Post-transcriptional mechanisms in type XVII collagen synthesis

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POST-TRANCRIPTIONAL MECHANISMS IN TYPE XVII COLLAGEN SYNTHESIS

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Cover: the 'birth" of type XVII collagen in the keratinocyte

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CHAPTER 1

INTRODUCTION

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ABSTRACT

The main function of the protein type XVII collagen, formerly known as BP180 or BPAG2, is anchoring the epidermis to the underlying dermis. The function of type XVII collagen can be impaired due to either an auto-immune reaction against or genetic deficiency of the molecule, which then leads to the development of blistering diseases.
Research over the years has mainly focused on understanding this adhesive function and less attention was given to understanding its basic regulation of transcription and translation. As it appeared that the protein may exert a very dynamic expression level and is also heterogeneous at both the mRNA transcript and at the protein level, it seems to make sense to study its basic features in detail. Also nearing the chances that gene therapy, applied to repair defective *COL17A1* alleles, will be successfully requires a thorough understanding of which factors control expression of the protein and its derivatives[1]. At this moment, however, many aspects are largely not understood.
In this chapter we try to highlight the major observations involving most functional aspects of type XVII collagen and to indicate what aspects need further investigation. Moreover, we will focus on other supposed functions of type XVII collagen. At the end of this introduction, we will point out the most intriguing subjects in connection with the aim of this study.

Heterogeneity of type XVII collagen at the mRNA and the protein level

The human gene coding for type XVII collagen, *COL17A1*, is located on the long arm of chromosome 10, in band 10q24.3. It spans 52 kb of genomic DNA and contains 56 exons that finally lead to a coding sequence of 4491 nucleotides[2-5]. This number of exons is substantially higher than the mean number per gene (8.8) in the human genome[6].
The coding sequence is highly conserved between species; *Mus musculus* (mouse) and *Homo sapiens* coding sequences showing a 86% overlap[4,7]. Other known (partial) *COL17A1* sequences include *Canis familiaris* (dog), *Mesocricetus auratus* (hamster), *Sus scrofa* (pig), and *Gallus Gallus* (chicken)[8-11].

By use of bullous pemphigoid and herpes gestationes patient sera the first cDNA clones that contained *COL17A1* sequences were obtained in 1990[2]. Within two years this led to the chromosomal assignment of the gene and the discovery of the complete ORF sequence, which showed on Northern blot as a 6 kb transcript[4,7]. For eight years it was assumed that this was the only transcript until in 2000 Molnar *et al* showed that alternative splicing generated two messengers that differed 0.6 kb in size[12].
This alternative splicing leads to two mRNA variants that have the same ORF but differ in their 3'UTR. One variant, hereafter referred to as the long variant, contains the full-length 3'UTR, whereas the second variant, hereafter called the short variant, lacks 610 nucleotides within the 3'UTR (Fig. 1). The long variant is the major transcript in normal human keratinocytes, whereas the short variant predominates in the squamous carcinoma cell line UMSCC-22B. In the long variant 3'UTR four stretches are found which are highly conserved (78-87%) between the human and the mouse sequence. The homology here is significantly higher than the mean homology found between 3'UTRs of human and mouse[13].
The function of this differential splicing is still completely unknown, but could involve functions that nowadays are attributed to 3'UTRs such as control of translation level, transcript stability, and subcellular mRNA localisation.
Extensive sequencing of ORF amplimers in many laboratories, partly as search strategies for *COL17A1* mutations in type XVII collagen deficient patients, has not unveiled any discrepancies at the coding sequence level. The sequence of the 5'UTR, however, is still elusive. The *COL17A1* 5'UTR of only one species has so far been found; the murine 5'UTR spans 306 nucleotides and the sequence is highly homologous to the human DNA sequence upstream of the first exon[7].

The protein was originally identified as a 180 kDa antigen that could be visualized by pemphigoid patient sera in Western blotting on epidermal cell extracts. Deduction

from the ORF sequence gives 155 kDa of amino acid sequence. It furthermore contains several kilodaltons of extracellular carbohydrate moieties and can be phosphorylated[14,15]. It has a type II transmembrane orientation, thus with a COOH-terminal ectodomain and an NH_2-terminal cytoplasmic domain. The ectodomain comprises two-third of the entire molecule and contains 15 collagenous subdomains (COL1-COL15) by which the molecular tail folds into a triple-helical structure. As a result the final shape of the trimer is a globular head with a central rod and a flexible tail (Fig. 1)[4,16,17].

Immunoelectron microscopy against two different epitopes in the ectodomain demonstrated that the ultrastructural localisation of these epitopes differed that way that it must be assumed that the flexible tail forms a loop in the lamina lucida, thus with the COOH-terminal end lying higher in the lamina lucida than the mid-portion of the tail[16].

This triple helix folding supposedly occurs in an N- to C-terminal way, which is the opposite of the classic fibril-forming of pro-collagens[18]. In this the folding process the three monomer chains may interact by a specific nucleation site in the NC16A subdomain, that precedes the fifteen collagen sub-domains, to allow for the formation of a proper stagger in the COL15 domain after which the folding can protrude in the C-terminal direction.

The main function of the cytoplasmic domain is to present a binding platform in the intracellular part of the hemidesmosome for other adhesion molecules, that will be discussed in more detail below.

The second form of type XVII collagen is the soluble 120 kDa ectodomain also called LAD-1 (Fig. 1)[14,19]. Originally the molecule was discovered in 1996 by Marinkovich *et al* when they immunoblotted sera of patients having linear IgA dermatosis[20]. Although first thought to represent a separate antigen, one year later Pas *et al* demonstrated that bullous pemphigoid and linear IgA dermatosis sera recognized a same 120 kDa antigen, and that this antigen was very similar to the ectodomain of type XVII collagen[14]. The form is also present as a separate homo-trimer and thus it does not form mixed trimers with the full-length type XVII collagen molecule[21].

The cleavage product was first demonstrated in the medium of cultured keratinocytes and antibody mapping showed that it only contained epitopes also present in the ectodomain of the full-length molecule. A polyclonal antibody against NC16A revealed that most of this domain must present in this cleaved form. Rotary-shadowed images showed that this molecule is composed of the central rod and the flexible tail, the globular head of the founder molecule is, as expected, lacking[19]. Pas *et al* suggested that the soluble form was generated either by alternative splicing or by proteolytic cleavage[14]. Five years later it was showed that the soluble form is probably shed from keratinocytes by metalloproteases, in particular ADAMs, a family of sheddases. Three members of the ADAM family, TACE, ADAM-9, and ADAM-10, can shed type XVII collagen in vitro, although TACE-deficient keratinocytes still showed a residual shedding of 60%. Moreover, silencing the gene

of the major sheddase, TACE, with siRNAs resulted only in reducing type XVII collagen cleavage to half of its original level and the actual contribution of ADAM-9 and ADAM-10 to the shedding of the ectodomain has not been investigated[22-24]. This suggests that the cleavage of type XVII collagen to its 120-kDa form may involve sheddases, but that other unidentified factors may also be necessary.
By deleting specific sequences within the NC16A domain it was defined that the cleavage site ranged from amino acid 528 to 547. Software prediction of secondary structures of these deletion mutants revealed that non-shed mutants formed a new amphipatic α-helix, which disturbed the tertiary structure of the homo-trimeric type XVII collagen molecule. One large deletion mutant showed no changes in secondary structure with respect to the original entire NC16A sequence and the non-cleavage here was explained by the residual NC16A being to short to allow cleavage[24].
The supposed shedding of the LAD-1 molecule may be accompanied by modifications to the amino acid sequence as autoantibodies in linear IgA disease preferentially target LAD-1 rather than the full-length protein. Moreover, sera of bullous pemphigoid patients, that in general recognise BP180 and/or BP230 may sometimes only target the LAD-1 molecule[14,25,26]. This suggests that the soluble ectodomain contains neo-epitopes that apparently are also immunogenic.

Besides LAD-1 a second soluble form has been described, LABD97, that is somewhat smaller, having a molecular weight of 97 kDa (Fig. 1). Originally it was described by Zone *et al* as an antigen in linear IgA dermatosis[27]. The 97 kDa form supposedly is a product of C-terminal cleavage of LAD-1, since both forms contain the NC16A domain[19,20]. Antibodies raised to the 97 kDa form locate it in the lamina lucida closely spaced to the ectodomain of type XVII collagen, and it has been suggested that it may form a complex with this molecule[28].
The two soluble forms of type XVII collagen have different N-termini; the N-terminus of LAD-1 appears to be seven amino acids upstream compared to that of LABD97 (Alanine-531 versus Leucine-524)[29,30]. The reason for this difference remains unclear. As mentioned above, the 97-kDa antigen may be proteolytically produced by cleavage of the C-terminal part of the 120-kDa antigen. It is conceivable that the same proteases cleave LAD-1 at the N-terminus. A hypothesis to explain the N-terminal difference may be that ADAMs determine their cleavage site on basis of fixed distance to the transmembrane domain rather than a specific recognition site. Slight differences in the molecular conformation of type XVII collagen in in vivo skin may influence the cleavage site. Another hypothesis is that the 97-kDa antigen is a product of distinctive cleavage processes[29]. The latter hypothesis seems the most favourable one.

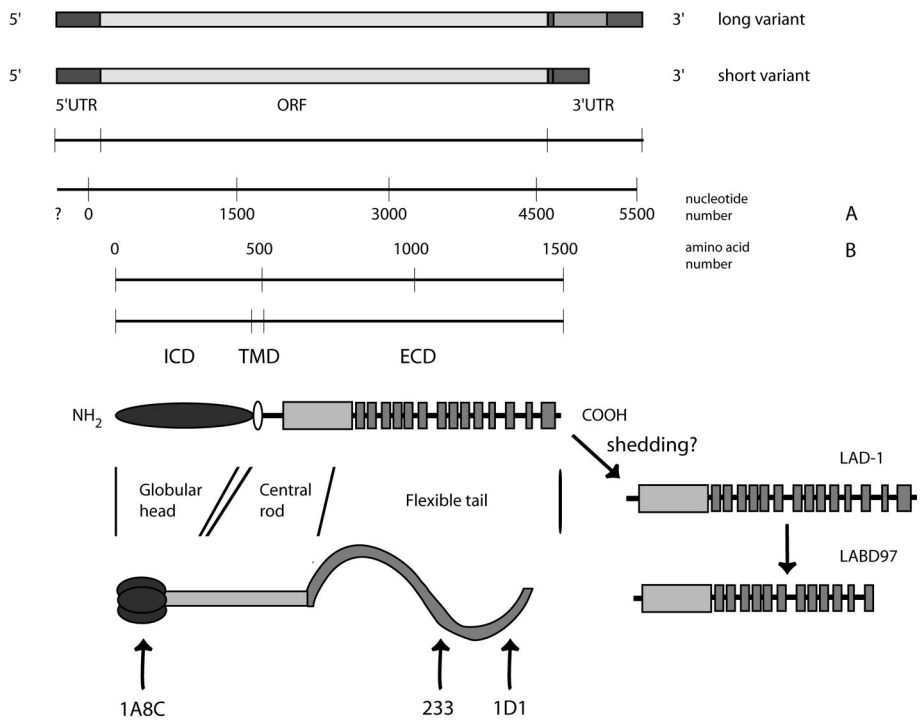

Figure 1. Heterogeneity of type XVII collagen at the mRNA and protein level.
Schematic depiction of both mRNA and protein structures of type XVII collagen. (**A**) *COL17A1* mRNA consist of two mRNA variants, one containing the full-length 3'UTR (in light and dark grey), hereafter referred to as the longer, whereas the second variant, hereafter called the short variant lacks 610 nucleotides within the 3'UTR (in dark grey). Moreover, the exact sequence of the 5'UTR is still unknown. (**B**) Type XVII collagen has a type II transmembrane orientation, thus with a COOH-terminal ectodomain and an NH_2-terminal cytoplasmic domain. The ectodomain comprises two-third of the entire molecule and contains 15 collagenous sub-domains (COL1-COL15: depicted in dark grey) by which the molecular tail folds into a triple-helical structure. Folding is initiated at the NC16A domain, after which a proper stagger is formed in the COL15 domain (in light grey). The folding can then protrude in the C-terminal direction. As a result the trimer takes on its final shape of a globular head with a central rod and a flexible tail. Epitopes detected by monoclonal antibodies 1A8C, 1D1, and 233 are depicted with arrows. All three monoclonal antibodies are suitable for immunohistochemistry, immunofluorescence and Western blot. The ectodomain can be shed of the cell membrane, possibly through proteolytic cleavage, resulting in a secreted form of 120 kDa (LAD-1), which can then be further cleaved to a second soluble form of 97 kDa (LABD97). UTR: untranslated region, ORF: open reading frame, ICD: intracellular domain, TMD: transmembrane domain, ECD: extracellular domain. Partly adapted from Hirako *et al*[17], for further references see text.

Whether the soluble forms have a functional meaning is still unknown although some suggestions have been done in the literature. Cleavage of the ectodomain may influence keratinocyte motility, detachment, and differentiation. Transfection of HaCaT keratinocytes with cDNAs of involved sheddases, TACE, ADAM-9, and ADAM-10, did not only enhance cleavage of the ectodomain but also altered cell motility. This evidence is, however, not very convincing since no significant differences in cell motility with control HaCaT keratinocytes were observed. Detachment may also influenced by shedding since it can be imagined that keratinocytes have lost binding partners after shedding[22]. In addition, Hirako *et al* suggested that shedding by metalloproteases contribute to hemidesmosomal turnover[23]. This would imply that the soluble forms are no more than garbage left behind by basal cells that have migrated to the upper layer. Others have suggested that cleavage of the ectodomain may render keratinocytes unresponsive for possible ligands of type XVII collagen as the soluble ectodomain may then modulate the activity of those possible ligands[31]. Moreover, the emerging N-terminus of the ectodomain may also contact other ligands on keratinocytes.
The only sound conclusion at this moment is that more detailed cell biological analyses are necessary to determine the role of shedding.

Location and function of type XVII collagen

The main function of type XVII collagen is the anchoring of the basal cells of the epidermis to the underlying basement membrane. Its adhesive function is exerted in combination with several other proteins that together form the hemidesmosome, to be precise the type I hemidesmosome (Fig. 2). The importance of type XVII collagen in this complex is emphasised by the severe blistering of the skin when the function of type XVII collagen is impaired by mutations in the *COL17A1* gene[32,33]. The protein may also be involved in motility of keratinocytes, since type XVII collagen deficient keratinocytes show increased movement compared to normal keratinocytes[34].
Furthermore, incubation of healthy human keratinocytes with IgG autoantibodies from sera of patients with pemphigoid diseases result in increased secretion of two specific cytokines, IL-6 and IL-8, whereas secretion of other cytokines is not altered. Pre-adsorption of these autoantibodies with recombinant stretches of the NC16A domain abolishes this secretion. These observations indicate a role for type XVII collagen in signal transduction in which the precise pathway still has to be resolved[35].
Type XVII collagen can be phosphorylated by protein kinase C (PKC) and this may cause disassembly of hemidesmosomes. When type XVII collagen was phosporylated by use of 12-O-tetradecanoylphorbol-13-acetate (TPA), the protein acquired a higher apparent molecular weight on SDS-PAGE, which could be abolished by a selective PKC inhibitor. Treatment of cells with TPA also changed the localisation of type XVII collagen from the cell surface to a smaller ring pattern in the cytoplasm[36,37]. Disassembly of hemidesmosome by phosphorylation of

another component of the hemidesmosome, integrin α6β4, has already been investigated and will be discussed in more detail below.

Hemidesmosomal structure

Hemidesmosomes are adhesion structures that provide a strong interaction between basal cells and the underlying basement membrane. They consist of at least six different proteins: the type XVII collagen, the plakin protein BP230, the α_6 and β_4 subunits of integrin $\alpha_6\beta_4$, tetraspanin/CD151 and plectin (Fig. 2)[4,38-44].
Hemidesmosomes form the bridge between cytoskeleton and the extracellular matrix components. Hemidesmosomes appear in two different types: type I hemidesmomes contain all components, whereas type II hemidesmosomes only consist of integrin $\alpha_6\beta_4$, tetraspanin, and plectin[45-47].

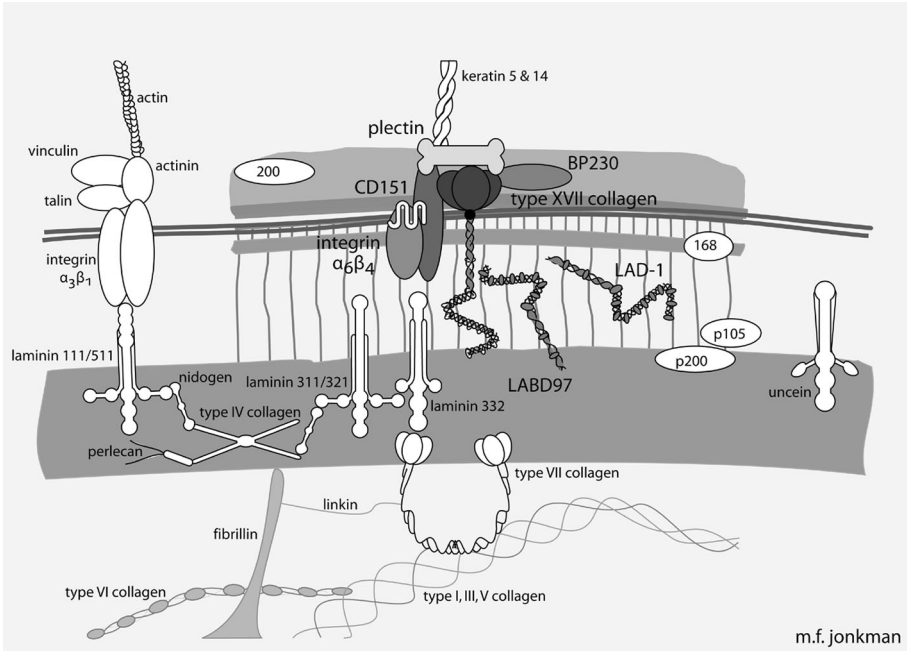

Figure 2. Schematic depiction of adhesion molecules at the basement membrane zone and hemidesmosome.
Drawing by prof. M.F. Jonkman.

