter, Marco Bruns, Jerome S. Perlish, E. Douglas MacDonald, Te-Cheng Pan,* Rupert Timpl,† and Mon-Li Chu*

Department of Dermatology, Mount Sinai School of Medicine, New York, New York; *Departments of Biochemistry and Molecular Biology, and Dermatology, Thomas Jefferson University, Philadelphia, Pennsylvania, U.S.A.; and the †Max-Planck Institute for Biochemistry, Munich, Germany

The purpose of this study was to determine whether nidogen, the linkage protein of the basal lamina, is of epidermal or dermal origin. The development of the basal lamina was studied in an *in vitro* skin model. Preputial fibroblasts seeded onto a nylon mesh attached, proliferated, and developed a rich extracellular matrix (dermal model). Preputial keratinocytes were added to the dermal model to form a keratinocyte dermal model that ultrastructurally resembled in many respects human skin. Ultrastructural analysis revealed early stages of dermal development, including an incomplete basal lamina, aggregates of dermal filamentous material connecting to the lamina densa, bundles of 10-nm microfibrils, formation of premature hemidesmosomes, anchoring filaments, and anchoring fibrils. The cell origin of nidogen was determined in the dermal model and in the epidermal and dermal components of the keratinocyte dermal model. Specific antibodies and a cDNA probe for nidogen were used for immunofluorescence microscopy, Western and Northern blots, and for *in situ* hybridization studies. Our data show that fibroblasts are the only source of nidogen during early basal lamina formation. Although fibroblasts can synthesize nidogen and deposit it in the dermal matrix, no basal lamina will form unless they are recombined with keratinocytes. This suggests that the epidermis plays a major regulatory role in the production and assembly of nidogen into the basal lamina. *J Invest Dermatol 105:597–601, 1995*

Basal laminae are sheetlike supramolecular structures that are deposited near cells in a polarized fashion (epithelial and endothelial cells) or surrounding the entire cell (muscle, fat, and nerve cells) [1]. Although the functions of the basal laminae are not fully understood, they appear to be involved in establishing and preserving tissue architecture, providing anchorage for adjacent cells, controlling cell migration and invasion, participating in branching morphogenesis of epithelia, and regulating blood filtration, and are also involved in receptor localization. The major components of the basal lamina are type IV collagen, laminin, heparan sulfate proteoglycan (perlecan), and nidogen (entactin). The exact molecular architecture of basal laminae is not entirely known, although there is evidence that type IV collagen as well as laminin can form homotypic polymers [2–4].

Another crucial element in the supramolecular assembly of the basal lamina is apparently the 150-kDa glycoprotein nidogen, which consists of three globular domains and some connecting structures [5]. Nidogen binds through these globular domains with high affinity to laminin, collagen IV, and perlecan, and thus allows the formation of ternary complexes [6,7]. This was interpreted to indicate that nidogen is the essential mediator for connecting the independent networks of collagen IV and laminin, which is required to form stable basal lamina structures. Further studies demonstrated a mesenchymal origin of nidogen during the embryonic development of many organs and its integration into basal lamina in the vicinity of epithelial cells that contribute laminin and collagen IV to these structures [8–10]. The general importance of such cellular cooperation was shown with antibodies that inhibit the laminin-nidogen interaction [11] and prevent the formation of new basal laminae during kidney tubulogenesis and lung branching [10]. Whether such cooperations also exist during skin development has not been examined.

Early transplantation studies with epidermal tissues suggested that keratinocytes are responsible for the formation of the basal lamina [12]. There is evidence that keratinocytes can synthesize laminin [13,14], type IV collagen [15,16], and perlecan [17], but nidogen has so far not been studied. Recently, a new culture system has been developed in which fibroblasts grown in a nylon mesh develop a rich extracellular matrix [18,19]. When such dermal models were recombined with keratinocytes, there was a rather striking reconstruction of the skin including an epidermis, basal lamina, anchoring zone, and a dermis [19–21]. It is interesting that laminin, type IV collagen, nidogen, and perlecan were localized at the epithelial stromal interface and also in the dermis. The purpose of this study was to determine the tissue origin (epidermal versus dermal) of nidogen in this new three-dimensional *in vitro* human culture system and to show that during early basal lamina formation, fibroblasts are the exclusive producers of nidogen.

