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Gene silencing in a human organotypic skin model

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Abstract

Here we present a simple and highly reproducible method which allows the study of the effects of a single gene knockdown in an organotypic skin model. Human keratinocytes (KC) were transfected with backbone-modified short interfering RNAs (siRNAs) specific for vascular endothelial growth factor (VEGF) and matriptase-1. Twenty-four hours later the transfected cells were seeded onto fibroblast collagen suspensions and allowed to build up a multilayered epidermis by culture at the air/medium interface for 7 days. Protein expression of both targeted genes remained down-regulated by more than 80% up to 8 days after transfection. As expected, VEGF knockdown by siRNA did not alter epidermis formation in our organotypic skin model. By contrast ablation of matriptase-1 led to aberrant KC differentiation and impaired filaggrin processing and resulted in an epidermal phenotype closely resembling that of matriptase-1 deficient mouse skin. Our results suggest that siRNA-mediated gene silencing is highly efficient in an organotypic skin model and readily allows the assessment of the roles of individual genes during terminal KC differentiation.

Keywords: RNAi; siRNA; Keratinocytes; Organotypic skin culture; Skin; Matriptase-1; VEGF; Knockdown

Specific silencing of genes in mammalian cells by RNA interference (RNAi) was described only a few years ago [1]. Since then this method has developed into a powerful tool allowing the evaluation of the contributions of individual gene products to complex biological processes in cells of higher organisms [2–5]. Thus RNAi technology represents an important *in vitro* alternative to the gene deletion approach in experimental animals, in particular in instances where it would result in a lethal phenotype. In contrast to its high efficiency *in vitro*, the use of RNAi *in vivo* has proved to be more difficult [1]. Major obstacles for *in vivo* applications are the instability of small interfering RNA (siRNA) in plasma and, importantly, the delivery into target organs [6,7]. Whereas the former problems can be overcome by backbone modifications of the siRNA [6,8], the difficulties concerning the targeting of specific organs are still not resolved.

Most of the published data on RNAi applications *in vitro* use conventional monolayer culture systems in short-term assays [9–11]. To improve on such models we set out to investigate whether RNAi technology could also be used in more complex *in vitro* cell culture systems. Three-dimensional organotypic skin cultures are widely used to study the signaling involved in terminal KC differentiation *in vitro* [12]. In this model system KC are grown on a support consisting of collagen and fibroblasts at the air liquid interface for up to 4 weeks. During this time KC start to form a multilayered, well-differentiated epithelium with a stratum corneum-like surface [12,13]. To investigate the effects of RNAi on this organotypic model, we chose to apply it for 2 genes strongly expressed by KC: VEGF and matriptase-1. VEGF is a central endotheliotropic cytokine participating in angiogenesis and regulating vascular permeability [14]. Its major source in the skin are epidermal KC [15]. Inactivation of the VEGF gene specifically in KC influences wound healing and tumor development in experimental animals but has no effect on

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epidermal development [16]. Matriptase-1, also referred to as membrane-type serine protease-1 (MT-SP1) and tumor-associated differentially expressed gene-15 (TADG-15), is a member of the type 2 integral membrane serine protease family [17,18]. Mice lacking matriptase-1 die within 48 h after birth due to an impaired skin barrier [19]. In fact the skin of these animals showed several abnormalities, in particular striking irregularities of the stratum corneum, characterized by dysmorphic and pleomorphic corneocytes [19]. More recently, it was shown that lack of matriptase-1 perturbs lipid matrix formation, cornified envelope morphogenesis, and stratum corneum desquamation, as well as a loss of proteolytically processed filaggrin monomer units and the NH_2-terminal filaggrin S-100 regulatory protein [20]. Skin from newborn matriptase-1 knockout mice transplanted onto SCID mice develops an ichthyosis-like phenotype with formation of a thickened and compact stratum corneum, severe acanthosis, and hyper- and weak parakeratosis [20].