Yeast-two-hybrid studies revealed several putative interactions between type XVII collagen and the other hemidesmosomal proteins.

Type XVII collagen interacts with BP230 through a region of 85 amino acids (145-230) in the N-terminal region and this sequence is both necessary and sufficient to incorporate BP230 into the hemidesmosome. BP230 is involved in this interaction by the NH_2-terminal Z-Y domain and to further strengthen the interaction additional binding sequences on type XVII collagen might be needed. BP230 itself then binds the specific basal cell intermediate filament keratins 5 and 14 by the B and C domains of the COOH-terminus[48,49].
The amino-terminal first 400 amino acids of type XVII collagen are needed for binding to the Z-Y domains of plectin. Surprisingly, in the same assays, binding of type XVII collagen to a component of the desmosome, desmoplakin, was observed[49].
Integrin $\alpha_6\beta_4$ is also bound by type XVII collagen. The β_4-subunit binds through half of its connecting segment and its FNIII repeat, both located in the C-terminus, to type XVII collagen. Two distinct binding sites in type XVII collagen are involved in this interaction: one in the first 230 amino acids and a second more C-terminally located in the region encompassing amino acids 231-401[49-51]. The α_6-subunit binds to the NC16A domain, to be precise amino acids 506-519, of type XVII collagen, since cells incubated with an antibody against this domain were unable to form hemidesmosomes. Noteworthy, which sequence of α_6-integrin is involved is unknown[52,53].
Binding of type XVII collagen to the most recent discovered component of the hemidesmosome, tetraspanin/CD151, has not been reported yet. So far, only interaction with integrin β_4 is seen, but considering the function of tetraspanin in the early formation of hemidesmosomes (pre-forms) actual interaction with tetraspanin must seriously be considered[44].
Type XVII collagen binding to non-hemidesmosomal proteins is also observed. Keratin 18 is bound by eleven amino acids (no. 15-25) in the N-terminal region of type XVII collagen. Almost the same region (amino acids 13-25) is responsible for the binding to P120-catenin, and binding to the isoforms 1-3 and not to 4 is observed. As type XVII collagen P120-catenin can be phosporylated, and this may alter their interaction and thus their signalling functions maybe modulated in this way. The C-terminus of type XVII collagen binds to laminin 5 and this interaction may be important for the organisation of laminin 5 in the basement membrane zone[34,54,55].

Type XVII collagen is not present in type II hemidesmosomes, as is BP230. These hemidesmosomes only consist of plectin, integrin α6β4, and tetraspanin and they are found in other tissues such as colon and mammary epithelium. Type II hemidesmosomes co-localise with the cytoskeleton and their organisation is probably controlled by actin[44-46,56]. The precise function of type II hemidesmosomes is still unknown. They may act as adhesion structures in cells that lack expression of type XVII collagen and BP230. In keratinocytes they are also thought to function as early complexes during the first rapid phase of wound healing. They would then

stabilise the epithelial-extracellular matrix interaction without disturbing the healing process[46,57].

Assembly of the hemidesmosome

Koster *et al* proposed a model for successive incorporation of the individual components into the emerging hemidesmosome[49]. They assume, that it starts with the interaction of integrin $\alpha_6\beta_4$ with plectin and tetraspanin/CD151, and also with the extracellular matrix protein laminin 5, which is the major and the most important lamina lucida component[40].
This is followed by incorporation of type XVII collagen into the complex. The binding involves interaction with the already present plectin, since in cultured keratinocytes that expressed a mutant form of the integrin β_4 incapable to interact with plectin, only hemidesmosome-like structures were observed that had less adhesive power[58,59]. The binding of type XVII collagen with plectin only is not sufficient strong to induce assembly hemidesmosomes when integrin $\alpha_6\beta_4$ is absent.
Finally, BP230 binds to two components of the complex, integrin $\alpha_6\beta_4$ and type XVII collagen thereby stabilising mature hemidesmosomes.
In this model a crucial role for plectin in the incorporation of type XVII collagen in the hemidesmosome is assumed, since in the aforementioned β_4-mutant no co-localisation of mutated β_4 with type XVII collagen was observed. Plectin, therefore, seems necessary to activate the cytoplasmic domain of β_4-integrin for binding of type XVII collagen. Although BP230 and plectin are members of the same family, the plakins, BP230 cannot take over the ligand-role of plectin.
This critical role of plectin is, however, not fully supported by *in vivo* data as cells of plectin-deficient mice do actually form hemidesmosomes, although rudimentary and in reduced number. In these mice, reduced expression of other hemidesmosomal components is observed[60].
A possible explanation for the discrepancy between skin and cell culture may be the presence of an *in vivo* ligand for type XVII collagen, whereas such a ligand is absent in cultured cells. In the latter case *in vitro* assembly of hemidesmosomes would rely on plectin. In support of an unidentified ligand is the observation that keratinocytes of patients with integrin $\alpha_6\beta_4$-deficiency also contain hemidesmosomes consisting of all components except integrin $\alpha_6\beta_4$. Moreover, in β_4-deficient keratinocytes, cultured on collagen-coated coverslips, punctuated expression of type XVII collagen, resembling hemidesmosomal structures, was observed[61]. Such hemidesmosomes may have been assembled by the interaction of type XVII collagen with the unidentified ligand in the epidermal basement zone[49,61].

Signal transduction by hemidesmosomal adhesion molecules

As mentioned before binding of immunoglobulin to type XVII collagen releases interleukins 6 and 8 from keratinocytes. Also tissue-type plasminogen activator is released after binding of type XVII specific IgG antibodies and this release is in part due to a signal transduction event[35,62].
Furthermore, Kitajima *et al* showed that phosphorylation of type XVII collagen on serine residues and destruction of hemidesmosomes occurred simultaneously after TPA stimulation of DJM-1 cells. This collapse of hemidesmosomes is also observed after binding of either monoclonal antibodies against type XVII collagen or after addition of sera of patients with bullous pemphigoid to the cells[36,63].
Thus, type XVII collagen obviously is involved in signal transduction but new data have not yet emerged. Since most evidence regarding hemidesmosomal signal transduction has come from studies on integrin $\alpha_6\beta_4$ we will focus on these here. It seems that signal transduction is especially important in regulating the disassembly of hemidesmosomes. Recently, the presence of both type XVII collagen and integrin $\alpha_6\beta_4$ in lipid rafts – subdomains of the plasma membrane enriched in cholesterol and glycosphingolipids- was reported[64,65].
Various signalling proteins concentrate in these rafts and these are all palmitoylated. Similar integrin $\alpha_6\beta_4$ will only be present in these rafts if also palmitoylated. Whether type XVII collagen is also palmitoylated in these lipid rafts has not been investigated. Currently, it is only known that disturbance of lipid rafts leads to increased shedding, although actual proof of shedding is limited[64].
In contrast, assembly of hemidesmosomes does not require palmitoylated integrin $\alpha_6\beta_4$, and therefore signal transduction and adhesion functions for integrin $\alpha_6\beta_4$ appear to be independent events[65].
Specific serine residues of integrin $\alpha_6\beta_4$ can be phosphorylated in lipid rafts via the EGF-R, which activates both Fyn and Yes. These Src family kinases phosporylate in turn integrin $\alpha_6\beta_4$. It is hypothesised that phosphorylation of integrin $\alpha_6\beta_4$ initiates disassembly of hemidesmosomes, and reverse, dephosphorylation causes assembly[66].
Moreover, EGF-R activates PKC through PLC-γ and inhibition of PKC may prevent EGF-R mediated disruption of the hemidesmosome[67-69]. It is not known whether this process is reversible.
Future research to the role of type XVII collagen in the disassembly of hemidesmosomes must reveal whether type XVII collagen is of equal importance here as integrin $\alpha_6\beta_4$. The presence of type XVII collagen in lipid rafts and the ability of keratinocytes to phosporylate type XVII collagen are strong indications that type XVII collagen also might have a functional role in the disassembly of hemidesmosomes.
This is especially important in understanding squamous cell carcinomas (SCCs). Here we see reduced ability to form hemidesmosomes in combination with increased integrin $\alpha_6\beta_4$ phosphorylation[66]. Whether type XVII collagen can act in the same way is currently unknown. In SCCs, however, we see disturbed expression of type XVII collagen and this expression varies with the stage of the tumor[70].

Hemidesmosomes have long been seen as stable structures, in line with their anchoring function. Tsuruta *et al* demonstrated that they are in fact very dynamic structures. The time needed for assembly was shown by the use of GFP-tagged β_4 integrin and type XVII collagen. Fluorescence after photobleaching was recovered by five minutes for β_4 integrin and somewhat longer for type XVII collagen. Due to this fast turnover, cells can rapidly re-organise their hemidesmosomes when necessary as in wound healing and division[71]. This dynamic behaviour fits in the proposed complex regulation.

Tissue distribution of type XVII collagen

Strong type XVII collagen expression is observed in various tissues with a prominent epithelial component other than skin. Multiple tissue RNA blots revealed expression in placenta, trachea, salivary and thyroid glands, colon, mammary, and prostate. No *COL17A1* mRNA expression was, however, observed in lung and kidney tissue, in which also epithelial cells are present. In contrast, tissue staining does reveal type XVII collagen in human bronchial epithelium[54,72].
Breast epithelia express type XVII collagen in the basal cells of the normal ducts and in glandular and surface epithelia of the endometrium mostly weakly intracytoplasmic and cell membrane-associated type XVII collagen expression was observed. The staining pattern was more diffusely distributed compared to expression in basal keratinocytes of the skin[73,74].
Surprisingly in mouse, *COL17A1* mRNA expression was observed in heart tissue in as well embryonic, neonate as adult stages. Expression of *COL17A1* mRNA was higher in adult and embryonic tissue[75]. So far, these findings have not been confirmed but it is very interesting considering the association of bullous pemphigoid (in which type XVII collagen is the key target antigen) with acute myocarditis[76]. However, we examined human heart tissue with monoclonal antibodies to type XVII collagen and found no binding (unpublished data).
Recently, Claudepierre *et al* found type XVII collagen expression in the retina, cerebellum, and the olfactory bulb. They suggest that type XVII collagen complexes with laminins in the central nervous system, although evidence for direct binding could not be established. This hypothetical complex may result in the stability or adhesion of synapses in the CNS[77]. Dissecting the functional role of type XVII collagen therefore does not only benefit our knowledge of cell biology of the skin but also of other organs.

Type XVII collagen and disease

Scientific interest became for the first time focused on type XVII collagen when it appeared to be an antigen in autoimmune bullous disorders. Today it is recognised as the most important antigen in the pemphigoid group of subepidermal bullous diseases. A further flurry of activity aroused when it was discovered that genetic deficiency of type XVII collagen results in the hereditary blistering disease non-Herlitz Junctional Epidermolysis Bullosa (nH-JEB). Additional interest comes from the fields of oncology and wound healing in which the hemidesmosomes are an important study subject considering its adhesive and signal-transduction functions.

Pemphigoid

The first disease in which type XVII collagen was found as one of the autoantigens was bullous pemphigoid (BP). Over the years it became clear that antibodies to the same protein also evoked other forms of pemphigoid. Today the presence of anti-type XVII collagen autoantibodies has been confirmed in pemphigoid gestationis (PG), linear IgA disease (LAD), ocular cicatricial pemphigoid (OCP), lichen planus pemphigoides (LPP), and mucous membrane pemphigoid (MMP). The antibodies bind to type XVII collagen and deposit in skin and/or mucosa and lead to dermal-epidermal separation. In direct immunofluorescence on tissue sections the deposition can be visualised as a linear staining pattern along the basement membrane (BMZ) (Fig. 3). Since the autoantigen role of type XVII collagen is best studied in BP, we here will further focus on the role of type XVII collagen in BP only[78,79].

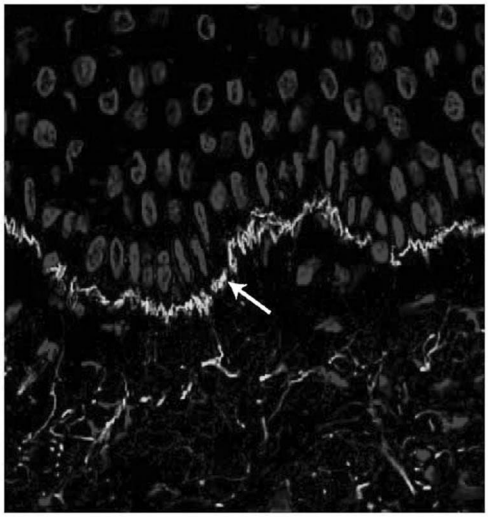

Figure 3. Autoantibodies bind to type XVII collagen and deposit along the BMZ in skin, leading to dermal-epidermal separation.
Direct immunofluorescence with antibodies against human IgG on skin tissue visualises the deposition as linear staining (arrow).

Evidence for the pathogenic role of the autoantibodies against type XVII collagen has been demonstrated both in *in vitro* and in *in vivo* studies. A fine example is the in vitro induction of separation of the epidermal and dermal compartment of skin by IgG autoantibodies from BP patient sera. Cryosections of healthy skin were first incubated with serum samples of BP patients, which was then followed by incubation with complement-containing serum and lymphocytes from healthy donors. This caused subepidermal separation and when the experiment was repeated with purified IgG from the same sera it became clear that this IgG was the initiating component of the separation process. When complement was omitted from the serum separation still occurred, when leukocytes were omitted no separation was observed anymore[80].

Moreover, injection of IgG antibodies against the murine homologue of type XVII collagen into neonatal mice caused all key features of BP. Histological examination showed broad subepidermal vesicle formation and near the edges of the blisters in this BP model large numbers of neutrophils were present. Immunological examination demonstrated that increasing the amount of injected antibodies resulted in higher titres, which, in turn, were associated with increased cutaneous disease activity[81]. The importance of both these studies was that they demonstrated that the autoantibodies were indeed pathogenic and not, as was also suggested, merely an epiphenomenon.

The precise mechanism of the subepidermal separation by the autoantibodies is largely unknown, although the BP mouse model suggests an important role for neutrophils in this process. In other autoimmune diseases the crucial role of neutrophils in immune-complex-induced inflammatory tissue destruction had already been established. In the pathogenesis of these diseases neutrophils are attracted by activated complement factors and upon engagement of their Fc receptors, proteases are released[82]. Therefore it was assumed that neutrophils are also the cause of tissue separation in the subepidermal autoimmune diseases. The presence of the neutrophil proteases 97-kDa gelatinase B and neutrophil elastase in the mouse BP is in line with this assumption.

Liu *et al* showed that mice deficient for these proteases did not form blisters when used in the BP model. Recruitment and function of the neutrophils in the skin of these mice was not impaired by the genetic deficiency of the proteases. Recently, it was also shown that in man these same proteases are also involved in blistering. Induction of dermal-epidermal separation by IgG autoantibodies of BP patients was abolished when elastase- and gelatinase B-specific inhibitors were used. Therefore, the authors stated that these proteases must be responsible for the blister forming in bullous pemphigoid, although the precise interplay between these proteases is still unclear and currently unknown proteases may additionally participate in the separation. Knowing the responsible proteases may open new approaches for patient treatment[83-85].

Man is not mouse. This is reflected in the cellular infiltrates that differ in cell type. In mouse neutrophils are attracted by the autoantibodies, whereas in human BP

eosinophils are observed as early infiltrates. This complicates a simple adoption of the mouse model data to explain blister formation in human. At this moment no causal link between the presence of eosinophils and subepidermal blistering in humans is established[81,83]. On the other hand, an *in vitro* experimental blister model demonstrated the functional activity of neutrophils in human BP[86]. Thus, it remains quite possible that the neutrophils, which act in a later phase of the pathogenesis, do have the same role in man as they have in mouse.
A lot of effort has been put in identification of the different autoantigenic epitopes on the type XVII collagen molecule. Current opinion is that two distinct important regions exist and both are located in the extracellular part of type XVII collagen. The first region is the NC16A domain that is situated just extracellular of the transmembrane domain. The second region is the carboxyl-terminal including the COL15 domain. By immuno-absorbing the NC16A-specific antibodies, it was shown that these indeed induce skin separation while autoantibodies against the COOH-terminus, used at a same titre, failed in attracting leukocytes and inducing separation. The NC16A autoantibodies can be further divided based on the subregion of the NC16A they recognise. Dividing the NC16A in 5 parts, NC16A-1 to NC16A-5, demonstrated that the NC16A-1 to -3 (aa 492-534) is the dominant target area. The NC16A-4 area was shown to be uniquely recognised by lichen planus pemphigoides sera[87]. As discussed before the soluble ectodomain is also a target for autoantibodies. Besides shared epitopes with the full-length molecule it also contains unique epitopes that may be neo-epitopes[14,25,80,88,89].

T-lymphocytes in both BP and LAD patients are specific to nearly the same amino acid sequences compared to the autoantibodies. NC16A responsive T-lymphocytes appeared CD4 positive and produced a mixed Th1/Th2 cytokine profile. CD4 positive T-lymphocytes are responsible for promoting antibody production by B lymphocytes[90,91].
Also the subclass of the autoantibodies determines the pathogenic pathways followed in inducing blisters. Most studies reported IgG4, a non-complement-fixing antibody, to be the predominant anti-type XVII collagen antibody with IgG1 as the second. Thus, already at this level different pathogenic routes may be followed. Above that also anti-type XVII collagen IgA and IgE antibodies are present. The IgA may be important as it attracts neutrophils. In MMP the presence of both IgA and IgG autoantibodies does mark a more serious and more chronic course of the disease than the presence of IgG autoantibodies alone[92]. Kromminga *et al* found in 88% of BP patients such IgA by immunoblotting of recombinantly produced protein[93]. In routine immunofluorescence analysis on esophagus and split-skin such high percentages are not seen, which suggest that in BP the IgA levels largely are very low. Furthermore, also anti-type XVII collagen IgE was reported and the serum levels of this IgE followed the severity of the disease[94]. IgE-coated mast cells were detected in perilesional skin of the BP patients. Moreover, type XVII collagen peptides were detected on these mast cells. In addition, histamine was released from

basophils of untreated BP patients after stimulation of these cells with a recombinant NC16A domain[95]. These findings underline the possibility that also IgE autoantibodies are involved in the pathogenesis of BP.