MATERIALS AND METHODS

Culture Systems The dermal model and the keratinocyte dermal model were grown at Advanced Tissue Sciences (La Jolla, CA) and shipped to our

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Reprint requests to: Dr. Raul Fleischmajer, Department of Dermatology, Box 1047, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

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laboratories on nutrient agarose. Keratinocytes and fibroblasts were isolated from human neonatal foreskin by sequential trypsin and collagenase digestion and then expanded into monolayer cultures [18,19]. Briefly, to obtain the dermal model, fibroblasts were seeded onto a nylon mesh in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/ml ascorbic acid. A feeding schedule for 26–27 d was established, alternating DMEM at 37°C with phosphate-buffered saline at 4–8°C [22]. To prepare the keratinocyte dermal model, keratinocyte cultures were grown in monolayers in keratinocyte serum-free medium (GIBCO, Grand Island, NY). Keratinocytes were then seeded onto the dermal model, submerged for 5–7 d, and then raised to an air-liquid interface [23] using DMEM supplemented with 5% fetal bovine serum, 100 μg/ml ascorbate, 0.5 μg/ml hydrocortisone, and a cholesterol-rich lipid supplement (Sigma, St. Louis, MO). Keratinocytes were kept at the air-liquid interface for 2–4 weeks.

Antibodies and cDNA Probes Affinity-purified nidogen antibodies were prepared from mouse EHS (Engelbreth-Holm-Swarm) tumor [24], and antibodies were raised in rabbits. Antibodies against human nidogen were also obtained with a recombinant product [5]. The probe for human nidogen was a 4.0-kb cDNA, as reported previously [25]. We also used a 1.2-kb cDNA for glyceraldehyde-3-phosphate dehydrogenase [26].

Electron Microscopy Samples from the keratinocyte dermal models were fixed in Karnovsky's solution for 4 h at room temperature, post-fixed for 1 h in ferrocyanide osmium tetroxide, and stained *en bloc* for 1 h in 1% phosphotungstic acid followed by 1 h in 3% uranyl acetate [27]. Samples were dehydrated and embedded in Spurr's resin. Meshes were then rotated at a 45° angle with the point of each square-shaped opening aligned with the knife edge. Semi-thin and ultra-thin sections were obtained in a Sorval MT-2b ultramicrotome and examined in a Jeol EM 100 electron microscope.

Immunofluorescence Microscopy Specimens of the dermal model and the keratinocyte dermal model were cut into 3–4-mm squares and frozen in Tissue Tech O.C.T. embedding compound (Miles Labs, Elkhart, IN). Frozen sections 5–6 μm thick were placed on polylysine-coated slides, fixed in cold acetone (−20°C) for 5 min, and subjected to indirect immunofluorescence microscopy as described previously [21]. Specimens were examined with a Nikon microscope equipped with epifluorescence illumination. Human embryonic skins (about 16–18 weeks) were obtained from spontaneous abortions and processed as described above. Antibodies were used in concentrations of 0.2 mg/ml. Controls consisted of pure rabbit IgG or serum from nonimmunized rabbits.

Western Blots Twenty-four dermal model and keratinocyte dermal model meshes (11 × 11 mm) were extracted in 0.15 M NaCl in 0.05 M Tris-HCl, pH 7.4, containing protease inhibitors (phenylmethylsulfonyl fluoride [3 mg/l], *p*-chloromercuribenzoate [3 mg/l], 0.5 mM iodoacetamide, and 10 mM ethylenediaminetetraacetic acid) [28]. The total extract was dialyzed at 4°C against water for 24 h and lyophilized. The dried extract was dissolved in Laemmli sample buffer, and comparable amounts of material were loaded onto a 4% to 20% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Daiichi gel; Integrated Separation Systems, Natick, MA). All samples were reduced with 2-mercaptoethanol before electrophoresis. Electrophoresis was performed in 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3, at a constant current of 50 mA per gel. Nidogen present in the extracts was identified by Western blot analysis using specific antibodies described above.