Applying RNAi technology in an organotypic skin model, we show here for the first time that epidermal phenotypes, comparable to those of corresponding knockout animals, can readily be reproduced *in vitro*.

Materials and methods

Cell culture. Human dermal fibroblasts were obtained from Cascade Inc. (Portland, OR, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS, PAA, Linz, Austria), 25 mM L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco).

KC derived from neonatal foreskin of single donors were purchased from Clonetics (San Diego, CA) and cultured in KC growth medium (KGM, Clonetics) which is a modified MCDB 153 medium supplemented with 0.1 ng/ml human recombinant epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.4% bovine pituitary extract, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B.

siRNA transfection. Stealth™ siRNAs (Invitrogen, Carlsbad, CA, USA) used are listed in Table 1. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with slight modifications. Third passage KC were grown to 70–80% confluency in a T75 culture flask. Lipofectamine 2000 (50 μl) was mixed with 65 μl of a 20 μM siRNA solution and 5 ml OPTI-MEM medium (Gibco). After 30 min at room temperature, KGM (20 ml) was added and the solution was poured onto the KC monolayer (25 ml) for 24 h. After transfection KC were trypsinized and seeded onto a fibroblast collagen gel as described below.

Preparation of organotypic skin cultures. In vitro organotypic skin cultures were generated as described previously with minor modification [12]. Briefly, a suspension of collagen type I (Vitrogen 100, Collagen Corporation, CA) containing 1×10^5 fibroblasts per ml was poured into cell-culture inserts (3 μm pore size; BD Bioscience, Bedford, MA, USA) and allowed to gel for 2 h at 37 °C in a humidified atmosphere. The gels were then equilibrated with KGM for 2 h, and 1.5×10^6 KC, either non-transfected or transfected with the respective siRNAs (see Table 1), in a total volume of 2 ml KGM, were placed on the collagen gel. After overnight incubation the medium was removed from both the inserts and external wells, and replaced in the external wells by serum-free KC defined medium (SKDM), consisting of KGM but without bovine pituitary extract and supplemented with 1.3 mM calcium (Sigma, Vienna, Austria), 10 μg/ml transferrin (Sigma), 50 μg/ml ascorbic acid (Sigma), and 0.1% bovine serum albumin (Sigma) for up to 7 days. The volume of culture medium was chosen so that KC were positioned at the air-liquid interface. SKDM was changed every second day.