It may seem disappointing that after two decades of research the exact pathogenesis of BP is still obscure. On the other hand it has been demonstrated that autoimmune mediated inflammation is rather complicated and in fact may consist of several distinct processes occurring simultaneously. Final victory will lie in cutting this 'Gordian knot'.

Hemidesmosomal Epidermolysis Bullosa

Genetic deficiency of either laminin 5 or type XVII collagen causes non-Herlitz-Junctional Epidermolysis Bullosa (nH-JEB)[32,33]. The underlying defect is mutation of one of the following genes: *LAMA3*, *LAMB3*, *LAMC2* (coding for the alpha, beta and gamma chain of laminin 5) or *COL17A1*. The subtype nH-JEB with pyloric atresia that is caused by mutations in either integrin β_4 or integrin α_6 is beyond the scope of this chapter. JEB is characterised by the impaired function and reduction of hemidesmosomes. The clinical phenotype includes generalized skin blistering, dental anomalies, universal alopecia, and nail dystrophy[32,61,96-98].
The classification is subject of some debate, since some researcher proposed the term Hemidesmosomal Epidermolysis Bullosa (HEB) in stead of JEB[99,100]. In addition, Pasmooij *et al* were able to distinguish the clinical more severe, generalized atrophic benign epidermolysis bullosa (GABEB), from milder localized atrophic benign epidermolysis bullosa (LABEB) phenotypes by immunofluorescent antigen mapping (Pasmooij *et al*, submitted). We will use the term HEB troughout this thesis.

Of the reported mutations most are nonsense and some are missense mutations. Nonsense mutations in *COL17A1* mRNA are thought to result in nonsense mediated mRNA decay (NMD), although this assumption is so far only supported by the up-regulation of mRNAs of NMD factors in HEB keratinocytes[101]. No data are available on the actual amount of mRNA decay in these patients. A lot of effort has been put in understanding phenotype-genotype correlation, but currently no clear correlation between the distinct mutations and the severity of the disease has been established[98].
Some missense mutations are located in one of the collagenous domains of type XVII collagen and that may lead to decreased stability of type XVII collagen. To what extent this influences proper functioning of type XVII collagen is also not fully known[102-104].

Very mild forms of HEB can be observed when one of the PTCs is removed by outsplicing of the mutated exon. In three reported cases splicing was in-frame,

involving respectively exon 22, 30, and 33, so a somewhat smaller, but obviously partly functional protein was expressed[105-107]. It is likely that the outspliced exons here coded for less essential protein sequences as the opposite was also observed. Chavanas *et al* described a case where the mutated exon 32 was also removed by exon-splicing. This patient however had all the classical HEB symptoms. No expression of type XVII collagen was seen in this patient. Chavanas *et al* suggested that the absence was caused by destabilisation of type XVII collagen due to the loss of possible proline hydroxylation sites. Two of these hydroxylation sites were lost by the deletion itself and due to the deletion two proline residues move from position Y to position X in the basic G-X-Y repeat[108]. In the X-position the proline residues are not hydroxylated and hydroxylation is a critical feature in the triple-helix stabilisation[109.]

However, no correlation between the number of lost X-position prolines and the phenotype of the patients could be found. Therefore, Pasmooij *et al* suggest that the involved exons may be important for ligand binding or signal transduction. If so, exon 32 has a more important function in this binding than the other three lost exons[105].

The exon 33 skipping, found by Ruzzi *et al*, was also present at a very low level in normal keratinocytes, indicating that this skipping may represent a constitutive alternatively spliced transcript that may be become upregulated under appropriate conditions[107].

The patient that had the exon 22 outspliced showed some features of another type of EB, the simplex type. Intracellular epitopes of type XVII collagen were hardly detectable, whereas the extracellular epitopes were present. This indicated that in this patient the shed ectodomain was present in the lamina lucida of the basement membrane zone. Apparently, in comparison to the classic HEB phenotype in which all epitopes are absent, the weakest point of the basement membrane had shifted from the lamina lucida to the intracellular part of the hemidesmosome here[106].

Three HEB patients are reported that were revertant mosaics. In these patients germ-line mutations were corrected in some of the somatic cells resulting in the re-expression of type XVII collagen in these cells. When the clusters of repaired cells are large enough patches of healthy skin are seen[110-112]. Small patches of revertant cells may not be sufficient enough to restore proper adhesive function since Pasmooij *et al* also find minor clusters in non-healthy skin. In combination with the finding that two out of eleven Dutch patients were mosaics they concluded that reversion probably happens more often and may be overlooked.

The big question here is of course whether somatic reversion is a driven process. However, calculating the chances that such a revertant mutation occurs revealed that the mosaic patches in these patients could also have resulted from random mutagenesis. Their most intriguing observation was that different repair mechanisms existed in the same patient. In both patients a second-site mutation abolished the

mutation in one area, whereas in another area of either one of the two patients respectively a back-mutation and gene-conversion refrained the original mutation[112].

Revertant gene corrections are naturally occurring phenomena and understanding them may benefit developing medical gene therapy. Since skin is an easy accessible organ not only *in vivo* but also *ex vivo* gene therapy is an attractive option. Several attempts using different approaches to correct type XVII collagen deficiency have already been made on cultured keratinocytes.
Trans-splicing between a β-galoctosidase construct with a *COL17A1* sequence containing a nonsense mutation and a *COL17A1* exon-construct resulted in restoring the correct coding sequence[113]. Transfection of these constructs into keratinocytes showed β-galactosidase expression in a low percentage of the cells, indicating that in these cells the nonsense mutation had been successfully replaced. Whether this approach is conceivable and efficient enough to restore endogenous *COL17A1* mRNA remains to be elucidated.
Seitz *et al* transduced HEB keratinocytes with a retroviral expression vector and also demonstrated type XVII collagen re-expression. When these cells were used to regenerate human skin on immune-deficient mice no clear clinical evidence of blister forming was seen. However, on the histological level subepidermal bulla formation was observed and the gene transfer approach in this study may not be durable enough for actual use in human[114].
In addition, many other aspects of gene therapy still have to be resolved before it can be successfully applied.
For effective gene therapy it will be necessary to transduce the epidermal stem cells, but at this moment still no effective label for identification of these cells is available. In *ex vivo* gene therapy replacing mutated skin with genetically engineered skin will need complete removal of the mutated stem cells at the targeted location in order to avoid competition with the repaired stem cells. Stem cells have been reported in the interfollicular epidermis, in the bulge region of the hair follicle, and in sebaceous glands, but it is still unclear what their respective function is in regeneration of the skin and its appendages[115].
Recently, it was reported that the bulge stem cells do not contribute to the epidermis in the absence of trauma, though[116]. After wounding, however, they rapidly respond by producing transient amplifying cells that populate the wounded area, but these disappear again after a few weeks[117,118]. This phenomenon may interfere with proper adhesion of the repaired graft and experimental graft studies may have to be performed that address this question. Due to their deeper location stem cells of the hair bulge will be more difficult to remove as those of the epidermis. Moreover, since surgical procedures to place *ex vivo* produced skin sheets may cause scarring and delayed wound healing other techniques must be sought[98].

Type XVII collagen in carcinogenesis

Several studies have addressed type XVII collagen behaviour in squamous carcinoma cell (SCC) carcinogenesis. When carcinoma cells become malignant and turn invasive they have to become detached from the basement membrane, so they need to lose their anchors.
Squamous carcinoma cells continue to express type XVII collagen in an aberrant manner expression (Fig. 4)[15,70,119]. Parika *et al* demonstrated that for oral SCCs the most intense staining was observed in higher grade tumours. She also demonstrated downregulation of collagen XVII in basal cells in mild dysplasias. This may reflect disturbed keratinocyte adhesion to the basement membrane.

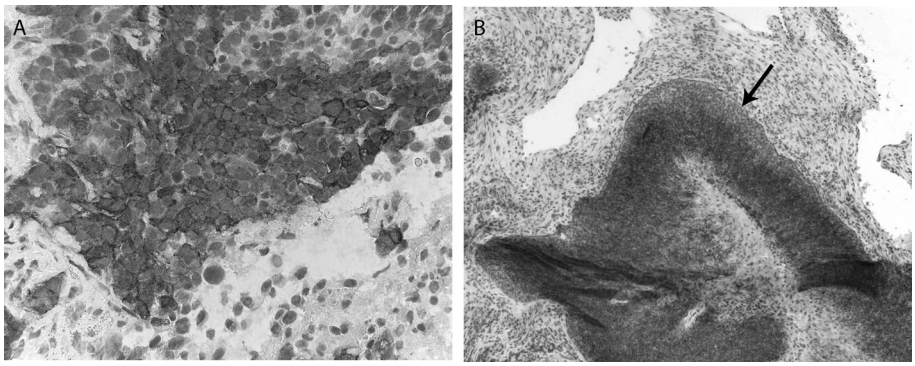

Figure 4. Aberrant type XVII collagen expression in squamous cell carcinoma.
Immunohistochemistry on moderate differentiated planocellular squamous cell carcinoma, using monoclonal antibodies against two different epitopes of type XVII collagen, shows strong expression in tumour nests (**A**) Cytoplasmic expression observed with 1A8C, an antibody against an intracellular epitope (**B**) An antibody against the extracellulair epitope 1D1 demonstrates, next to expression on the basement membrane (arrow), also similar strong expression in tumour cells (magnification: A: 400x, B 100x; H.H. Pas and H. van Goor, unpublished results).

Also ameloblastomas and basal cell carcinomas (BCC) demonstrate aberrant expression. In ameloblastomas, apart from expression in basal and suprabasal cells of the tumour nests, a diffuse intracellular staining was sometimes detected in the central cells of the neoplastic islands. In BCCs a similar diffuse cytoplasmic staining was observed in some central and peripheral cells of the tumour islands[120].
Bahadoran *et al* used an antibody to the soluble shedded type XVII collagen molecule and found faint or absent staining in tumours[121]. This might parallel the more cytoplasmic staining of the full-length molecule as seen by Parikka *et al* and could reflect an absence of shedding. Unfortunately no double staining with an antibody to the full-length molecule was performed.

In line, in another study of BCCs downregulation of *COL17A1* mRNA was shown as demonstrated by semi-quantitative RT-PCR. Downregulation of *COL17A1* mRNA was accompanied by a similar decrease in mRNA coding for other basement membrane components as BP230, integrin $\alpha_6\beta_4$, and laminin $\beta3$[120,122].
Also in neoplastic glandular epithelium of endometrial adenocarcinomas, type XVII collagen is expressed in variable degrees with increased synthesis observed in lower grade adenocarcinomas. As in other carcinoma types, cytoplasmic staining was observed, but here this was also seen in normal endometrium. Remarkable intensified expression was observed in foci of dispersed epithelial cells, and the authors wondered if this increased synthesis was a repair effort on enhanced matrix degradation due to metalloproteinases[74].
All above data demonstrate that type XVII collagen is actively regulated during the development of neoplasia, and during the invasive phase and the maturation of the tumour cells. Understanding why type XVII collagen is downregulated in mild dysplasia but upregulated in later phases as in SCCs will lead to better understanding of tumour development.

Wound healing

When basal cells start migrating to cover wounded surfaces they lose their hemidesmosomes, but after wound closure hemidesmosomes rapidly reassemble[123]. The knowledge on the exact role of type XVII collagen in this process is limited. Studies revealed that in the basal cells of the leading edge type XVII collagen is cytoplasmic rather than cell membrane bound[123,124].
This contrasts with the expression of the other membrane-bound hemidesmosomal molecule, integrin $\alpha_6\beta_4$, that is still observed at the cell membrane, although more uniformly and not only basal. This suggests different roles for these molecules. The cytoplasmic localisation of type XVII collagen is a fascinating observation as this is also observed in distinct phases of developing carcinoma (see above). Why and by what mechanisms type XVII collagen remains cytoplasmic rather than become organised in hemidesmosomes is yet unaccounted[57,123].

Unknown aspects of type XVII collagen: transcription and translation

In 1967 Jordon *et al* showed for the first time that the epidermal basement membrane contained an autoantigen that led to a blistering disease[125]. Nineteen years later Labib *et al* demonstrated the existence of an autoantigen of 180 kDa at the BMZ[126]. Today, after again a same period, we have gathered an impressive amount of information on this protein. As has become apparent in the preceding pages, many aspects of type XVII collagen have been thoroughly investigated. Studies have

been performed to its role in healthy tissue and cells and even more to its role in the pathogenesis of a range of diseases. Although these studies have provided a wagonload of information about type XVII collagen, certain aspects still are not fully understood. In the following paragraphs we will point out these aspects and we will argue the importance for further research.

Full-length type XVII collagen protein is additionally processed into two soluble forms, possibly by shedding of the cell membrane[14,19,27]. However, the involved sheddases could not completely account for the total of soluble forms, so the search for additional mechanisms is required[22]. In addition, no function is known for these other type XVII collagen proteins. Suggested functions include that both molecules may act as ligand or as a receptor for a yet unidentified ligand in the basement membrane.

Whether the alternative splicing of the *COL17A* gene into the two different mRNA forms is somehow connected with the appearance of the two soluble ectodomain forms remains elusive, and no evidence exists to support this hypothesis. Due to the fact that this alternative splicing entails the 3'UTR sequence, the function of the *COL17A* mRNA splicing may concern regulation of translation, since 3'UTRs are known to be capable of influencing translation levels, transcript stability, and mRNA transcript localisation. For instance, *cis*-acting sequences in the 3'UTR confer instability to mRNA transcripts which can be overcome by binding of *trans*-acting protein factors and mRNA transcript localisation is an important regulation mechanism for a variety of reasons:

1 to produce a local high concentration of protein
2 to segregate specific RNAs to particular organelles or subcellular structures.
3 to initiate cell lineages by sequestering localized mRNAs within a specific blastomere.
4 to produce a gradient of morphogen[127].

The latter two are especially important in embryonic development.
A further lack is the sequence of the 5'UTR. UTRs have been accepted as a major players in the complex process of initiation of protein synthesis[128]. Considering the proved involvement of both the untranslated ends of mRNA in numerous important mechanisms related with protein expression, we will focus on characterisation of both the 5'UTR and the 3'UTR of *COL17A1* mRNA in part of this thesis.

To achieve more information about the transcription and translation of type XVII collagen is of cardinal importance, seeing the participation and influence of type

XVII collagen in many processes in the epidermis, and other tissues as well. The deregulated expression of type XVII collagen in the pathogenesis of carcinomas and in wound healing is completely uncomprehended. Here, expression of type XVII collagen is not only decreased or increased, but also a shift from cell membrane to cytoplasmic expression is observed[15,57,70,119,123]. These changes may be regulated through 5'UTR or 3'UTR sequences of the *COL17A1* mRNA.
Moreover, also the involvement of type XVII collagen in the normal homeostasis of the keratinocytes needs attention. Its ligands are probably only partly known and we also do not know the function of the lateral pool of protein observed in the basal cells. It may be connected with sequence of incorporation of the hemidesmosomal components in emerging hemidesmosomes –as the subcellular location at which this occurs is still unknown- but it is also possible that it is connected in completely unknown interactions with neighbouring keratinocytes[49].
Circumstantial evidence in favour of type XVII collagen as signal transduction molecule can be found but hard data have not yet been presented[35]. Also the function of type XVII collagen in non-epithelial cells has barely been investigated[54].

Many studies have focused on the role of type XVII collagen in the pathogenesis of blistering diseases. They have confirmed the main function of type XVII collagen: the anchoring of the basal cell to underlying basement membrane. In HEB it is clear that absence of type XVII collagen leads to easy separation of dermis and epidermis. On the other hand it has also evolved that lower than normal levels, or even mutated forms of the protein, may still confer certain stability to the skin. Answering questions, as what is the minimal needed transcript level and how important are the protein subdomains for adhesion, will be important to come to appropriate gene therapy. Therefore we will also address the *COL17A1* mRNA transcript levels in HEB patients, and compare these with the clinical phenotypes.

Aim of the thesis

In this thesis we attempt to lay a basic fundament for understanding the regulation of transcription and translation. We will address the possible function of the alternative splicing by further characterisation of the untranslated regions of the *COL17A1* transcripts. The two starting points on which we direct our research will therefore be the undiscovered sequence of the 5'UTR of *COL17A1* mRNA and the unexplored function of the alternative 3'UTR ends. Furthermore, we will study *COL17A1* mRNA transcript levels in type XVII collagen deficient HEB patients to better understand the relationship between transcript levels, protein expression, and functional adhesion.

Hence, in **chapter 2**, we will address the unknown sequence of the 5'UTR and demonstrate that it is of a rather complex nature. **Chapters 3 and 4** are dedicated to the functional meaning of the two alternative 3'UTRs. In the first of these two chapters we investigate the effect of the alternative spliced 3'UTRs on the translation respective transcript levels. In the other chapter, the subcellular localisation of both *COL17A* mRNA transcripts is determined and, moreover, the implication of this subcellular localization of type XVII collagen protein is examined. In **chapter 5**, the mRNA levels of both *COL17A1* transcripts are determined in keratinocytes of a panel of HEB patients. Also the influence of nonsense mediated mRNA decay on these transcript levels is investigated by blocking of the NMD machinery by cycloheximide. In **chapter 6**, the findings of these studies are summarised and we will discuss what the implications of our findings are for further study of type XVII collagen.

CHAPTER 2

TWO MAJOR 5'UNTRANSLATED REGIONS FOR *COL17A1* mRNA

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In press

ABSTRACT

Type XVII collagen is an important structural component of keratinocyte hemidesmosomes and its functional loss in genetic or autoimmune disease results in blistering of the skin. In neoplastic tissue aberrant expression is seen dependent on the stage of the tumor. While the sequence of the type XVII collagen encoding gene –*COL17A1*- is now completely elucidated, the sequence of the 5' untranslated region (UTR) of the mRNA is still unknown. Since UTRs can modulate translation efficiency, the determination of the UTR sequence is indispensable for understanding the regulation of translation of *COL17A1* mRNA. To resolve the sequence of the 5'UTR of *COL17A1* mRNA and to analyse the promoter region for transcription motifs 5'Rapid Amplification of cDNA ends (RACE) followed by sequence analysis and ribonuclease protection assays (RPA) were performed. RACE and sequence analysis revealed the presence of six different 5'UTRs for the *COL17A1* mRNA. The start points of these six transcripts differ but no alternative exons are used. The longest 5'UTR starts 220 nucleotides before the open reading frame, whereas the shortest UTR is only 89 nucleotides in length. RPA confirmed the RACE results and furthermore demonstrated that the 5'UTRs with lengths of 102 and 220 nucleotides are the two major transcripts. Transcription motif analysis of the 5' region of the *COL17A* gene demonstrated several binding sites for transcription factors including the Sp1 and Activating Protein-1 (AP-1) families. We conclude that *COL17A1* mRNA is alternatively transcribed, which may result in complex regulation of type XVII collagen.