Protein bands were transferred to nitrocellulose, treated with antibody and ^{125}I-protein A (NEN, Boston, MA), and exposed to x-ray film at −20°C. In addition to the dermal model and keratinocyte dermal model samples, preputial keratinocyte and fibroblast monolayers from the same source (Advanced Tissue Sciences) were processed as described above.

***In Situ* Hybridization** Plasmids containing the cDNA subclones were digested with the appropriate restriction enzyme to liberate the cDNA inserts. After digestion, cDNA inserts were purified by excision of slices of low-melting agarose-containing DNA bands following electrophoretic separation. The DNA in low-melting agarose was then directly labeled by random primer extension to a specific activity of 10^8 cpm/μg using ^{33}P-dATP [29]. The radiolabeled probes were then purified from unincorporated nucleotide either on Sephadex G-50 or by selective precipitation with ammonium acetate/ethanol [29].

Frozen sections of dermal model and keratinocyte dermal model were post-fixed in 4% paraformaldehyde, dehydrated with increasing concentrations of ethanol, and air dried. After rehydration in 2 × sodium citrate/sodium chloride buffer (SSC), the sections were treated with 100 μg/ml of proteinase K (in 2 × SSC) for 15 min at room temperature, then incubated with pre-blocking agents I and II following the Oncor Sciences (Gaithersburg, MD) *in situ* hybridization protocol. The cDNA probes were dissolved in Oncor hybridization buffer (50% formamide/2 × SSC; Oncor Sciences) in the presence or absence of 100 ng of unlabeled homologous probe. A total of 5–10 × 10^6 cpm in a volume of 40 μl was added to each slide. The slides were coverslipped and incubated in a humidified sealed container overnight at 45°C. Sections were washed twice in 2 × SSC; incubated for 30 min in 50% formamide, 2 × SSC at 45°C; and then washed once in 2 × SSC and 1 × SSC, respectively. After dehydration with increasing ethanol concentrations, the slides were air dried and immersed in Kodak autoradiographic emulsion (diluted 1:1 with deionized water). In all experiments, slides were exposed in the dark at 4°C for 5 d before development.

Northern Blot Total RNA was isolated from fibroblasts and keratinocyte monolayers and 24 meshes of dermal model. Twenty-four meshes of the keratinocyte dermal model were treated with thermolysin (1.2 mg/ml) to separate the epidermis from the dermis. Total RNAs were isolated using acid guanidinium thiocyanate/phenol/chloroform [30]. RNA samples were separated on a 1% agarose gel containing 6% formaldehyde, transferred to hybond N membrane (Amersham, Chicago, IL), and hybridized to the cDNA probe labeled with ^{32}P by nick-translation. Hybridization was performed in 4 × SSC (1 SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 100 μg/ml salmon sperm DNA at 65°C overnight. The membrane was washed to a final stringency of 0.1 × SSC, 0.1% SDS at 60°C, and then exposed overnight to Kodak XAR film at −70°C in the presence of intensifying screens.

RESULTS

Ultrastructure of 2- and 4-Week Keratinocyte Dermal Model Cultures Four-week dermal model cultures revealed a connective tissue structure consisting of fibroblasts in parallel arrangement separated by chambers with a rich extracellular matrix, containing collagen fibrils 30 to 80 nm in diameter, 10-nm microfibrils, and monolayers of fibroblasts in the upper and lower edges of the specimen, as reported previously [20,21,31]. Two-week keratinocyte dermal model specimens revealed a well-organized epidermis and an absent or incomplete lamina densa, which was present in areas with and without hemidesmosomes, suggesting that these structures probably do not precede the development of the lamina densa. There were numerous vesicles connecting to the cell plasma membrane facing the extracellular space. The lower border of the keratinocyte dermal model culture, in contact with the plastic support, revealed a monolayer of fibroblasts in parallel arrangement, consisting of elongated cells with multiple pinocytotic vesicles and a well-developed rough endoplasmic reticulum. However, the narrow intercellular spaces revealed small amounts of interstitial matrix and no evidence of a basal lamina.