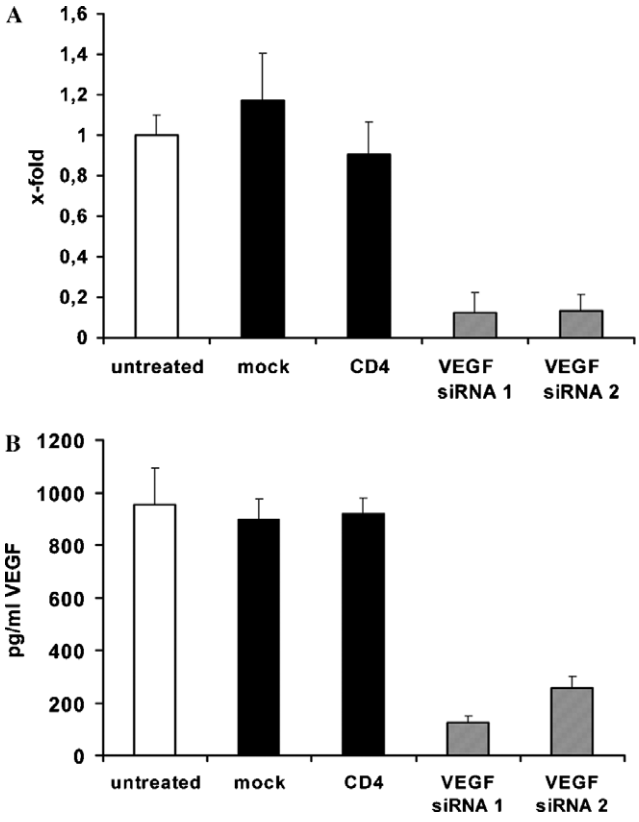

Fig. 1. Efficient inhibition of VEGF secretion by VEGF siRNA. (A) mRNA from organotypic skin cultures at day seven was prepared and analyzed by RT-PCR. VEGF mRNA production in VEGF deficient organotypic cultures was reduced by 90% and 89% as compared to untreated organotypic skin cultures. (B) Cell culture media from organotypic skin cultures at day seven were collected and subjected to VEGF ELISA. Mock treatment or CD4 siRNA transfection did not influence VEGF secretion in organotypic skin cultures. Transfection of two different VEGF siRNAs inhibited VEGF secretion by 86% and 75%.

Table 1
Sequences of used Stealth™ siRNAs

Gene	Accession No.	Position[a]	Sequence of RNAi
CD4	NM_000616	nt 319	CCAACCAGAUAAAGAUUCUGGGAAA
Matriptase-1	NM_021978	nt 1084	UCCUGCUCAUCACACUGAUAACCAA
Matriptase-1	NM_021978	nt 1835	GGUGAACGUCGUCACUUGUACCAAA
VEGF	NM_003376	nt 1332	GGAGUCCAACAUCACCAUGCAGAUU
VEGF	NM_003376	nt 1625	GCAAGGCGAGGCAGCUUGAGUUAAA

[a] Indicates the starting position of used siRNAs.