INTRODUCTION

Type XVII collagen is a structural component of the hemidesmosome. Hemidesmosomes participate in connecting epithelial basal cells to the underlying basement membrane[129]. They consists of at least six different proteins: the already mentioned type XVII collagen, the plakin protein BP230, the α_6 and β_4 subunits of integrin $\alpha_6\beta_4$, tetraspanin/CD151 and plectin[4,38-44]. Recent studies demonstrated that hemidesmosomal components are also involved in signal transduction and thereby are able to influence cell growth, motility and differentiation[67,130].

Type XVII collagen is a target molecule in blistering diseases. Autoimmunity against type XVII collagen results in various forms of pemphigoid that may effect skin, mucous membranes of mouth and genitals, and the eyes[131]. Due to its SDS-PAGE estimated molecular weight of 180 kDa the protein is known here as BP180. Genetic deficiency of type XVII collagen causes Hemidesmosomal epidermolyis Bullosa[33,88,132].

Type XVII collagen is a type II transmembrane protein, that is, with the collagenous carboxyl-terminal ectodomain outside the cell and the amino-terminal domain at the inside. The ectodomain contains 15 collagenous subdomains (COL1-COL15) by which the molecular tail folds into a triple-helical structure[4]. Type XVII collagen is expressed in skin and in other epithelial tissues as mammary, salivary and thyroid glands, colon, prostate, testis, placenta, thymus, and the retina of the eye[54,77]. Aberrant expression of type XVII collagen at different stages of carcinogenesis is observed, starting with downregulation in mild dysplasias and changing to overexpression as the tumor further evolves[70.]

The type XVII collagen gene, *COL17A1*, maps to the long arm of chromosome 10 at position 10q24.3. It spans 52 kb of genomic DNA and contains 56 exons that finally lead to a coding sequence of 4491 nucleotides[2-5].

In a previous study we demonstrated that by differential splicing of exon 56 two mRNA transcripts are produced that differ 610 nucleotides in length of the 3'UnTranslated Region (3'UTR)[12]. Here we extend these studies by characterising also the 5'UTR of *COL17A1* mRNA.

5'UTRs are important in regulating translation of their mRNAs. Features of UTRs that influence the translation efficiency include the length of the UTR, the presence of secondary structures, the presence of internal ribosome entry sites (IRES), and the existence of small open reading frames (ORFs) and ATGs upstream of the start codon[133.] In this study we performed 5' Rapid Amplification of cDNA Ends (RACE) in combination with Ribonuclease Protection Assay (RPA) and demonstrate six different starting sequences.

MATERIAL AND METHODS

Cell cultures

The squamous cell carcinoma line UMSCC-22B, derived from a tumor of the hypopharynx, was cultured in DMEM (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (Cambrex, Maryland, USA), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37 °C with 5% CO_2[134]. Keratinocytes were cultured from patches of healthy skin of breast reductions. The epidermis was separated from the dermis after overnight incubation with 2.4 U/ml dispase at 4°C followed by incubation of the epidermis with 0.025% trypsin, 0.01% EDTA at 37°C for 10 minutes. The harvested keratinocytes were cultured under serum-free conditions using Keratinocyte-Serum Free Medium (SFM; Gibco)[135]. This medium was supplemented with 25 µg/ml Bovine Pituitary Extract (BPE) and 0.1 ng/ml recombinant Epidermal Growth Factor (rEGF) (all from Gibco).

Total RNA and mRNA isolation

Cells were grown to subconfluence and then harvested by extraction with 1 ml/15 cm^2 Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated according to the manufacturer's protocol. In brief, the cells were lysed with Trizol, chloroform was added and after centrifugation total RNA was precipitated from the aqueous phase with isopropyl alcohol. PolyA$^+$ RNA was purified from the total RNA with the mRNA Isolation Kit from Roche (Mannheim, Germany), that is based on poly(T)-conjugated magnetic beads. To remove all traces of ribosomal RNA the protocol was performed twice. This double purified mRNA was used in all RACE and RPA experiments.

5'RACE

The GeneRacer™ Kit from Invitrogen was used according to the manufacturer's instructions. For each RACE experiment 250 ng mRNA from SCC cells was used. cDNA was prepared with Superscript Reverse Transcriptase (Invitrogen) in the presence of the gene-specific primer R0511. After cDNA-synthesis, PCR was performed with the GeneRacer™ 5'primer and gene-specific primer R0437. The resulting amplimers were cleaned with the QIAquick PCR purification kit from Qiagen (Hilden, Germany). Next, nested-PCR was performed with the GeneRacer™ 5'Nested primer and gene-specific primer R0323. After agarose-gel electrophoresis bands were cut out of the gel and the amplimers were isolated with the Qiagen Gelextraction Kit (Qiagen). Subsequently, amplimers were sub-cloned into the pCR4-TOPO vector and sequenced. All primers used are depicted in Table 1.

Table 1. Primers used in the 5'RACE and long template PCR experiments.

Primer	*Primer location**	*Sequence 5' → 3'*
R0511BP2	511-487	GACTCCGTCCTCTGGTTGAAGAAG
R0437BP2	437-413	CTGTAACTAGAGGTGGAGGCATGG
R0323BP2	323-300	CCTCGTGTGCTTCCAGTTGAGTT
F-115BP2	-115- -96	ATGTTTGGAAGCAAAGAGTTGAAAG
F0028BP2	28-47	CAGGAGAAGAAAGAGAGAGG
R5101BP2	5101-5080	GCCAACTGACTAGAGAATGC
R5388BP2	5388-5369	GTCTTGAGCTGATGCTGTGT

*Numbering according to Giudice[4].

Labeling of RNA probes

[α-^{32}P]-labeled RNA probes were synthesised using Uridine 5'-[α -^{32}P]triphosphate according to the following procedure. DNA fragments were generated by PCR on either genomic SCC DNA or SCC cDNA. The cDNA was produced from SCC mRNA using Superscript reverse transcriptase according to the manufacturer's protocol (Invitrogen). PCR products were cloned into the pCR4-vector (TOPO™ TA cloning kit, Invitrogen). Plasmids were linearized with either NotI or BcuI, depending on the type of RNA polymerase used in the subsequent transcription reactions. Antisense RNA probes were generated by *in vitro* run-off transcription on linearized plasmid. T7 and T3 RNA polymerase (MBI Fermantas, Vilnius, Lithuania) were used for transcribing linearized plasmids. Forty µCi [α -^{32}P]UTP (800 Ci/mmol; Amersham Biosciences, Braunschweig, Germany) was added to the transcription mix and the reaction was incubated at 37°C for 2 hours, after which template was digested with 1 U DNase I for 15 minutes at 37°C. The reaction was stopped by the addition of 1 µl 0.5 M EDTA. Probes were purified by running on a 5% TBE-Urea polyacrylamide gel at 150 V for 1 hour. After excision of the probes from the gel, the probes were eluted out of the gel by incubation in probe elution buffer (0.5 M NH_4-acetate, 1mM EDTA, 0.2% SDS) at 37°C overnight. Purified probes were quantified by liquid scintillation counting.

Ribonuclease protection assay

Total RNA (10 μg) or mRNA (600 ng) was hybridised overnight at 42°C with $4*10^4$ cpm [α-^{32}P]-labeled probe in hybridisation buffer (40 mM PIPES, 400 mM NaCl, 1mM EDTA, 80% formamide pH 6.4). Next, single-stranded RNA was digested for 1 hour at 37°C with 2000 units RNase T1 (Roche) in RNase digestion buffer (10 mM Tris-HCl, 5 mM EDTA, 300 mM NaOAc pH 7.5), followed by proteinase K (Fermentas) digestion of the RNases. After ethanol-precipitation, samples were loaded onto an 8% acrylamide/8 M Urea gel. After running, the gel was exposed to X-ray film with intensifying screen at -80°C.

Long template PCR

cDNA of keratinocytes was amplified with the Expand Long Template PCR system of Roche. PCRs were performed with four different primer combinations (Table 1). Forward primer -115BP2 was developed against the most 5'end of the longest 5'UTR, whereas forward primer 0028BP2 was made to amplify all 5'UTR variants of *COL17A1* mRNA. One reverse primer (R5388BP2) was created against a sequence behind the insert in the 3'UTR, while another reverse primer (R5101BP2) was directed against a sequence in the insert. PCR was performed in buffer 1 of the PCR system at varying annealing temperatures (58°C for both forward primers with R5101 and 62°C with R5388) with a $MgCl_2$ concentration of 2.25 mM. For the first ten cycles elongation was executed at 68°C for 4 minutes. After 10 cycles each successive cycle was prolonged with an additional twenty seconds and amplification was done for a total of 35 cycles. Final elongation was performed for ten minutes. Amplimers were analysed on a 0.8% agarose gel (RESponse™ Research PCR agarose; Bioplastics, Landgraaf, the Netherlands)

In silico promoter analysis

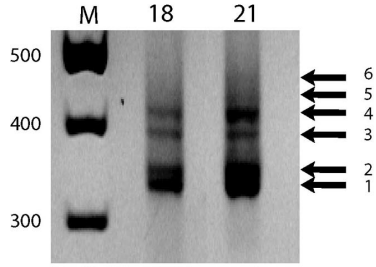
The promoter region of the *COL17A1* gene was analysed with the Alibaba 2.1 software[136]. This software is used for predicting transcription factors and uses the Transfac 4.0 database[137].

RESULTS

Six alternative 5'UTR *COL17A1* mRNA ends

Nested PCR was performed on cDNA obtained in the RACE procedure and this resulted in the amplification of six amplimers of different size. Four of these, the smaller ones with lengths between 330 and 450 bps, are clearly visible after 18 cycles of PCR. The two longer ones become visible after 21 cycles (Fig. 1). When the tobacco acid pyrophosphatase, that removes the 5' cap-structure from full-length mRNA, was omitted from the RACE experiments then no bands were observed indicating that all bands indeed originate from mature mRNA (not shown). Sequencing of the obtained PCR-products revealed that all six bands indeed

represented *COL17A1* mRNA transcripts but that these differed in the length of their respective 5'UTRs. All sequences aligned with the DNA sequence (clone RP11-16H23, Genbank accession number: AL138761) and no gaps due to alternative splicing were observed. The respective transcription start sites, the lengths of the different 5'UTRs and their GC content are shown in Fig. 1. The relative intensity of the RACE products decreased with increasing length. This is a well-recognised problem and thought to be due to secondary structures, often present in 5'UTR that hamper the reverse transcriptase causing premature termination of cDNA near the 5' end.



band#	size (bps)	5'UTR startpoint	5'UTR length	5'UTR start sequence	%GC
1	330	17	89	gaagaaaaca	40
2	350	4	102	ctgcaaatttt	40
3	390	-28	133	tggctgggtc	39
4	420	-64	169	aacatttttg	39
5	450	-95	200	gaaagaaaca	39
6	470	-115	220	atgtttggaa	39

Figure 1. 5'RACE demonstrates six different 5'ends for *COL17A1* mRNA of keratinocytes.
Nested PCR was performed for 18 and 21 cycles and amplimers were analysed on 2% agarose gel. M is a 100 bps DNA ladder. Arrows indicate the different bands and the numbers correspond with the 5'UTRs in the table on the right. This table shows the size of the PCR-amplimers, the lengths of sequenced 5'UTRs, the starting sequences of the 5'UTRs, and the percentage GC of each 5'UTR. Starting sequences were verified in two independent experiments.

Ribonuclease protection assay confirms the existence of six different transcripts

To independently verify the existence of six different *COL17A1* 5' ends we performed ribonuclease protection assays (RPA). The [α-32P]-probe spanned nucleotides from position -233 to 149. After digesting single-stranded RNA the remaining double-stranded RNA was visualised by gel electrophoresis. Again six fragments were detected and the lengths coincided with the lengths as expected from the 5'UTRs identified in the RACE experiments (Fig. 2). The same results were found in both keratinocytes and the UMSCC-22B carcinoma cells, both with total RNA and with double purified mRNA. The RPA experiments demonstrated that two of the six UTRs are expressed at higher levels. These start at positions +4 and –115 (bands 2 and 6, Fig. 2). We calculated the expression levels of all transcripts by intensity scanning of the autoradiograms, and after correction for the number of

labeled uridine residues in each transcript it appeared that the two major transcripts accounted for at least 75% of the total signal.
Comparing the expression levels of these two major transcripts between normal keratinocytes and UMSCC-22B cells showed a preference of the SCC cells for the larger variant. In SCC cells the ratio of the larger variant versus the shorter variant was approximately 1.2 while in keratinocytes it was 0.9.

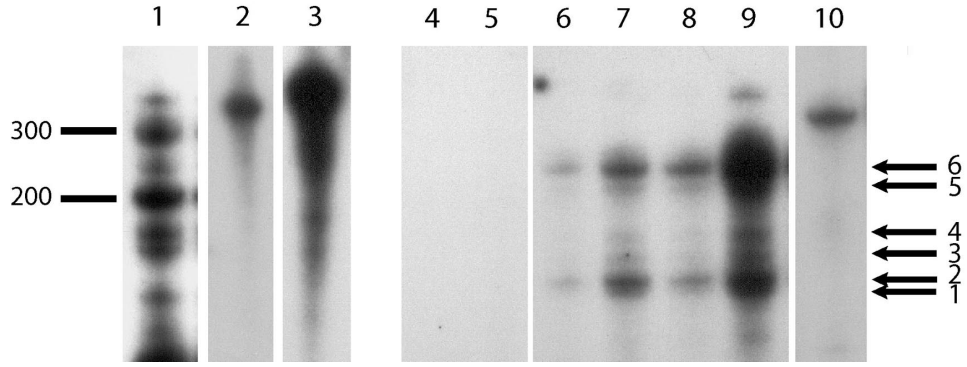


Figure 2. RPA experiments confirm the existence of the six different ends of the 5'UTR.
Lane 1: RNA marker, lane 2: Undigested Probe 28 to 348, lane 3: Undigested Probe –233 to 149. RPA experiments are shown in lanes 4 to 10 with probe –233 to 149 in lanes 4-9 and with probe 28 to 348 in lane 10. Lane 4: control yeast total RNA (10 μg), lane 5: no substrate RNA, lane 6: SCC total RNA (10 μg), lane 7: NHK total RNA (10 μg), lane 8: SCC mRNA (600 ng), lane 9: NHK mRNA (600 ng), lane 10 SCC total RNA (10 μg). Numbers of arrows correspond with Fig. 1

COL17A1 mRNA 5'ends show no prevalence for a particular 3'UTR variant

As the *COL17A1* mRNA is also alternatively spliced at the 3' end we performed long template PCR to study if the two individual 3'UTR variants belonged to a particular unique major 5'UTR end. If the reverse primer was chosen behind the unique 3'UTR sequence and the forward primers in the two major 5'UTR regions this resulted for both forward primers in two bands (Fig. 3, lanes 1 and 2). In the event that the reverse primer was chosen against the unique insert sequence of the long 3'UTR then for both forward primers the expected amplimer of the appropriate length was observed (Fig. 3, lanes 3 and 4). This undoubtedly demonstrates that the long 3'UTR variant has alternative 5' ends. Although strictly speaking it does not definitively prove that the shorter 5'UTR is also shared by the two 3'UTRs the intensities of the amplimers in these experiments do not suggest otherwise.

Motif analysis of the 5' region of the *COL17A1* gene.

Analysis of the sequence as far as 500 nucleotides upstream of the translation start revealed several transcription motifs (Fig. 4). Five Sp1 motifs are present (positions:

+48 to +55, –25 to –18, –163 to –150, –205 to –196, and –240 to –231) and furthermore two AP-1 related transcription motifs at positions –138 to –129 and –419 to –409. Interestingly, also a C/EBP del site, a motif controlling keratinocyte-specific gene expression[138], 414 nucleotides (position: –317 to –308) before the translation start site is found. The MRF4 site (position: –183 to –173) at 279 bases in front of the start codon is reported to bind muscle specific regulatory factors, such as Myf-3, 4 or 5 and MyoD[139]. Furthermore, two TATA-binding protein motifs (positions –19 to –8 and –273 to –264) are present.

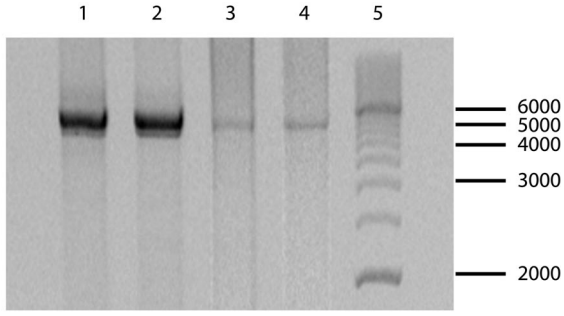


Figure 3. Long template PCR shows a similar distribution of the two major *COL17A1* 5'ends over the two 3'UTR variants in mRNA of keratinocytes.
Four different primer combinations were used. Lane 1: F0028BP2 to R5388BP2, lane 2: F-115BP2 to R5388BP2, lane 3: F0028BP2 to R5101BP2, lane 4: F-115BP2 to R5101BP2, lane 5: O'RangeRuler™ 100bp+500bp DNA Ladder (Fermentas)

DISCUSSION

In this study we demonstrate by RACE and by RNase protection experiments the existence of six alternative 5'UTR ends for the mRNA coding for type XVII collagen. Two of these, with lengths of 102 and 220 nucleotides are the major transcripts and together account for at least 75% of the cellular *COL17A1* mRNA level. Notably, the two TBP-sites found are in front of these two transcripts. The other four, with lengths of 89, 103, 169, and 200 nucleotides, are expressed at substantially lower levels.
The existence of two major transcripts at both the 5' and the 3' end raises the question if the differential transcription at both ends is somehow connected. Previously we showed that normal keratinocytes preferentially express the longer 3'UTR variant while in UMSCC-22B cells this was reversed[12]. Here again we see a similar phenomenon for the +4 and the –115 5'UTR. Normal keratinocytes express relatively more of the shorter +4 5'UTR while in UMSCC-22B cells more of the -115 5'UTR is found. However, the ratios for the shorter versus the longer variant at the 5' end and those at the 3' end did not match. In line, long template PCR demonstrated that a particular 3'UTR variant is not linked with a distinct 5'UTR.