Localization of Nidogen at the Dermoepidermal Junction and in the Dermis Indirect immunofluorescence microscopy of the dermal model showed that nidogen was strongly present throughout the entire space in a rather uniform distribution **(Fig 1*a*)**. In the 2-week keratinocyte dermal model, where keratinocytes were in contact with the dermis for 2 weeks, nidogen showed a distinct linear distribution at the dermoepidermal junction, although the rest of the dermis also showed nidogen deposits within or around fibroblasts near the basal lamina **(Fig 1*b*)**. In the 4-week keratinocyte dermal model, most of the nidogen staining was noted at the dermoepidermal junction and in fibroblasts, whereas the rest of the dermal matrix showed only little staining **(Fig 1*c*)**. Fetal human skin (16–17 weeks old) showed the nidogen restricted to the dermoepidermal junction, around hair follicles and capillaries, whereas it was absent in the entire dermal matrix (not shown). This suggests that the presence of nidogen in the model dermis represents an initial transitory stage in the formation of the basal lamina.

Evidence for a Mesenchymal Origin of Nidogen Three different approaches were used to determine the origin of nidogen in the *in vitro* skin model. Western blots for nidogen revealed a broad band in the range of 80–100 kDa in the extracts of the dermal model, keratinocyte dermal model, and in fibroblast monolayers **(Fig 2)**. The presence of multiple nidogen fragments in the gels (molecular weight of nidogen 150 kDa) is due to endogenous proteolysis, which is particularly high for human nidogen. How-

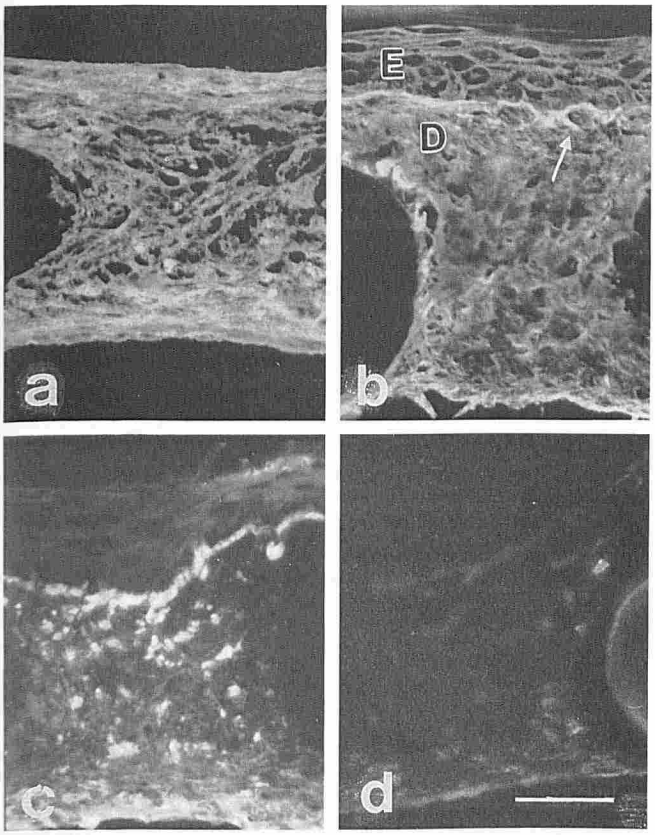

Figure 1. Nidogen originates in the dermis. Indirect immunofluorescence microscopy with nidogen antibodies. *a*) Dermal model. *b*) Keratinocyte dermal model, 2 weeks. E, epidermis; D, dermis. *c*) Keratinocyte dermal model, 4 weeks. *d*) Dermal model stained with purified rabbit IgG. *Bar*, 50 μm.

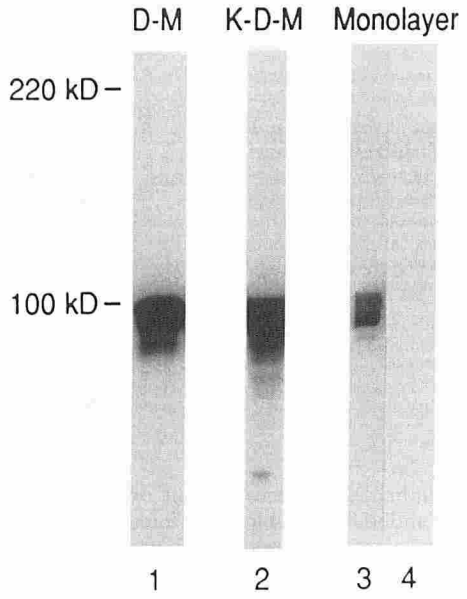

Figure 2. Dermal origin of nidogen by Western blot. Western blot using nidogen antibodies after separation of extracted tissue proteins in a 4% to 20% SDS-PAGE gel. *Lane 1*, dermal model (D-M); *lane 2*, keratinocyte dermal model (K-D-M); *lane 3*, fibroblast monolayer; *lane 4*, keratinocyte monolayer.