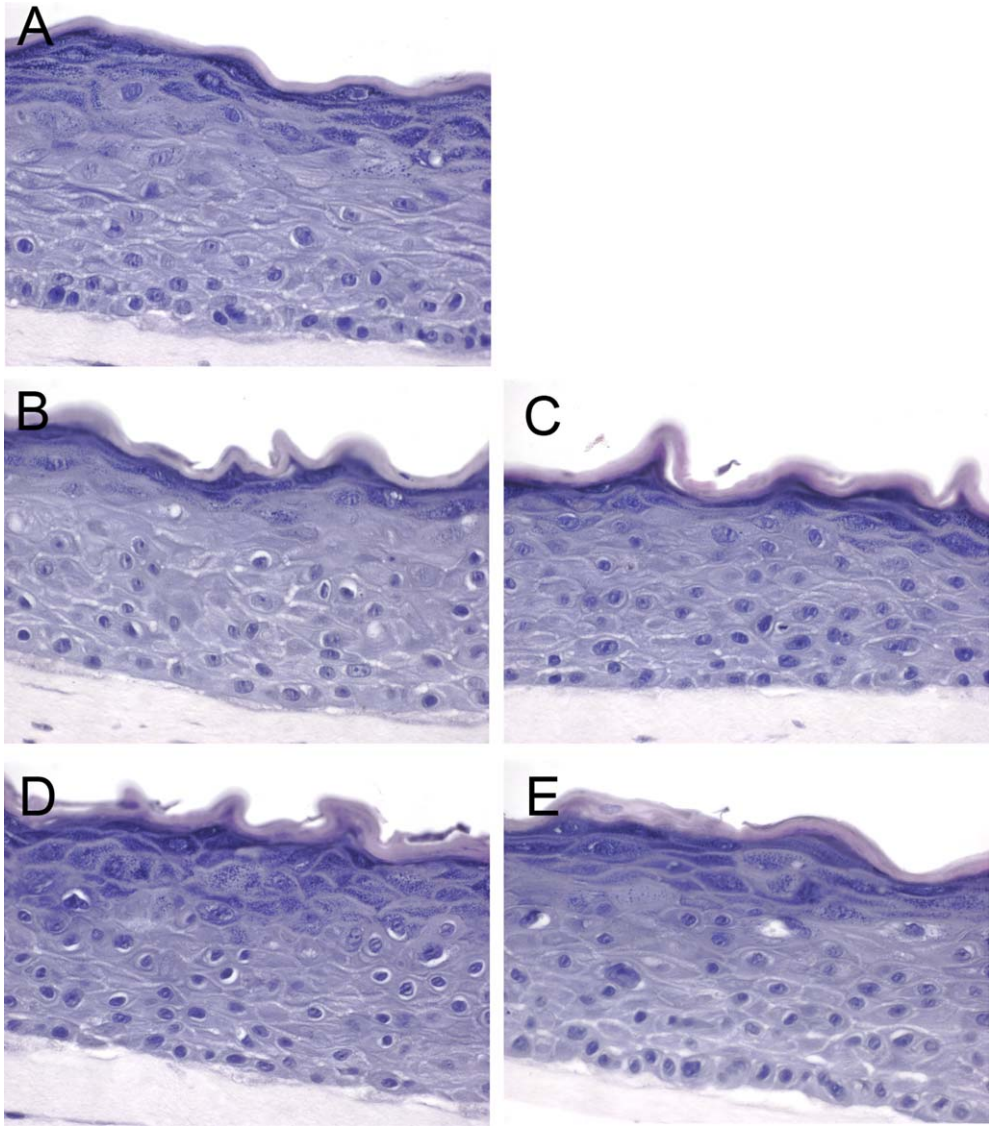

Fig. 2. Organotypic skin cultures established from KC transfected with VEGF siRNAs show a normal morphology. H&E staining of organotypic skin cultures either untreated (A), mock treated (B), transfected with CD4 siRNA (C) or deficient of VEGF (D,E) revealed no differences in the differentiation of KC in the organotypic skin cultures.

Analysis of the gene knockdown efficiency. Efficiency of the knockdown of VEGF expression was measured by determining secreted VEGF in the supernatants of the organotypic skin cultures by ELISA (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Knockdown of matriptase-1 expression was monitored by Western blot analysis. Western blot analysis was performed as described previously [21] with minor modifications. Organotypic skin cultures were lysed in SDS–PAGE loading buffer. After sonication and centrifugation proteins were size fractionated by SDS–PAGE through an 8–18% gradient gel (Amersham Pharmacia Biotech, Uppsala, Sweden) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Immunodetection was performed with a rabbit anti-matriptase-1 antiserum (dilution 1:3000; Bethyl Laboratories, Inc., Montgomery, TX, USA), followed by a HRP-conjugated goat anti-rabbit IgG antiserum (dilution 1:20,000; Pierce, New York, NY, USA). An identical blot was reacted in parallel to an irrelevant rabbit serum as negative control. Reaction products were detected by chemiluminescence with the ChemiGlow reagent (Biozyme Laboratories Limited, South Wales, UK) according to the manufacturer's instructions.

RNA isolation and cDNA preparation. After lysis by TRIzol® Reagent (Invitrogen) and RNA extraction following the manufacturer's instruction, cDNAs were transcribed with the GeneAmp® Kit using MuLV-reverse transcriptase and oligo(dT) primers (Applied Biosystems, Foster City, CA) as indicated in the instruction manual.

Quantitative real-time PCR. mRNA expression was quantified by real-time PCR with LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. The primers for VEGF (forward: 5′-CCCTGATGAG ATCGAGTACATCTT-3′, reverse: 5′-ACCGCCTCGGCTTGTCAC-;3′), matriptase-1 (forward: 5′-AGGTGTTCCAATGGGAAGTG-3′, reverse: 5′-CGTCGCTACAGTCCTCCTTC), and β-2-microglobulin (B2M, forward: 5′-GATGAGTATGCCTGCCGTGTG-3′, reverse: 5′-CAATCCA AATGCGGCATCT-3′) were designed as described previously [22]. The relative expression of the target genes was calculated by comparison to the housekeeping gene β2M using a formula described by Wellmann et al. [23]. The efficiencies of the primer pairs were determined as described in [22].