Only in one other species the 5'UTR of the *COL17A1* mRNA has currently been identified; in mouse the 5'UTR has a length of 293 nucleotides[7]. The possibility of a second 5'UTR there can however not be excluded, since in 5' primer extension analysis an additional shorter transcript was observed that was then hypothesised to be due to either premature termination or to be a transcription start site. Our data indicate that alternative transcription would be a serious option, also in view of the high human-mouse 5'UTR sequence homology. Alignment of the genomic sequences covering the *COL17A1* 5'UTR region gives an 83 percent identity, substantially higher than the mean 67% identity for mouse and human 5'UTRs. This high homology is comparable with the homology found for human and mouse coding sequences[13].

Alternative 5'UTR splicing has also been reported for other hemidesmosome associated adhesion molecules. Human keratinocytes express four plectin mRNA isoforms all different in the 5' end[58], and in mouse an even more complex structure with eleven alternative mRNA transcripts is found[140]. The mRNA for the β3 chain of laminin 5 consists of two isoforms - B3A and B3B- also with different 5'UTRs. Only the B3A coded chain is expressed, whereas the B3B transcript is thought to fulfil a regulatory function[141].

Several aspects of the 5'UTR sequence can influence translation of their coding ORF, including the length, the guanine plus cytosine (GC) content, and upstream ORFs or ATGs. Negative length effects of 5'UTR have been reported but are usually seen for UTRs over 250 nucleotides in size[142]. As the lengths of the type XVII collagen 5'UTRs are between 89 and 220 nucleotides, it is unlikely that this effects translation efficiency.

Sequences with a high GC content may form stable secondary structures that hamper translation[133]. The average GC content of mammalian 5'UTRs is 56 to 63%, which is considerably higher than those of the *COL17A1* transcripts[142]. All six 5'UTRs have low GC contents of around 40%, making it unlikely that they form inhibitory secondary structures of significant stability. The low GC percentage was unexpected given the lengths of the *COL17A1* 5'UTRs, since Pesole *et al* reported GC contents over 80% in 5'UTRs with an average length of 200 nucleotides[143]. Whether this low GC content has any functional meaning remains to be solved.

Upstream AUGs in 5'UTRs can also influence translation efficiency[144,145]. Only one upstream start codon is found. It is the first triplet of the transcript with the longest 5'UTR at position –115. This start codon results in an uORF of 129 nucleotides, which potentially could inhibit the translation machinery. However, the Kozak context of this AUG is very weak and therefore it is unlikely that any translation starts here[146.]

The presence of an IRES in the 5'UTR can initiate cap-independent translation that can be important under conditions of cellular stress. IRES consist of complex secondary structures close to the start codon[147,148]. The low GC content of the *COL17A1* 5'UTRs likely precludes the folding of any such structures. Therefore it seems unlikely that type XVII collagen translation is under IRES-type regulation.

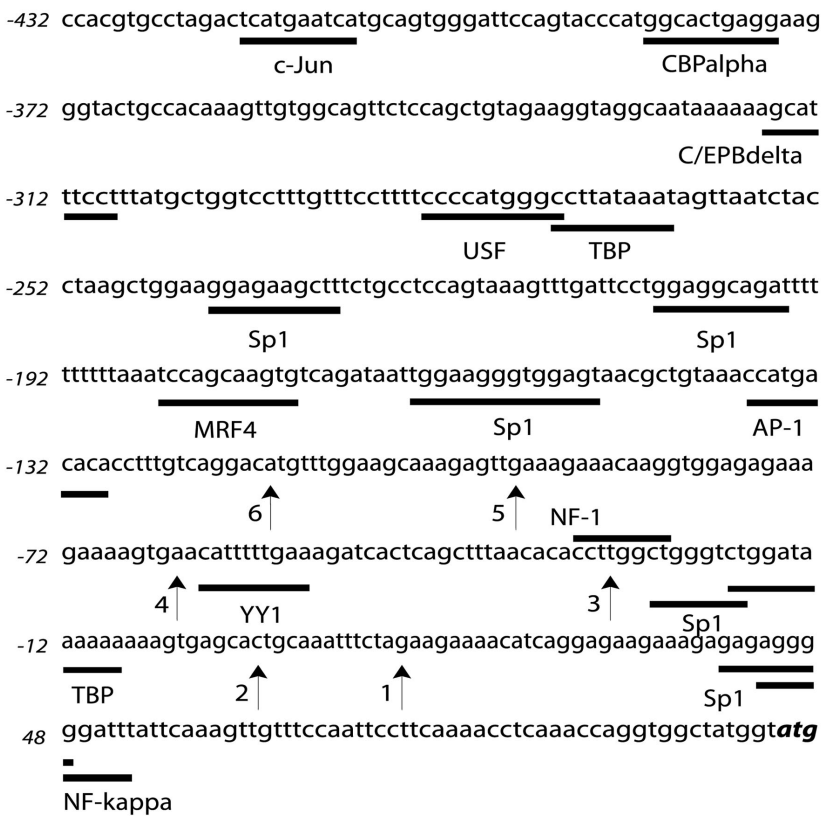


Figure 4. Motif analysis of the 5'region of the *COL17A1* gene demonstrates various motifs that are transcription factor binding sites.
Motifs are indicated by underlining of the sequences. The start codon according to Giudice is exhibited in bold and the start points of the six different 5'UTRs of type *COL17A1* mRNA are indicated with arrows (numbers correspond to the numbers in Fig. 1). Nucleotide numbering is according to Giudice[4] . The nucleotides at positions 1, 2 and 3 (TGA) are different than those published by Giudice (CCG). This TGA triplet is also found in clone RP11-16H23 (Genbank accession number: AL138761) and was invariably found in all of our sequenced PCR products. TBP: TATA-binding protein, CBP: CREB- binding protein, YY1: Yin and Yang 1 aka NMP-1; nuclear matrix protein 1, MRF4: muscle regulatory factor, C/EPB: CCAAT/enhancer-binding protein, USF: upstream stimulating factor.

AP-1 binding proteins are important for transcription of genes that are involved in skin homeostasis[149]. These proteins, the Fos, Jun and ATF family, are active in many biological processes of which the majority are present in skin. The two AP-1 sites found may well be important for transcription regulation of the *COL17A1* gene. The potential importance of these binding sites is already demonstrated for the other

transmembrane hemidesmosomal component, β_4 integrin. The promoter of the β_4 integrin binds the AP-1 transcription factors Fra-2 and Jun D[150]. Mutation of the AP-1 binding site results in diminished promoter activity. Furthermore, AP-1 induced change of gene expression is also speculated to be especially important when rapid action in keratinocytes is required as in the complex and dynamic process of wound healing[151]. This is emphasised by the increased expression of AP-1 proteins seen in wound-healing models and upregulation of c-Fos in renal epithelium after damage[152-154]. Type XVII collagen shows also altered expression during wound healing[124]. Likewise the up- and downregulation of type XVII collagen observed during development of squamous cell carcinoma may be subject to the same regulation.

The MRF4 site at position –173 to –183 is a motif that binds transcription factors involved in regulation of gene expression in muscles. Although type XVII collagen is recognised as a typical epithelial protein Kondo *et al* found evidence of *COL17A1* mRNA in mouse heart. The expression level of this mRNA differed through the developmental stages of the heart[75]. The MRF4 transcription factor may be involved in control here.

In summary, the *COL17A1* 5'UTR consists of six alternative transcripts that are expressed at different levels. These UTRs and the promoter region contain several potential regulation sites. Considering the aberrant expression of type XVII collagen in neoplastic tissue, its specific expression in wound healing and its involvement in signal transduction[35], the function of these transcripts and their regulation has to be subject of further research

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CHAPTER 3

TYPE XVII COLLAGEN PRODUCTION IS EFFECTED BY THE *COL17A1* 3'UNTRANSLATED REGION DUE TO CHANGES IN mRNA STABILITY

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To be submitted

ABSTRACT

Type XVII collagen is a transmembrane component of the hemidesmosome encoded by the *COL17A1* gene. Transcription generates two alternative mRNAs, both with the full coding sequence but with alternative 3'Untranslated Regions (UTRs). Compelling evidence suggests that 3'UTR sequences might have various regulatory functions including translational control, mRNA stability, and mRNA localization. We investigated the influence of the two alternative 3'UTRs on the protein expression levels and stability of the mRNA transcript.
Luciferase assays were performed with the alternative 3'UTRs of type XVII collagen cloned behind the luciferase coding sequence of the pGL3-plasmid. These constructs were transfected into different cell lines and luciferase expression was determined. Transfection with the full-length 3'UTR construct invariably led to a higher luciferase expression than transfection with the short variant construct. Furthermore, mRNA degradation after actinomycin D arrest was measured by real time RT-PCR experiments to monitor the stability of the two alternative mRNA transcript variants. These mRNA stability assays demonstrated decreased stability of the short 3'UTR variant in comparison with the long one.
These results suggest that the type XVII collagen 3'UTRs are involved in mRNA stability and that keratinocytes are able to direct the type XVII collagen protein level by differential expression of the alternative transcription variants.

INTRODUCTION

The *COL17A1* gene encodes the type XVII collagen protein that is an important transmembrane component of the hemidesmosome[3,7]. Hemidesmosomes are build from at least six different proteins, the transmembrane proteins type XVII collagen, α_6- and β_4-integrin, tetraspanin/CD151, and the intracellular plakin proteins BP230 and plectin[4,38-44]. Hemidesmosomes are indispensable structures for connecting the epidermal basal cells to the underlying basement membrane[129]. Genetic deficiency of the protein, as in Hemidesmosomal Epidermolysis Bullosa (HEB), leads to fragile skin and generalized blistering, alopecia, nail dystrophy, and enamel pits[33,88,132]. In HEB mechanical stress induces dermal-epidermal separation at the level of the lamina lucida.

Type XVII collagen has a type II orientation with the globular head at the cytoplasmic side of the membrane. The extracellular tail contains 15 interrupted collagenous domains by which the protein folds into a triple helix[4,18,24]. The protein is 1497 amino acids long and on SDS-PAGE runs as a 180 kDa protein. A second soluble 120 kDa form is found that consists of the shed extracellular domain[14,19,155].

Immunofluorescence of skin shows a distribution of type XVII collagen along the basilar surface of the basal cells and also along their lateral site[17]. Expression is not restricted to skin since other epithelial tissue as mammary, salivary and thyroid glands, colon, prostate, testis, placenta, thymus, and retina also express type XVII collagen[54,77]. In different stages of carcinogenesis aberrant expression of type XVII collagen is observed, starting with downregulation in mild dysplasias and changing to overexpression as the tumor progresses[15,70,156].

Transcription of the *COL17A1* gene generates two alternative mRNAs, both with the full coding sequence but with different 3'Untranslated Regions (3'UTRs). Differential splicing of exon 56 results in the transcription of two *COL17A1* mRNA transcripts, which differ by an insertion of 610 nucleotides in the 3'UTR. In normal human keratinocytes (NHK) the long transcript is expressed at a higher level than the short variant, whereas in the squamous carcinoma cell line UMSCC-22B the short transcript predominates[12]. Why keratinocytes have two mRNA variants, both containing the coding sequence, is unclear, but part of the answer must lie in the function of the 3'UTRs.

Compelling evidence shows that 3'UTR sequences harbor various regulatory functions including translational control, mRNA stability, and mRNA location. These forms of gene regulation may influence biological processes such as cell development[157,158]. Translational control is achieved by regulatory elements in the 3'UTR that govern the temporal expression of the mRNA in question and several control mechanisms have been identified. *Trans*-acting factors, mostly RNA binding proteins, bind to specific sequences - *cis*-acting elements - within the 3'UTRs and effect the stability of the transcript. A well-characterized *cis*-acting element is the adenylate/uridylate (AU)-rich element (ARE). Binding of *trans*-acting factors to

these AREs accelerate mRNA decay by exposing the mRNA transcripts to the exosome[159,160]. In contrast, binding of the Hur protein of the Elav/Hu family of RNA binding proteins, has an opposite effect and stabilizes the transcript[161]. Other non-ARE *cis*-acting elements found are mainly C and/or U-rich[162,163]. Binding of short non-coding RNAs to complementary sequences in 3'UTRs also influences translation[164].
In a first characterization of the alternative *COL17A1* transcripts we investigated whether the two 3'UTR variants are subject to different decay and whether the translation level of the coding sequence depends on the 3'UTR type.

METHODS

Plasmid construction

The 3'UTR of type XVII collagen was amplified by RT-PCR using gene specific primers that had an additional XbaI recognition sequence synthesized at the 5' end (Eurogentec, Seraing, Belgium). For RT-PCR, cDNA of normal human keratinocytes (NHKs) and UM-SCC22B cells were used. PCR products were ligated into the pCR4 TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and subsequently digested with XbaI (Fermentas, Vilnius, Lithuania). After electrophoresis the digested fragments were isolated from agarose gel with the Gel Purification kit from Qiagen (Hilden, Germany). Isolated DNA fragments were ligated into the unique XbaI recognition site of the pGL3-control vector (Promega, Madison, WI) with T4 DNA ligase (Fermentas). Plasmids were checked for the correct sequence by DNA sequence analysis. For schematic depiction of the constructs see Fig. 1.

Cell cultures

NHKs were cultured from patches of healthy skin from mamma reductions. The epidermis was separated from the dermis after overnight incubation with 2.4 U/ml dispase at 4°C followed by incubation of the epidermis with 0.025% trypsin, 0.01% EDTA for 10 min. The harvested keratinocytes were cultured under serum-free conditions using Keratinocyte-Serum Free Medium (SFM) (Gibco, Paisley, UK)[135]. The type XVII collagen deficient keratinocyte cell line, hereafter referred to as t-mar, was generated by immortalizing keratinocytes of a HEB patient, homozygous for the 2342delG mutation[165]. The transformation was accomplished at the Fred Hutchinson Cancer Research Center (Seattle, WA) using the LXSN16 E6,E7 retroviral vector[166]. This retroviral vector contains E6 and E7 coding sequences from human papilloma virus and a neo resistance gene. We used no drug selection as the uninfected cells would differentiate and only infected cells proliferate. After immortalization and selection the cells were tested for retrovirus production to ensure that the cells did not produce any virus. Immunoblotting and immunofluorescence confirmed the absence of measurable type XVII collagen production. The t-mar cells were cultured under the same conditions as NHKs.

CHO-K1 cells were cultured in Ham's F12 medium (Gibco) supplemented with 10% fetal calf serum (Cambrex, Verviers, Belgium), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco). All cells were incubated at 37ºC and 5% CO_2.

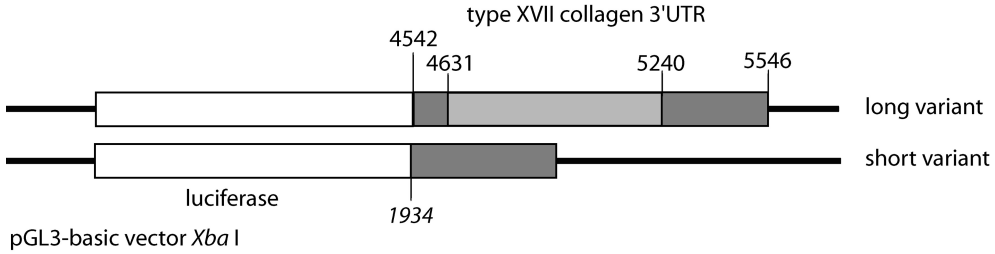

Figure 1. Schematic depiction of the luciferase-*COL17A1* 3'UTRs constructs in the pGL3-control vector.
Numbers correspond to the *COL17A1* mRNA sequence (roman) and the pGL3-sequence (italic).

Cell transfection

All three cell types described above were transfected with poly-L-ornithine (Sigma, St. Louis, MO, USA) followed by a DMSO-shock. In detail, cells were plated in a 12-wells plate in a density of $1*10^5$ cells/well and incubated overnight in their respective culture media. Transfection mixtures of 1.1 µg of reporter gene DNA plasmid and 0.25 µg transfection efficiency control pRL-TK plasmid (Promega) or 2.0 µg of a 1:1 mixture of the two different luciferase-*COL17A1* constructs in 100 µl HS-buffer (150 mM NaCl, 20 mM HEPES) containing 0.33 µl poly-L-ornithine were pre-incubated at RT for minimal 10 and maximal 15 minutes. The medium on the cells was changed and the transfection mixture was added. After four hours incubation the medium was removed and 250 µl room temperature culture medium containing 25% DMSO was pipetted onto the cells. After four minutes the cells were washed with 0.5 ml HBSS (Gibco), 1.0 ml culture medium was added and the cells were incubated a further 24 or 48 hours at 37ºC.

Luciferase activity assay

The luciferase levels obtained after transfection were measured in the Dual-Luciferase Reporter system (Promega), according to the manufacturer's protocol. Briefly, cells were washed once with 0.5 ml HBSS and then lysed in 250 µl 1x Passive Lysis buffer on a shaker for a minimum of 30 minutes. The luciferase activity of the samples was then determined on an Anthos Lucy I luminometer by adding 20 µl cell lysate to 100 µl LARII. Light output was recorded for 10 seconds and subsequently 100 µl of Stop&Glo buffer was added to establish the Renilla luciferase activity due to the co-transfected pRL-TK plasmid. This Renilla luciferase activity was used to calculate transfection efficiency. Data are expressed as relative light units (RLU).

mRNA stability experiments

To analyze mRNA stability with the help of real time RT-PCR experiments, CHO cells were transfected with a 1:1 mixture of the long variant and the short variant luciferase-*COL17A1* vector constructs. To determine mRNA stability in Northern blotting experiments, CHO cells were transfected with either one of the luciferase-*COL17A1* vector constructs. After 24 hours incubation at 37ºC, actinomycin D (Sigma; 10 µg/ml) was added to inhibit transcription. Plates were incubated again and at certain time points individual plates were harvested by putting 0.5 ml Trizol/4 cm^2 (Invitrogen) on the cells. The RNA was then isolated according to the manufacturer's protocol.