ever, no reactive band could be obtained from the keratinocyte monolayer. These data strongly suggest a mesenchymal localization of nidogen. Northern blot analysis detected an mRNA of 6.0 kb for nidogen in the dermal model. After thermolysin treatment, the keratinocyte dermal model revealed a similar mRNA transcript in the dermal component, whereas the epidermis was negative. Extracts of monolayer cultures revealed nidogen mRNA in the fibroblast cultures, but it was not present in keratinocytes **(Fig 3)**. *In situ* hybridization studies of the keratinocyte dermal model specimens showed that only the dermal component contained mRNA for nidogen, whereas practically no nidogen was present in the epidermis, thus correlating well with the Northern blot data. *In situ* hybridization of the dermal model showed that fibroblasts in the upper and lower monolayers also expressed mRNA for nidogen, although some staining was also noted within the dermal matrix **(Fig 4)**.

DISCUSSION

Several previous studies have shown that the formation of basement membranes is very often dependent on mesenchymal-epithelial interactions, which are particularly obvious during embryonic development and could still exist in adult tissues [10,32]. In the present study, we have used the keratinocyte dermal model culture, which mimics in many aspects skin development [18–21]. A particular advantage of the model is the possibility to initiate basement membrane formation by adding keratinocytes to a preformed fibroblastic extracellular matrix, which allows examination of the early molecular events of the process. We have concentrated here on nidogen synthesis because of its functional versatility [7]. Antibody studies and mRNA analysis clearly identified fibroblasts as the exclusive source of nidogen, in agreement with data observed in many developmental studies. These studies have initially shown a transient deposition of nidogen in the dermis of embryonic rats [33], in mammary glands [34], and in reticulin fibrils of lymph nodes [35]. *In situ* hybridization of various mouse embryo organs including ganglia, tongue, jaw, heart, liver, stomach, kidney, and lung [8,10,36] also emphasized a major mesenchymal origin of nidogen. Its final destination, however, is the epithelial and other

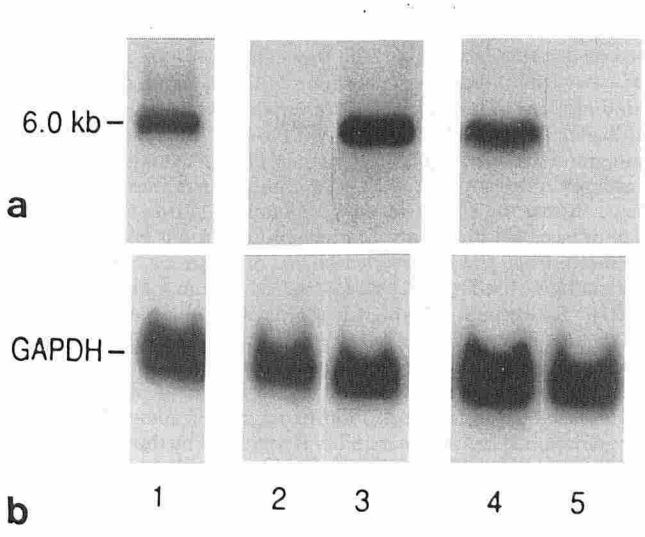

Figure 3. Dermal origin of nidogen mRNA by Northern blot analysis. The epidermis was separated from the dermis of a keratinocyte dermal model by thermolysin digestion. A 30-μg sample of total RNA in each lane was electrophoresed on a formaldehyde-1% agarose gel, transferred onto nylon membrane, and hybridized with ^{32}P-labeled cDNA probes for human nidogen (*a*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*b*). *Lane 1*, dermal model; *lane 2*, keratinocyte dermal model, epidermis; *lane 3*, keratinocyte dermal model, dermis; *lane 4*, fibroblast monolayer; *lane 5*, keratinocyte monolayer.