Filaggrin processing. To analyze filaggrin processing, organotypic skin cultures were homogenized by ultrasonication in 1 M NaSCN, 50 mM Hepes, and 10 nM EDTA, pH 6.8, containing 20 μg/ml phenylmethylsulfonyl fluoride and 0,1% iso-propanol on ice. Filaggrin and its precursors were precipitated from these lysates by addition of 10 volumes of ice-cold water followed by centrifugation at 12,000*g* according to a protocol

published by [24]. Pellets were dissolved in SDS–PAGE loading buffer and subjected to Western blot analysis as described above. Membranes were incubated with a mouse anti-filaggrin monoclonal antibody (2 μg/ml; Neomarkers, Lab Vision Corporation, Fremont, CA, USA) followed by a HRP-conjugated goat anti-mouse IgG antibody (dilution 1:10,000; Amersham Pharmacia Biotech, Uppsala, Sweden). Reaction products were detected by chemiluminescence with the ChemiGlow reagent (Biozyme Laboratories Limited, South Wales, UK) according to the manufacturer's instructions.

Immunostaining. Immunostaining was performed on 5 μm thick sections of formalin fixed, paraffin-embedded organotypic cultures. After deparaffinization and hydration, sections were pre-treated with protease XXIV (0.5 mg/ml, Sigma) for 10 min at 37 °C. After three washes with PBS (Gibco) staining was performed with fast red staining kit (Neomarkers, Lab Vision Corporation, Fremont, CA, USA) according to the manufacturer's instructions. The slides were incubated with either anti-matriptase-1 (1 μg/ml; Bethyl laboratories, Montgomery, Texas, USA) or normal rabbit IgG (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS containing 2% BSA overnight in a humidified chamber at 4°. Nuclei staining was done with haematoxylin (Sigma). Slides were mounted with Aquamount (Merck, Darmstadt, Germany).

Results

Knockdown of VEGF in an organotypic skin model

Transgenic animals, in which the VEGF gene has been specifically inactivated in KC, show normal skin development and no obvious defects of epidermal differentiation [16]. Knockdown of VEGF expression in organotypic skin culture prepared from KC transfected with two different siRNAs targeting VEGF (Table 1) blocked VEGF mRNA expression by 90% and 89% (Fig. 1A), and release of VEGF protein by 86% and 75% as compared to non-transfected controls (Fig. 1B). Neither mock treatment with Lipofectamine 2000 alone nor transfection of KC with a siRNA targeting CD4, which is not expressed by KC, influenced the expression or release of VEGF (Fig. 1A and B). H&E staining of paraffin sections revealed no morphological difference of the newly formed epidermis between cultures established from untreated KC (Fig. 2A) or KC treated with either Lipofectamine 2000 alone (Fig. 2B) or together with CD4 (Fig. 2C) or VEGF siRNA (Fig. 2D and E). These experiments demonstrate that transfection of siRNA by itself does not disturb epidermal development of the organotypic skin model.

Knockdown of the serine protease matriptase-1 in an organotypic skin model

In contrast to transgenic animals with inactivation of the VEGF gene in KC, matriptase-1 deficient mice show a severe epidermal phenotype [19,20]. Using the same protocol as above two different siRNAs targeting matriptase-1 (Table 1) were transfected into KC. Transfection of KC with matriptase-1 siRNAs led to a downregulation of matriptase-1 mRNA by 87% and 86% after 7 days in the organotpyic skin culture. Western blot analysis confirmed that matriptase-1 protein expression was also reduced by 98% as compared to untreated and mock-transfected KC as well as KC transfected with CD4 siRNA (Fig. 3). Histology of the cultures transfected with both matriptase-1 siRNAs (Fig. 4D and E) but not of controls (Fig. 4A–C) revealed distinct changes of epidermal morphology with an increase in stratum corneum thickness for matriptase-1 siRNA 1: 3.46-fold $\pm$ 0.52, $p < 0.0001$, and for matriptase-1 siRNA 2: 2.76 $\pm$ 0.23, $p < 0.0001$ as compared to the untreated control organotypic skin culture. In addition some dispersed parakeratotic nuclei were regularly visible (Fig. 4D and E). Immuno-histochemical staining of organotypic skin cultures established from either untreated (Fig. 4F) or mock treated KC (Fig. 4G), as well as KC transfected with CD4 siRNA (Fig. 4H), shows pronounced matriptase-1 staining in the granular layers. By contrast, in organotypic skin cultures from KC transfected with matriptase-1 siRNAs, matriptase-1 staining was nearly completely abolished (Fig. 4I and J). The phenotype of organotypic skin cultures derived from matriptase-1 siRNA transfected KC thus reproduced features of the epidermal phenotype of skin transplanted from matriptase-1 knockout animals on SCID mice [20]. Furthermore, similar to matriptase-1 knockout animals, filaggrin processing was altered in organotypic skin