Real time PCR

Two-hundred ng total RNA was used for synthesis of first-strand cDNA with SuperScript III RNase H minus reverse transcriptase (Invitrogen) in a 20 µl final volume containing 300 ng of random hexamers (Invitrogen) and 40 units of RNase OUT (Invitrogen).
Combinations of unlabeled PCR primers and Taqman® minor groove binder (MGB) probes (FAM™ dye-labeled) for use in real-time RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands). The targets included the short variant *COL17A1* transcript (Hs00996062), and the long variant *COL17A1* transcript. This latter set was specially developed by Assay-on-Design of Applied Biosystems against the unique long variant 3'UTR sequence.
For each gene the final concentration of primers and MGB probes in TaqMan PCR MasterMix (Applied Biosystems) was respectively 900 and 250 nM, and 1 µl of cDNA was added to the PCR-mix. TaqMan real-time RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Amplification was performed with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15s at 95°C and 60s at 60°C. Triplicate real-time RT-PCR analyses were executed for each sample. Threshold cycle values (C_t) were determined with the help of SDS2.2.2 software and were averaged for each sample. The two *COL17A1* mRNA variant specific primers sets were tested for non-specific annealing and amplifying by real-time RT-PCR analyses of pCR4-vectors (TOPO™ TA cloning kit, Invitrogen) in which either one of the two 3'UTR variants of type XVII collagen was cloned. The primer set specific for the full-length 3'UTR variant was not able to detect any plasmid containing the shorter 3'UTR variant and *visa versa*, indicating the specificity of the two primer sets.
Difference in expression between the two constructs was determined by the following formula: $\delta C_t = C_t$(long variant)- C_t(short variant).

RNA probes

Digoxigenin (DIG)-labeled antisense RNA probes were synthesized using the DIG-Labeling mix (Roche, Mannheim, Germany) according to the manufacturer's protocol with some minor modifications as described below. DNA fragments were generated by PCR on SCC cDNA that was produced by transcribing SCC mRNA with Superscript reverse transcriptase (Invitrogen). PCR products were cloned into vector pCR4 (Invitrogen). Plasmids were linearized with restriction enzymes NotI or BcuI (Fermentas), depending on the RNA polymerase, respectively T3 or T7 (Fermentas), that was used in the subsequent

transcription reaction. Probes were then generated by *in vitro* run-off transcription on linearized plasmid. For detection of Luciferase-*COL17A1* mRNA the following antisense probe was used: nucleotides 1266-1684 on basis of Genbank accession number U47296 and for detection of *Cricetinae gen*. sp. β-actin this antisense probe was used: nucleotides 741-1155 on basis of Genbank accession number AF014363.

Northern blot

Two µg total RNA (CHO cells) was separated on 1.2 % agarose- 6% formaldehyde gel and transferred to a positively charged Nylon membrane filter (Amersham, Braunschweig, Germany) in 10X SCC (1.5 M NaCl, 150mM NaCitrate). After transferring the RNA was covalently immobilized by 5 min 305 nm UV cross-linking. Pre-hybridization was carried out in a roller bottle for 1.5 h at 68°C in DIG Easy Hyb buffer (Roche). DIG-labeled probes (100 ng/ml in DIG Easy Hyb) were hybridized to the membrane overnight also at 68°C. The hybridized membrane was washed twice in 2×SSC, 0.1% SDS at room temperature followed by two washes in 0.5×SSC, 0.1% SDS at 68°C. Next, the membrane was blocked in blockbuffer (Roche) for 1.5 h and hybridized probes were detected with anti-digoxigenin Fab fragments conjugated with AP (Roche; 1:20,000 in blockbuffer). Chemiluminescence was developed with CDP-Star (Roche) 1:500 in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5).
Density of the bands was determined with the help of Biorad Quantity One Software. Luciferase-*COL17A1* expression was corrected for uneven mRNA loading by dividing the density of Luciferase-*COL17A1* mRNA by the density of the β-actin mRNA expression.

***In silico* analysis**

Sequences encoding the complete 3'UTR of type XVII collagen or ESTs of mouse (GenBank accession number: NM_007732), rat (XM_219976), bovine (CK849716), pig (AY277629), chicken (M60172) and human (NM_000494) were retrieved from Genbank. Sequences encoding the complete mRNA of *COL4A6* (NM_033641), *COL4A5* (NM_000495), *COL1A2* (NM_000089), *COL17A1* (NM_000494), and *COL16A1* (NM_001856) were also retrieved from the Genbank. The software program ClustalW and the internet tool MultAlin[167] were used to align the sequences.

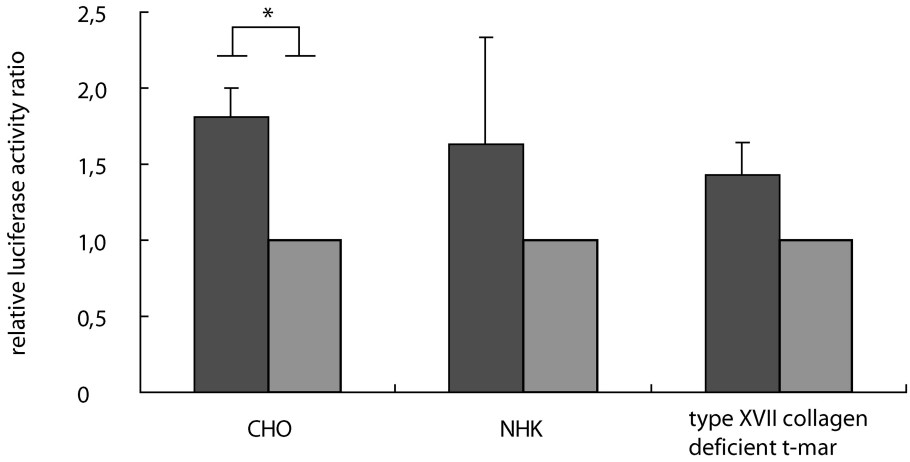

Figure 2. Luciferase expression after transfection of pGL3 vector- *COL17A1* 3'UTR constructs in different cell types.
Dark grey bars show the relative luciferase expression of the construct with the long 3'UTR compared with the expression of the short 3'UTR construct (light grey bars), that is set at one. Data are mean and standard deviation of three independent experiments all performed in triplicate. *P< 0.025 compared to mean expression of the short construct with the help of Student's t-test, paired, two-tailed.

RESULTS

The long variant *COL17A1* 3'UTR upregulates reporter protein expression.

The luciferase expression level after transfection of the pGL3 constructs depended on the variant of the *COL17A1* 3'UTR that was placed behind the luciferase coding region. In all cell types transfection with the longer 3'UTR construct invariably led to a higher luciferase expression than transfection with the short variant construct. In CHO cells this difference was significant and the luciferase levels were almost twice as high with the long variant construct (Fig. 2). Keratinocytes, both normal and immortalized type XVII collagen-deficient, had a low transfection efficiency that led to lower expression ratios, but here transfection of the long variant construct also resulted in higher luciferase protein levels. Due to the low transfection efficiency of keratinocytes the standard deviation became relatively high and the P-values did not achieve the 0.05 level but ended at 0.15 and 0.20. However, when we analyzed the level of the mRNA transcripts 24 hours after transfection we invariably observed that the short transcript was at an approximately 25% lower level than the long transcript in Northern blotting experiments, well in line with the shorter transcript being more liable to enhanced decay (not shown).

Table 1. Difference between quantitative PCR C_t-values of long luciferase-*COL17A1* construct and short luciferase-*COL17A1* construct at indicated time points.

Time point (minutes)	*$\delta C_t = C_t$(long variant)- C_t(short variant)*	*P-value*
0	0.05 ± 0,10	-
30	-0.30 ± 0,10	0.005
60	-0.17 ± 0,91	0.435
90	-0.39 ± 0,03	0.038
180	-0.27 ± 0,13	0.016

Data are the mean and standard deviation of two independent experiments in triplicate. P-value compared to t=0 determined with Student's t-test, paired one-tailed.

Rapid decay of reporter mRNA containing the short variant 3'UTR.

CHO cells were transfected with a 1:1 mixture of the luciferase-vectors with the long and with the short variant 3'UTR construct. Twenty-four hours after transfection mRNA transcription was blocked by adding actinomycin D. The fate of the transcripts was then followed by real-time RT-PCR experiments. Table 1 shows $\delta Ct = C_t$(long variant)- C_t(short variant) for 3 hours after blocking of transcription and demonstrates that this δC_t increased shortly after the addition of actinomycin D. This designates that the luciferase-*COL17A1* construct mRNA transcript containing the short variant 3'UTR declines more rapidly than the transcript with the long variant 3'UTR.

In a separate experiment the effect of the mRNA transcription blocking was also studied by Northern blotting. These experiments confirmed that mRNA of the short variant construct decayed more rapidly as the mRNA of the long variant construct was more stable over time. The control β-actin mRNA appeared stable over the 3 hours time course (Fig. 3).

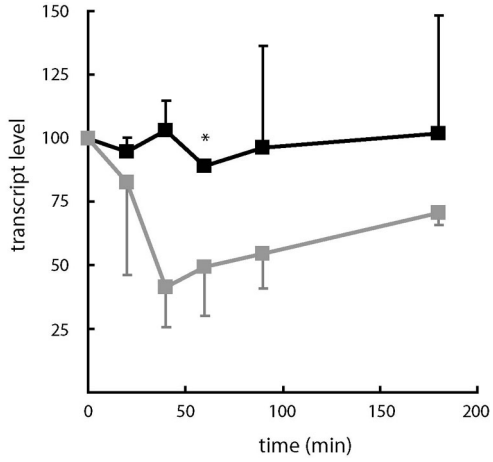

Figure 3. Luciferase-*COL17A1* construct mRNA decay in CHO cells after treatment with actinomycin D.
Total RNA was isolated at indicated time points after addition of actinomycin D at t=0 and mRNA expression is determined with Northern blotting. The ratios between luciferase mRNA and β-actin mRNA are depicted with the t=0 time point set at 100 for each construct. Black rectangles: long variant 3'UTR construct, grey rectangles: short variant 3'UTR construct. Each time point shows the mean and standard deviation of the density of the normalized luciferase-*COL17A1* construct mRNA expression of three independent experiments. *P<0.05 compared to mean expression of the short construct with Student's t-test, paired, one-tailed

COL17A1 3'UTR contains highly conserved sequence stretches.

Alignment of human and mouse *COL17A1* 3'UTR unveiled four stretches having over 80% homology[12]. Alignment of these four stretches against the other identified *COL17A1* 3'UTRs now demonstrated three regions of high conservation that are all present in the unique 610 nucleotides sequence of the long variant transcript (Table 2, Figs. 4 and 5A). At position 5051-5090 a C-rich sequence is present that shows large overlap with the Poly(C)-Binding Protein (PCBP) binding consensus sequence[168] (Figs. 5B and 5C).

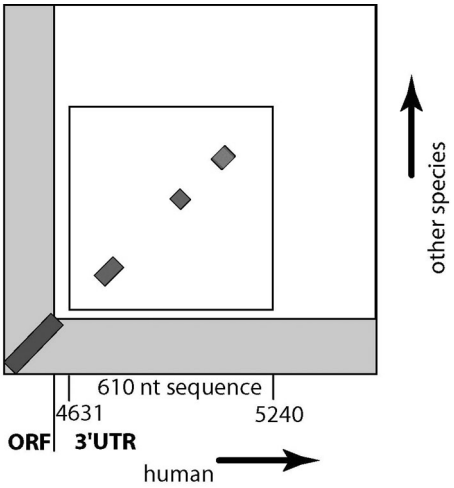

Figure 4. Graphical alignment of human versus other species *COL17A1* 3'UTR.
The 3'UTR area is in white. The rectangles inside the 610 nt area depict the three high homology stretches, and the light grey rectangle herin indicates the putative PCBP binding site sequence. Numbering is according to Guidice[4].

DISCUSSION

In this study we investigated the influence of the alternative spliced *COL17A1* 3'UTR forms of on the translation levels and stability of the transcripts. Reporter gene analysis demonstrated that the mRNA transcript with the long 3'UTR leads to higher protein synthesis compared to the alternative short 3'UTR transcript. This effect was seen independent of the cell-type that was transfected, and thus denotes a general mechanism rather than a keratinocyte-specific mechanism. Type XVII collagen-deficient keratinocytes displayed the same difference in expression as normal keratinocytes indicating that the presence of the type XVII collagen itself apparently does not effect the difference in translation efficiency. In mRNA stability assays we witnessed that the short variant was less stable. This shorter half-life may explain the lower reporter protein synthesis and translation regulation by modulation of mRNA stability has already been shown for several other genes[169-175].
Alignment of human and murine 3'UTR sequences by Molnar *et al* had revealed the presence of four high homology stretches[12]. When we compared these stretches with the now characterized *COL17A1* 3'UTRs of other species we found three short stretches with very high identity. Without selective pressure 30% conservation is expected[176]. As the conservation of these three stretches equaled that of coding sequences they apparently are essential to proper functioning of the transcript. Most probably these stretches function in post-transcriptional regulation.

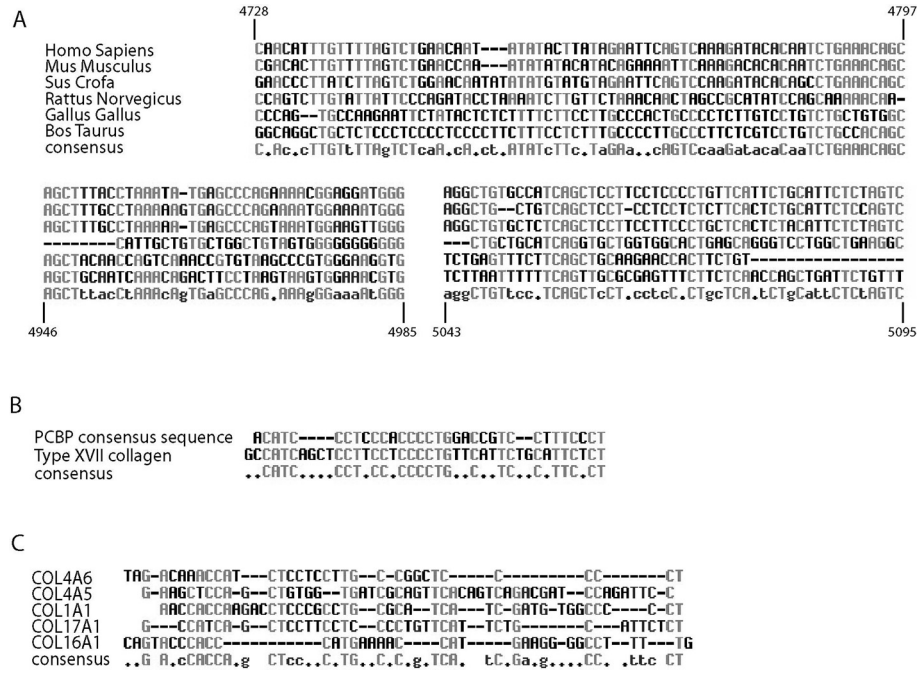

Figure 5. Alignment of high homology stretches of the *COL17A1* long variant 3'UTR
(A) Alignments of three homologue stretches found in the 3'UTR. Light grey capitals correspond with 70% homology or more and black lower case characters with 50% or more.
(B) Overlap of the PCBP binding consensus sequence with the C-rich sequence in the *COL17A1* 3'UTR. Light grey capitals show perfect overlap.
(C) Alignment of the hypothetical PCBP site of *COL17A1* with other collagen 3'UTR sequences. Light grey capitals correspond with 80% homology or more and black lower case characters with 60% or more.

Interestingly, a potential Poly(C)-Binding Protein (PCBP) binding site (nucleotides 5052-5089), highly homologous to the consensus CU-rich binding sequence[168], is located in the third high homology stretch. The PCBPs bind to pyrimidine-rich sequences in the 3'UTR and this is believed to represent a general mechanism for stabilization of long-lived cellular mRNAs. This mechanism is characterized in detail in many proteins such as the α-globin and the androgen receptor[162,177,178]. PCBP binding sites have been found before in other type collagen mRNAs, and simple software alignment readily revealed homology of this putative type XVII collagen binding site with other collagen sequences. Actual PCBP control has already been proved for collagen types I and III in cardiac fibroblasts[173], and in activated hepatic stellate cells (HSCs) where collagen α1(I) mRNA is stabilized by the PCBP αCP, a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family, which leads to increased type I collagen expression[179]. The PCBP mechanism however seems more complicated than simple binding of one regulatory protein, and the

exact mechanism is not yet clear. Thiele *et al* already demonstrated that three different RNA-binding proteins – hnRNP A1, E1, and K- are involved in post-transcriptional control of collagen I and III[173].
Furthermore, studies on α-globin mRNA in murine erythroid cells and non-erythroid fibroblasts demonstrated a cell-type dependency and the likely involvement of three PCBP proteins independently binding to the α-globin 3'UTR[177]. In both cell types mRNA stabilization was observed but, whereas in erythroid cells αCP1 binds to the mRNA, no expression of this particular PCBP was seen in non-erythroid cells[178,180]. A similar demand – existence of different PCBPs- evolved from studies on type I collagen wherein quiescent HSCs, in contrast with in activated stellate cells, no binding to the *COL1A1* mRNA was observed despite the presence of αCP[179].