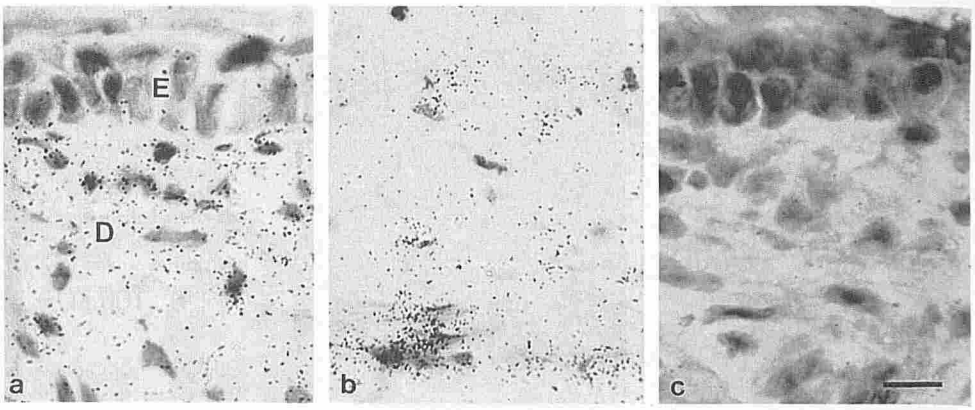

Figure 4. Dermal origin of nidogen by *in situ* hybridization. Tissue sections were incubated overnight with a ^{33}P-labeled cDNA probe for nidogen. *a*) Keratinocyte dermal model. E, epidermis; D, dermis. *b*) Dermal model. *c*) Control treated with excess unlabeled cDNA probe. *Bar*, 12.5 nm.

basement membranes, as seen in adult organs. If this process is prevented by antibodies that block the interaction between laminin and nidogen, then nidogen relocalization and the whole developmental process are interrupted [10].

With the dermal model substrate, we could show that fibroblasts alone produce large amounts of nidogen and deposit the protein in a rather uniform fashion in the interstitial matrix. The addition of keratinocytes to the model induces a time-dependent redistribution of nidogen. After 2 weeks of incubation, when most keratinocytes have already attached to the dermal equivalent, the interstitial nidogen staining was reduced and in part substituted for by staining at the dermoepidermal junction. After 4 weeks, the relocalization process has progressed further and resulted in a spot-like appearance of remnant nidogen deposits in the interstitium, which suggests restriction around individual fibroblasts. A similar massive and transient dermal deposition of nidogen was reported previously for embryonic rat skin [33], which indicated a similar relocalization during normal development. This process is apparently completed in the skin of 16–17-week-old human fetuses which, as shown here, have nidogen exclusively in basement membrane zones.

The presence of keratinocytes not only triggers nidogen accumulation at the dermoepidermal junction, but also decreases nidogen deposition in the interstitial matrix, as indicated from strongly reduced antibody staining. The first effect could be due to a supply of basal lamina ligands, by either keratinocytes or fibroblasts that bind nidogen. These ligands were previously identified as laminins, collagen IV, perlecan, and fibulins, and have been shown to integrate themselves into larger complexes through connections made by nidogen [5–7,32,37]. Preliminary data in our laboratories have shown the cell surface assembly of collagen IV in the matrix site of basal keratinocytes (in preparation). In this context, it is also of interest that the laminin γ-1 chain contributes a high-affinity site for nidogen binding [11] and is shared by most laminin isoforms except for laminin-5, which is a typical component of anchoring filaments. The reduction of interstitial nidogen very likely cannot be entirely explained by its accumulation in the basement membrane. Keratinocytes may therefore down-regulate nidogen synthesis by a negative feedback loop or trigger proteolytic systems for degradation. The latter possibility is indicated by the high sensitivity of nidogen to tissue proteases, including several matrix metalloproteinases [38]. Such mechanisms undoubtedly will be of general relevance, and the keratinocyte dermal model substrate could be an appropriate model for further experimental approaches.

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