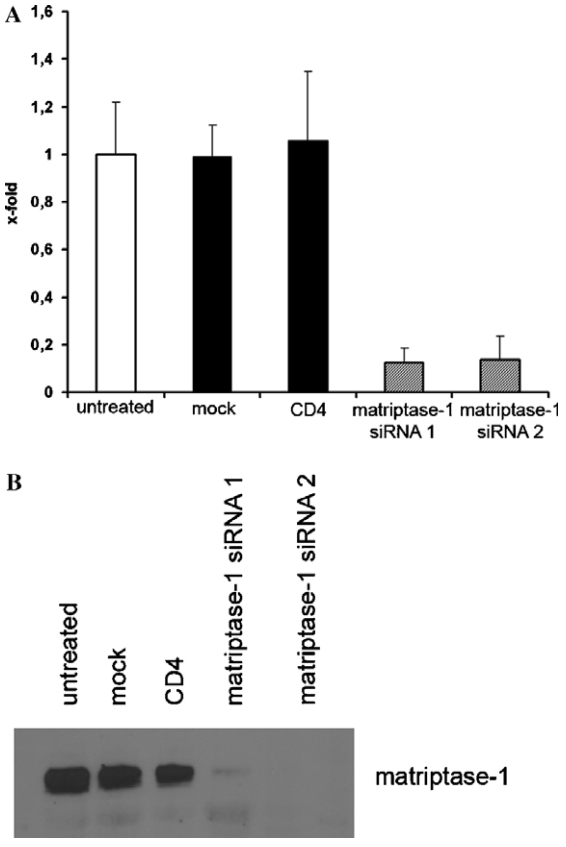

Fig. 3. Matriptase-1 expression by KC can be efficiently blocked by transfection with matriptase-1 specific siRNAs. In contrast to untreated, mock treated or CD4 siRNA treated organotypic skin cultures, the expression of matriptase-1 was nearly completely abolished by transfection of KC with two different siRNAs targeting matriptase-1, as shown by (A) real-time PCR and (B) Western blot analysis. Equal loading of protein was controlled by Ponceau stain of the same blot (data not shown).