Table 2. Percentage identity of the human *COL17A1* long variant 3'UTR sequence with other species *COL17A1* 3'UTR

Overall 3'UTR sequence				*high homology stretches*		
				4728-4797	*4946-4985*	*5053-5095*
species	*length*	*matches*	*% identity*	*% identity*	*% identity*	*% identity*
Homo Sapiens	1005	IR*	IR	IR	IR	IR
Mus Musculus	864	638	73	87	88	92
Bos Taurus	831	419	50	80	83	87
Gallus Gallus	790	431	55	83	83	87
Sus Crofa	574	440	76	86	85	78
Rattus Norvegicus	771	408	53	81	88	87

*irrelevant

In summary, we found that the longer *COL17A1* mRNA variant leads to higher protein expression than the short variant. This is most likely the result of higher stability of the longer transcript. Keratinocytes thus seem able to control the type XVII collagen protein level by differential expression of the *COL17A1* gene. The conserved potential PCBP binding site suggests that RNA-binding proteins might stabilize the longer *COL17A1* transcript and will be subject of further research.

CHAPTER 4

SUBCELLULAR LOCALISATION OF TYPE XVII COLLAGEN IS DETERMINED BY THE ALTERNATIVE *COL17A1* 3'UNTRANSLATED REGIONS

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To be submitted

ABSTRACT

The *COL17A1* gene codes for type XVII collagen, a structural component of the hemidesmosome. Alternative splicing leads to two major transcript populations that differ by 610 nucleotides in the 3'Untranslated Region (3'UTR). UTRs can be involved in control of various cellular mechanisms including post-transcriptional regulation of translation, transcript stabilisation, and subcellular mRNA transcript localisation. In this study we investigated whether the 3'UTR sequence effects the localisation of the two major mRNA transcripts using green fluorescent protein (GFP) reporter assays in various cell types, and fluorescent *in situ* hybridisation (FISH) of *COL17A1* mRNA in cultured cells and on skin sections. Transfection of a GFP-vector with the long 3'UTR variant cloned behind the GFP coding region directed GFP to perinuclear compartments, whereas the short 3'UTR variant led to a more random distribution of the recombinant protein. FISH demonstrated that the *COL17A1* mRNA localisation has a similar distribution, since the long *COL17A1* mRNA transcript variant is located perinuclear and the short *COL17A1* transcript variant is observed throughout the cytoplasm.

These observations demonstrate that the *COL17A1* 3'UTR, next to regulation of mRNA stability, is also important for the localisation of the mRNA transcript and thereby determines the subcellular location of type XVII collagen synthesis in the cell.

An additional observation in the FISH experiments was that in skin not only the basal cells but also suprabasal cells express *COL17A1* mRNA. Immunofluorescense demonstrated that non-differentiated cells in the suprabasal layer also expressed type XVII collagen with a granular distribution throughout the cytoplasm. The suprabasal expression suggests that type XVII collagen has other functions than mere anchoring of the basal keratinocytes to the basement membrane.

INTRODUCTION

Type XVII collagen is a structural component of the hemidesmosome and is encoded by the *COL17A1* gene[3,7]. Hemidesmosomes are structures that connect the epithelial basal cells to the underlying basement membrane[129]. They consist of at least six different polypeptides: type XVII collagen, the plakins BP230 and plectin, integrin $\alpha_6\beta_4$, and tetraspanin/CD151 and plectin[4,38-44]. Type XVII collagen itself is a type II orientated protein that protrudes for two-third out of the cell in the shape of a flexible hooked tail[4,16].

In skin, type XVII collagen is mainly expressed along the basal surface of the epithelial basal cells, but expression is also seen along the lateral surface of these cells[19]. Other tissues that express type XVII collagen include mammary, salivary and thyroid glands, oesophagus, colon, prostate, testis, placenta, thymus, and retina[54,77]. Deviant expression is observed at different stages of carcinogenesis and a correlation between over-expression and tumour-progression may exist[15,70,156].

Type XVII collagen is a target molecule in both autoimmune and genetic variants of blistering diseases. Genetic deficiency of type XVII collagen leads to Hemidesmosomal Epidermolysis Bullosa[33,88,132]; a condition characterised by skin fragility due to inadequate anchoring of the epidermal basal cells to the dermal matrix. From studies on the autoimmune blistering diseases it evolved that type XVII collagen is not only present as the full-length 180 kDa transmembrane form, but also as a cleaved 120 kDa soluble form that is further processed to a 97 kDa form[19,21,181]. This latter form localises to the lamina lucida underneath hemidesmosomes[28].

Also at the mRNA level more transcript forms are found, at least six, all with apparently the same coding region but with different 5' and 3'Untranslated Regions (UTRs)[12] (Van Zalen *et al*, in press). Whether this heterogeneity at the transcript level is related to the appearance of the different protein forms is unknown. So far, some evidence has been presented that favours generation of the soluble 120 kDa form from the full length protein by proteolytic shedding[22]. However, if this occurs at the hemidesmosome or at another location is unknown.

In a previous study we demonstrated that the two alternative 3'UTRs, generated by differential splicing of exon 56, influence both the transcript stability and the translation level. Keratinocytes are thus able to control the type XVII collagen protein level by differential expression of the *COL17A1* gene (Van Zalen *et al*, unpublished data).

Next to post-transcriptional regulation of translation and transcript stabilisation, the 3'UTR may also localise the mRNA to specific subcellular sites. This enables encoded proteins to be translated close to their place of action. Such specific localisation can be effectuated by different stability in different subcellular locations but also by active transport of mRNAs. Both mechanisms are accomplished by binding of *trans*-acting factors to *cis*-acting signals -"zipcodes"- within the 3'UTR that

can be sequence- but also structural-based motifs[182-184]. Regional stability of mRNA transcripts is achieved by protection from degradation after binding of protective *trans*-acting factors to the *cis*-acting elements[185]. In the mRNA targeting mechanism actual transport of transcripts takes place along cytoskeleton elements followed by delivery and anchoring at the place of action. Two classes of cytoskeleton elements have been shown to be used: actin microfilaments for short distances and microtubuli for long-distance transport[186,187].
Since basal keratinocytes are polarised cells with respect to the localisation of type XVII collagen protein the location of production might also be controlled. In this study we investigated whether the 3'UTR sequence effects the localisation of the two major mRNA transcripts. Moreover, we explored whether this localisation of the mRNA transcripts directs type XVII collagen protein production to distinct subcellular compartments.

MATERIAL AND METHODS

Cell culture

The squamous cell carcinoma line UMSCC-22B, originally derived from a tumour of the hypopharynx, was cultured in DMEM (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (Cambrex, Verviers, Belgium), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) at 37°C with 5% CO_2[134]. Normal human keratinocytes were cultured from patches of clinically unaffected donor skin of breast reductions. The epidermis was separated from the dermis after overnight incubation with 2.4 U/ml dispase at 4°C followed by incubation of the epidermis with 0.025% trypsin, 0.01% EDTA at 37°C for 10 minutes. The harvested keratinocytes were cultured under serum-free conditions using Keratinocyte-Serum Free Medium (SFM) (Gibco) according to Mitra and Nickoloff[135] also at 37°C with 5% CO_2. The medium was supplemented with 25 μg/ml Bovine Pituitary Extract (BPE) and 0.1 ng/ml Recombinant Epidermal Growth Factor (rEGF) (all from Gibco). The type XVII collagen deficient keratinocyte cell line, hereafter referred to as t-mar, was generated by immortalizing keratinocytes of a HEB patient, homozygous for the 2342delG mutation[165]. The keratin 14 deficient keratinocyte cell line, hereafter referred to as t-witt, was generated by immortalizing keratinocytes of a HEB patient, homozygous for the *KRT14* 1842-2A-->C splice-site mutation[188]. These transformations were accomplished at the Fred Hutchinson Cancer Research Center (Seattle, WA) using the LXSN16E7 retroviral vector[166]. This retroviral vector contains E6 and E7 coding sequences from human papilloma virus and a neo resistance gene. We used no drug selection, as in prolonged culture only the infected cells would proliferate while non-infected cells would cease growing due to differentiation. After immortalization and selection the cells were tested for retrovirus production to ensure that the cells did not produce any virus. Immunoblotting and immunofluorescence confirmed the absence of measurable type XVII collagen

production in t-mar cells. The t-mar and t-witt cells were cultured under the same conditions as NHKs.

Plasmid construction

The *COL17A1* 3'UTR was amplified by RT-PCR using gene specific primers that had an additional XbaI recognition sequence synthesized at the 5'end (Eurogentec, Seraing, Belgium). For RT-PCR, cDNA of normal human keratinocytes (NHKs) and UM-SCC22B cells were used. PCR products were ligated into the pCR4 TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and subsequently digested with XbaI (MBI Fermentas, Vilnius, Lithuania). After electrophoresis the digested fragments were isolated from agarose gel with the Gel Purification kit from Qiagen (Hilden, Germany). Isolated DNA fragments were ligated into the unique XbaI recognition site of the phMGFP-vector (Promega, Madison, WI) with T4 DNA ligase (Fermentas). Plasmids were checked for the correct sequence by DNA sequence analysis. For schematic depiction of the constructs see Fig. 1.

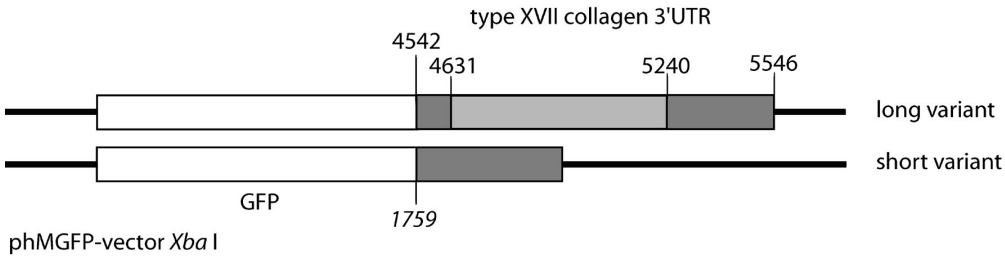

Figure 1. Schematic depiction of the GFP-*COL17A1* 3'UTRs constructs in the phMGFP- vector.
Numbers correspond to the *COL17A1* mRNA sequence (roman) and the phMGFP-sequence (italic).

Cell transfection

All cell types but SCC were transfected with poly-L-ornithine (Sigma, St. Louis, MO, USA) followed by a DMSO-shock. In detail, cells were plated in a 12-wells plate in a density of $1*10^5$ cells/well and incubated overnight in their respective culture media. Transfection mixtures of 2.0 µg of phMGFP-vector in 100 µl HS-buffer (150 mM NaCl, 20mM HEPES) containing 0.33 µl poly-L-ornithine were pre-incubated at RT for minimal 10 and maximal 15 minutes. The medium on the cells was changed and the transfection mixture was added. After four hours incubation the medium was removed and 250 µl RT culture medium containing 25% DMSO (Sigma) was pipetted onto the cells. After four minutes the cells were washed with 0.5 ml HBSS (Gibco), 1.0 ml culture medium was added and the cells were incubated for a further 48 hours at 37°C.
SCC cells were transfected with DOSPER (Roche, Mannheim, Germany). In brief, SCC cells were seeded in a density of $1*10^5$ cells/well and incubated overnight in DMEM. Transfection mixtures of 2.0 µg vector and 5 µl DOSPER in a total volume of 100 µl HS-

buffer were incubated for 15 minutes at RT. After replacing of the medium the transfection mixture was added and the cells were incubated for a further 48 hours at 37ºC. After 24 hours, another 0.5 ml medium was added, to preclude starvation of the cells.
After 48 hours, cells were washed with PBS and fixed in 4% formaldehyde in PBS, followed by washing with again PBS. Next, cells were mounted in glycerol/PBS (1/1 v/v) and for all cell types a representative number of cells was photographed.

Table 1. Location and region of the probe sequences as used in the FISH experiments

*Location**	*region*	*Forward primer (5'→3')*	*Reverse primer (5'→3')*	*labeling*
230-626	coding	AAACAGCCTCTCTTGG TGGA	CTCACACTTGCCGAT CGACT	DIG
3679-4131	coding	GTGTGGTCCAGCATC AGCGT	GCCGATGTCAGTGCC ATAGG	DIG
4649-5101	non-coding	CACTTAGGTCCAAGG TCTCC	GCCAACTGACTAGAG AATGC	biotin

*Numbering according to Giudice[4].

RNA probes

Digoxigenin (DIG)- and biotin-labeled RNA probes were synthesized using the DIG- or Biotin Labeling mix (Roche), according to the manufacturer's protocol with some minor modifications as described below. DNA fragments were generated by PCR on SCC cDNA that was produced by transcribing SCC mRNA with Superscript reverse transcriptase (Invitrogen). PCR products were cloned into vector pCR4 (Invitrogen). Plasmids were linearized either with restriction enzyme NotI or with BcuI (Fermentas), depending on the type of RNA polymerase, T3 or T7 (Fermentas), that was used in the subsequent transcription reaction. Probes were then generated by *in vitro* run-off transcription on linearized plasmid (for sequences and location see Table 1).

***In situ* hybridisation**

Cell fixation

Cryosections of 4 μm thickness were cut from skin biopsies of healthy individuals and these were fixed in 4% formaldehyde in PBS at 37°C for 24 hours. Next the sections were subsequently washed with 17 mM potassiumdihydrogenphosphate, 52 mM sodiumphosphate, 1.5 M Nacl pH 7.2 (PBS), deionized water, ethanol and then air-dried. Sections were either used immediately or else stored at –20°C.
Cultured cells were grown on LabTek Chamber Slides (Nunc, Wiesbaden, Germany) to subconfluence. After three washings in PBS, cells were fixed in 4% formaldehyde/PBS (Merck, Darmstadt, Germany) for 20 minutes cells at RT. After again three washes in PBS they were treated with 0.2 M HCl for 10 minutes at RT, and then rinsed in 10 mM Tris-HCl pH 7.4, 150 mM NaCl (TBS) for three times.
Tissue permeabilisation for both the cryosections and the cultured cells was achieved by incubation in 500 ng/ml Proteinase K (Fermentas) in TBS at RT for 1 h. After

permeabilisation, cells were rinsed in TBS and directly used for the actual *in situ* hybridization. Before actual *in situ* hybridisation, cultured cells were treated in acetic anhydride (0.25% v/v; Merck) for 10 minutes at RT and fixed again in 4% formaldehyde/PBS for 10 minutes at RT. In between cells were washed with PBS.

In situ hybridization

Slides were pre-incubated in hybridisation buffer (40% deoinized formamide (Ambion), 4xSCC (600 mM NaCl, 60 mM NaCitrate, pH 7.0), 10 mM Ultrapure EDTA (USB corporation, Cleveland, OH, USA) 10% Ultrapure dextran sulphate (USB), 0.05% polyvinylpyrrolidon, MW 360,000 (USB), 0.05% Ficoll™ PM400 (Amersham Biosciences AB, Uppsala, Sweden), 0.05% Acetylated BSA (Sigma), 0.01% hearing sperm DNA (Roche) at 55˚C for at least 1 hour.
This was followed by overnight hybridisation at 55˚C with respectively 50 µl of 2 ng/µl DIG – and/or biotin-labeled probe (Table 1) in hybridisation buffer when using Labtek slides or 25 µl of 4 ng/µl when using skin sections. After rinsing in TBS, sections were treated with 1 µl RNAse A (Fermentas) in 100 µl RNAse buffer (10 mM Tris-HCl pH 8.0, 0.5 M NaCl, 1 mM EDTA) at 37˚C for 30 minutes, and then incubated in pre-warmed wash buffer (50 mM Tris-HCl, 150 mM NaCl pH 9.7) at 55˚C for another 30 minutes. Next, sections were blocked for at least 1 hour in block buffer 1 (0.05% blocking reagent (Roche), 0.1 % Triton X-100, 0.1% SDS in maleate buffer pH 7.5) at RT.
DIG-labeled probes were detected by incubation of the slides with α-DIG F_{ab} fragments conjugated with POD (Roche, 150 U/ml 1/20 in block buffer 2 (0.02% blocking reagent, 0.1% Triton X-100, 0.1% SDS in maleate buffer pH 7.5) for 24 hours at RT. Hereafter, slides were washed with TBS twice. Biotin-labeled probes were detected by incubating the sections with streptavidin-HRP (DAKO, Glostrup, Denmark, 0.40 mg/ml, 1/50 in buffer 2) for 1 h at RT. Thereafter, sections were incubated with goat biotinylated α-streptavidin (Vector Laboratories Inc. Burlingame, CA, 0.50 mg/ml 1/100 in buffer 2) at 37˚C for 30 minutes, followed by incubation with again the first streptavidin-HRP antibody for 1 h at RT. In between incubations, slides were washed with TBS twice.

TSA reaction

Tyramide reagent, conjugated with either Alexa Fluor ® 488 or 568 (Molecular Probes, Leiden, the Netherlands), was diluted in amplificationbuffer/0.0015% H_2O_2 (Molecular Probes) and brought onto the sections for either 5 minutes on skin sections (only Alexa ® 488) or 2 minutes for both Alexa Fluors on cultured cells at 37˚C in the dark. Before and after the tyramide reaction, slides were washed in pre-warmed PBS for three times. For double staining of sections, the full procedure was first executed for the DIG-labeled probes, including the TSA reaction. After that, residual HRP-activity was quenched by incubation in 1% H_2O_2 in PBS for 1 hour, followed by washing in TBS for three times. Next, the biotin-labeled probes were detected with the other wavelength TSA reagent. After the TSA reaction, nuclei were counterstained with 1 µg/ml bisbenzimide (SERVA, Heidelberg, Germany) in PBS for 5 minutes. The slides were rinsed with PBS and then mounted in PBS/glycerol (v/v 1/1).

Immunofluorescence microscopy

Immunofluorescent slides or Labteks were examined with a Leica DMRA microscope equipped with switchable filter cubes for selective incident light fluorescence. For detection of Alexa Fluor ® 488 or GFP-protein fluorescence expression we used a Chroma high Q filter set containing (a) exciter filter HQ 480/40, (b) dichroic beam splitter Q 505 LP, and (c) emission filter HQ 535/50. For Alexa Fluor ® 568 and LSRC fluorescence we used a Chromas Texas red highQ filter set with (a) exciter HQ 560/55, (b) beamsplitter Q 595 LP, and (c) emission filter HQ 645/75. Bisbenzimide staining was observed with (a) exciter HQ 340-380, (b) beamsplitter Q 400 LP, and (c) emission filter LP 425.
Three objectives were used: an HC PL 20x/0.50, a PL APO 40x/0.85, or an HCX/PL FLUOTAR 63x/0.90 dry objective in combination with an HC PLAN 10x/20 objective.
Representative areas of each slide were photographed using a DFC350 FX camera (Leica Microsystems AG, Wetzlar, Germany) for all filter sets, and pictures were overlaid with the help of Leica Application Suite software using the image overlay module.