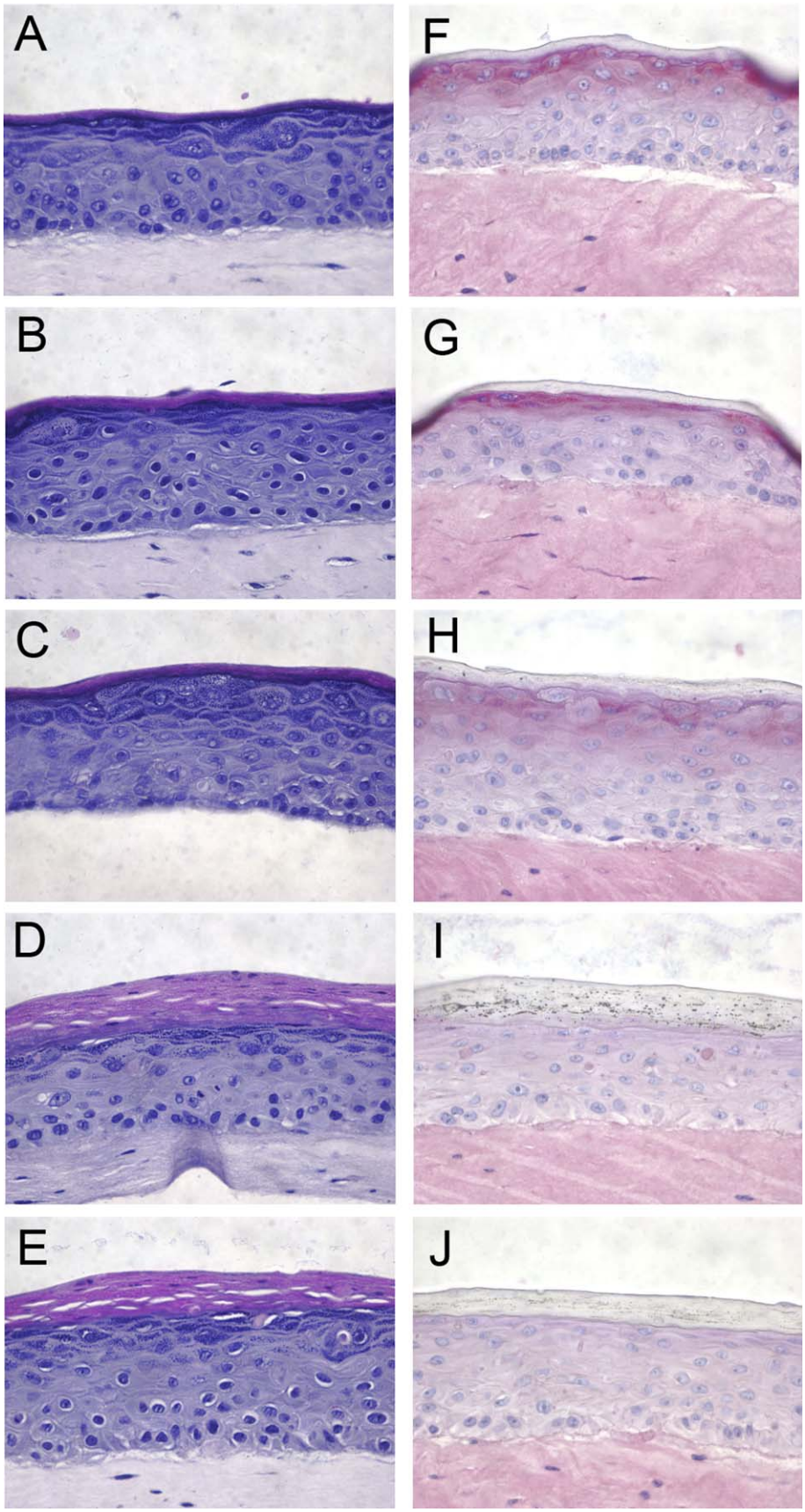

Fig. 4. Matriptase-1 deficient organotypic skin cultures show an impaired KC differentiation. H&E staining did not show differences between organotypic skin cultures established with untreated (A) mock (B) or CD4 siRNA (C) treated KC. In contrast, organotypic skin cultures derived from KC deficient for matriptase-1 showed hyper- and parakeratosis (D,E). Immuno-histochemistry revealed pronounced matriptase-1 staining in the granular layers of control organotypic skin cultures (F–H) whereas in organotypic skin cultures from matriptase-1 siRNA transfected KC (I,J) matriptase-1 was virtually absent.

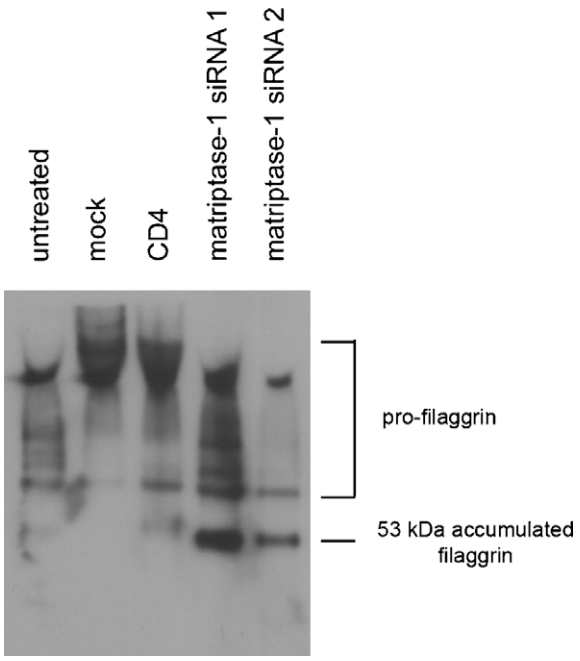

Fig. 5. Filaggrin processing is altered in matriptase-1 deficient organotypic skin cultures. Western blot analysis revealed a 53 kDa filaggrin protein species which accumulated in matriptase-1 knockout organotypic cultures but not in untreated, mock or CD4 siRNA treated organotypic skin cultures. Equal loading of protein was controlled by silver stain of an identical gel (data not shown).

cultures with matriptase-1 deficient KC. By Western blot analysis with a monoclonal antibody directed against the N-terminus of the filaggrin molecule, we detected a protein with a molecular mass of 53 kDa in lysates from matriptase-1 deficient but not control organotypic skin cultures (Fig. 5). Accumulation of a similar protein was also found in epidermis of matriptase-1 knockout animals [20]. As deduced from the size and the reactivity with monoclonal antibody (directed against the N-terminus of human filaggrin) this protein corresponds to that which has previously been identified as the N-terminal part of the filaggrin molecule, consisting of a Ca^{2+}-binding protein of the S-100 family together with the truncated first filaggrin repeat [25]. This cleavage product represents a processing intermediate which is rapidly converted into the mature S-100 protein [25]. Therefore the 53 kDa intermediate is hardly detected by Western blot analysis of normal epidermis [25]. Thus our data show that knockdown of matriptase-1 in KC in an *in vitro* organotypic skin model by RNAi technology reproduced both the epidermal phenotype of the matriptase-1 deficient mice and the alteration of adequate filaggrin processing.

Discussion

The results presented here demonstrate for the first time that siRNA-mediated knockdown of genes in KC in an organotypic skin model leads to effects on KC differentiation comparable to that seen in corresponding knockout animals: In the case of VEGF knockdown, no altered epidermal phenotype was observed [16]. By contrast, in the case of matriptase-1 knockdown terminal differentiation of KC was affected, manifesting as thickening of the stratum corneum and incomplete removal of the nuclei from end differentiated KC. This phenotype recapitulates the phenotype of the matriptase-1 knockout animal [19,20]. A highly interesting finding was that the effects of siRNAs were sustained for at least 7 days in our organotypic skin model. A possible explanation is that KC, once put on the collagen/fibroblast matrix, stop dividing (our own unpublished observation), and thus siRNAs may be retained at relevant concentrations within the transfected cells. One advantage of using the RNAi technology in an organotypic skin model is that it allows the study of the direct role of target genes in epidermal development/differentiation without the difficulties imposed in animal models either by associated mortality [26] or inflammation [27]. Furthermore, the organotypic model used here can also be extended by other cell types present within the skin such as Langerhans cells, melanocytes, endothelial cells, etc. It is therefore conceivable that in the future even more complex cellular interactions may be studied in this model system with the help of RNAi methodology. Last but not least an important advantage of the approach presented here is that human cells are used. Therefore the use of RNAi in an organotypic model represents an important intermediate step in the evaluation of the feasibility and safety of using siRNA for clinical applications. This is particularly interesting for therapies of hereditary epidermal diseases with single gene defects such as epidermolysis bullosa [28]. In conclusion our data demonstrate that the use of siRNA technology in an organotypic skin model is able to reproduce functional changes comparable to those observed in animal knockout models. This approach might therefore help to reduce the number of animal experiments and will facilitate the testing of the potential of siRNAs in therapies of skin diseases in a preclinical setting.

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