Immunofluorescence staining of proteins

Cryosections of 4 µm thickness were cut of skin biopsies of healthy donors. Sections were incubated with monoclonal 1A8C (1/50 diluted in PBS containing 1% ovalbumin) against the intracellular part of type XVII collagen, either alone or in combination with a rat IgG2a anti-Hsc-70 antibody (Stressgen Bioreagents, Victoria BC, Canada), diluted 1/1800. As second antibodies were respectively used goat anti-mouse IgG conjugated with Alexa Fluor ® 488 (Molecular Probes) and a donkey anti-rat IgG antibody conjugated with LRSC (Jackson Immunoresearch, Cambridgeshire, UK), both in a 1/100 dilution. In between, sections were washed with PBS for three times and antibodies were incubated for 30 minutes at RT. Sections were counterstained with 1 µg/ml bisbenzimide (SERVA) in PBS for 5 minutes. After rinsing with PBS, slides were mounted in PBS/glycerol.

RESULTS

GFP-reporter gene is translated at different cellular locations dependent on the *COL17A1* 3'UTR variant.

Normal human keratinocytes and keratinocyte cell lines were transfected with a GFP reporter gene plasmid with either the long *COL17A1* 3'UTR or the short *COL17A1* 3'UTR cloned behind the GFP coding sequence (Fig. 1). Normal human keratinocytes transfected with the long variant construct showed a perinuclear distribution of fluorescent protein in most of the transfected cells, whereas cells transfected with the short variant construct displayed a more randomly located fluorescent signal in the majority of the cells (Figs. 2A and 2B).
Transfection with the empty GFP-vector resulted in random localisation comparable to the transfection with the short 3'UTR variant (Figs. 2C and 3A). Fischer exact test

demonstrated the difference to be highly significant. The expression levels with both variants appeared similar and the localisation was independent of the passage number of the keratinocytes.
To investigate whether the boundary of the cytoplasm was the determining factor in the localisation of GFP protein by the *COL17A1* 3'UTR, fluorescent microscopy images of transfected cells with GFP constructs were overlaid with light microscopy images of the same cells (Fig. 4). These pictures clearly demonstrate that the localization of the GFP protein is not hampered by the volume of the cytoplasm and that the 3'UTR therefore determines its location.
This localisation effect appeared to be independent of the cell type transfected. In all used cell lines, UMSCC-22B, t-witt, and also t-mar that contains about 3% *COL17A1* mRNA compared to normal keratinocytes, GFP protein expression was observed predominantly perinuclear after transfection of the long *COL17A1* 3'UTR construct and randomly after transfection of the short variant or the GFP-vector control. In all cell types this difference in localisation was significant (Figs. 3B, 3C and 3D).

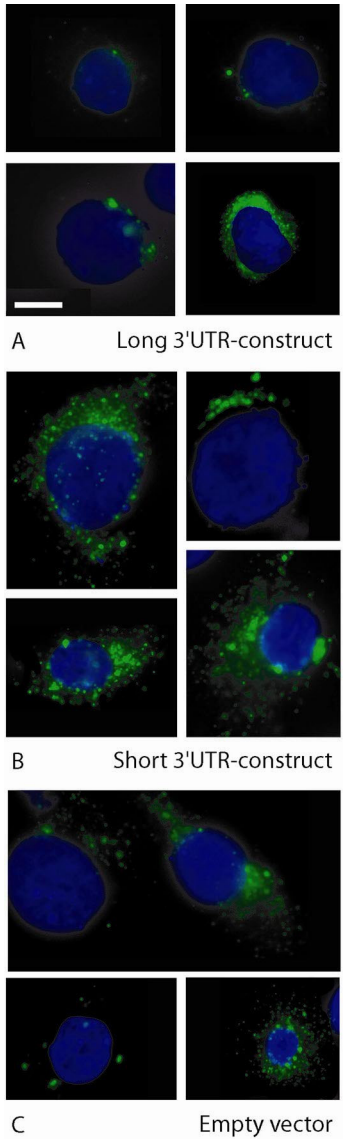

Figure 2. GFP is expressed at different locations in the cell depending on the *COL17A1* 3'UTR variant.
(A and B) Keratinocytes transfected with the long variant 3'UTR-GFP construct express GFP protein perinuclear, whereas keratinocytes transfected with the short variant 3'UTR construct show random localisation of GFP protein. (C) Transfection with the empty GFP-vector gives a same distribution as the short variant 3'UTR -GFP construct. Four representative pictures for each GFP-construct out of three independent experiments are shown, and in these experiments a total of 239 keratinocytes were examined.

The alternative *COL17A1* mRNA transcripts are targeted to distinct subcellular locations in the cytoplasm of keratinocytes

The difference in subcellular location observed in the reporter gene analyses made us investigate whether the localization of the two major alternative *COL17A1* mRNA transcripts differed similar in subcellular localization in keratinocytes. Therefore we performed two colour FISH in which *COL17A* mRNA in cultured keratinocytes was hybridized with several probes against different regions of the *COL17A1* mRNA (Table 1). Two DIG-labeled probes were made against sequences in the coding region, both hybridizing with both the long and the short variant. These were stained with Alexa Fluor ® 568 and thus appeared as red fluorescence. A third biotin-labeled probe was created against a sequence in the unique insert in the *COL17A1* 3'UTR and hybridized only with the long variant. This was stained with Alexa Fluor ® 488, which is a green fluorescent molecule.

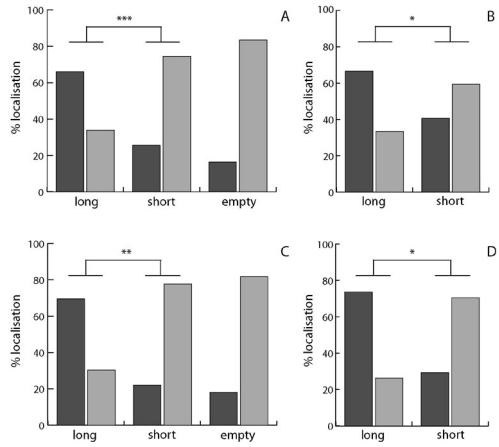

Figure 3. Distribution of perinuclear and random GFP expression after transfection of the GFP constructs in different cell types.
Cytoplasmic localisation of GFP-protein was judged as either perinuclear or random in a representative number of cells for each transfected cell type. Dark grey bars express perinuclear localisation and light grey bars express random localisation (percentages). (A) Normal human keratinocytes, n = 239 (B) UM-SCC22B cells, n = 74 (C) t-mar cells, n = 50 (D) t-witt cells, n = 36. Fisher exact test revealed significant difference in GFP localisation after transfection of the long variant 3'UTR construct and the short variant 3'UTR construct. ***P<0.0001, **P<0.01, *P<0.05.

FISH with all antisense probes simultaneously demonstrated the same location of the *COL17A1* transcripts as above, since green fluorescence was again mainly observed near the nucleus and red fluorescence was seen throughout the cell (Figs. 5D and 5E). In an overlay of both images this is seen as a light green color around

the nucleus (Fig. 5F). The red fluorescence seen throughout the cytoplasm confirmed the more extensive spreading of the short transcript.

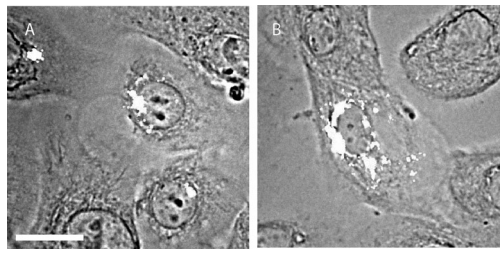

Figure 4. Perinuclear and random expression of GFP.
Overlays of GFP-expression images with light microscopic images of the same keratinocytes shows (**A**) the perinuclear GFP-localisation by the long variant *COL17A1* 3'UTR -GFP construct (**B**) and the random localisation of GFP-protein by the short variant 3'UTR-GFP construct. Bar is 10 μm.

When the second - anti-biotin- antibody after hybridizing of both DIG- and biotin-labeled probes was omitted, then no green fluorescence was observed, demonstrating that any residual HRP-activity after staining of the first - anti-DIG-antibody was totally abolished (not shown). Therefore, the green fluorescence is solely the result of the biotin-labeled probe targeting the long *COL17A* mRNA transcript. Control hybridizations with matching sense probes were negative (Fig. 5C).
In the squamous carcinoma cell line UM-SCC22B a shift in the alternative splicing ratio of *COL17A* mRNA is observed, such that the short transcript becomes the dominant form[12] (Van Zalen *et al*, unpublished results). Hence, to confirm the distinct locations of the alternative *COL17A1* mRNA transcripts, especially that of the short variant, we applied the same double FISH technique on cultured UM-SCC22B cells. This demonstrated that the short transcript is indeed spread in the cytoplasm, although due to the small extent of the cytoplasm in carcinoma cells we saw in most cells only two small concentric rings (Figs. 6D, 6E, and 6F). The inner ring that enclosed the nucleus is home to the long *COL17A* transcript, whereas the red fluorescence in the outer ring thus denominates the expression of the short *COL17A1* transcript throughout the cytoplasm. Hybridizations with single probes and sense probes confirmed the correctness of the signals (Figs. 6A, 6B, and 6C).

COL17A1 mRNA expression in human skin

Surprisingly, during investigation of *COL17A1* mRNA expression in human skin sections, we observed that *COL17A1* mRNA is not only expressed in the basal layer of the epidermis but also in the suprabasal layers (Fig. 7A). FISH on skin slides of a HEB patient, who had less than 5% *COL17A* mRNA expression (Van Zalen *et al*, unpublished data), was used to confirm the specificity of the hybridisation signal

(Fig. 7B). *COL17A1* mRNA expression was absent in all layers of the epidermis in this HEB patient, indicating that the technique is valid, and thus confirming that indeed suprabasal mRNA expression is present in the epidermis. Moreover, also hybridisation of the corresponding sense probes on skin sections was negative as expected (Fig. 7C).
The suprabasal expression was unexpected since type XVII collagen expression is thought to be present in basal keratinocytes only. In basal cells it is reported predominantly on the basal cell surface with some lateral expression around the entire cell membrane[17]. Since we found *COL17A1* mRNA expression also in the suprabasal layers we decided to explore type XVII collagen protein expression in skin sections by use of the 1A8C monoclonal that stains only the full-length molecule but not the soluble form. Next to the expression at the basal surface of the basal keratinocytes, granular perinuclear and cytoplasmic expression is observed in the basal keratinocytes and also in suprabasal keratinocytes (Fig. 8).
Immunofluorescence in skin sections of a HEB patient showed the absence type XVII collagen expression herein (not shown). Double-staining with an anti-Hsc70 antibody, together with 1A8C showed the suprabasal type XVII collagen expression was only present in keratinocytes that had not differentiated yet (Fig. 9C). The anti-Hsc70 antibody stains the heat shock cognate protein Hsc70 that is specifically expressed in differentiated suprabasal keratinocytes[189,190] (Fig. 9B).

DISCUSSION

In this study we have investigated whether the alternative splicing of the *COL17A1* 3'UTR has any influence on the localization of the *COL17A1* transcripts and type XVII collagen by GFP-reporter protein expression and mRNA FISH studies. The long *COL17A1* 3'UTR variant directed its translated protein into perinuclear compartments, and not, as maybe expected, close to the cell membrane, whereas protein production by the short variant is more randomly dispersed. This protein localization results from differential targeting of the two alternative mRNA transcripts. The long *COL17A1* mRNA transcript focuses near to the nucleus whereas the short transcript spreads throughout the cytoplasm.

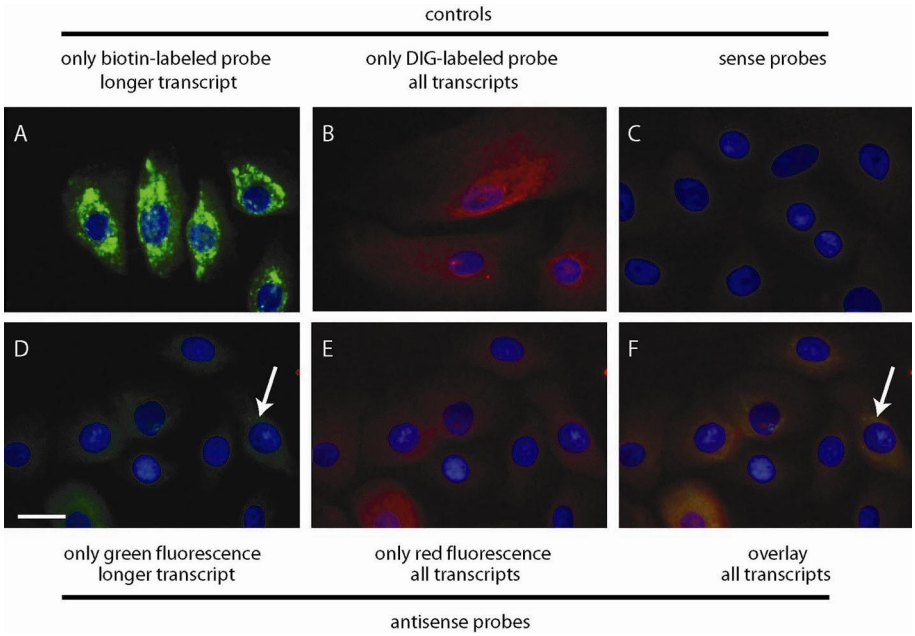

Figure 5. FISH demonstrates that the long and short *COL17A1* transcripts are found at different locations in cultured keratinocytes.
COL17A1 mRNA is detected with probes against different sequences in the *COL17A1* mRNA (Table 1). Red fluorescence evolves from two different DIG-labeled probes that hybridize with the coding region, and thus with both transcript variants. Green fluorescence represents the biotin-labeled probe that is directed against the unique insert in the 3'UTR of *COL17A1* mRNA and thus visualises only the long variant. **A** shows fluorescence from the biotin probe and thus demonstrates that the longer *COL17A1* mRNA is located perinuclear. In **B** only the fluorescence after hybridisation of the DIG-probes is displayed, and this shows the distribution of all *COL17A1* mRNA variants, that appears to extend throughout the whole cytoplasm. The cytoplasm thus harbours predominantly the short variant. Note the strong expression near the nucleus in **B**, caused by the difference in expression of both transcripts, which is in favour of the long variant in normal human keratinocytes. In **E, D,** and **F** probes are hybridised simultaneously, and locations of both *COL17A1* mRNA transcripts here is in agreement with the single colour hybridizations. Again a light green colouring is seen around the nucleus depicting perinuclear expression of the long *COL17A* transcript and a red colouring throughout the cytoplasm indicating the random location of the short *COL17A1* mRNA. **F** shows that the corresponding sense probes are negative. Bar is 20 μm.

An additional observation was that *COL17A1* mRNA was also expressed in suprabasal keratinocytes. This was rather unexpected considering that type XVII collagen is primarily seen as an adhesive hemidesmosomal protein that is important for anchoring of the basal cells to the extracellular basement membrane matrix.
Immunofluorescence confirmed that the type XVII collagen protein is indeed suprabasally expressed and thereby confirmed that *COL17A1* mRNA is present in

these layers. Double staining with Hsc-70 showed that these cells had not yet differentiated. The absence of the typical linear basement membrane staining of type XVII collagen in the more suprabasal layers indicates that tangential cutting cannot explain the observed patterns.
Since no hemidesmosomes are observed in suprabasal cells type XVII collagen must exert another function herein. Supposed additional functions for type XVII collagen include involvement in signal transduction and ligand or receptor activity, and our data undoubtedly show the need for further research into these options[22,35].

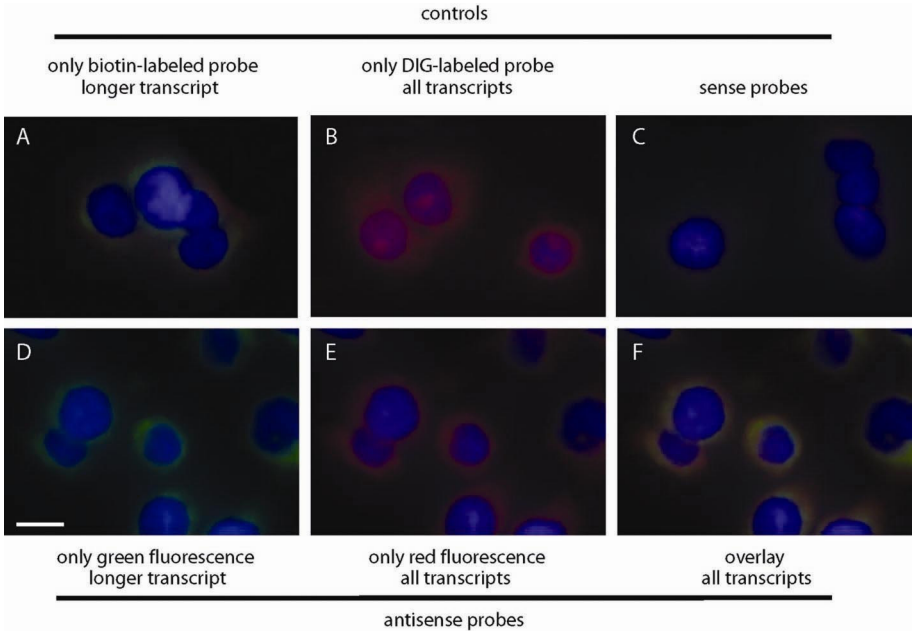

Figure 6. FISH on the squamous carcinoma cell line UM SCC 22B confirmed the distinct locations of both alternative *COL17A1* transcripts.
The SCC cells were hybridized as in Fig. 7. Note the smaller extent of the cytoplasm in these cells leading to fluorescence appearing as two concentric rings around the nucleus (**D**, **E**, and **F**). The inner ring denotes expression of the long transcript, whereas the larger red ring indicates expression of the short *COL17A1* transcript. Controls (**A**, **B**, and **C**) are similar as in Fig. 7.

We were surprised by the staining patterns of the basal cells. Type XVII collagen is present as granules trough the cell and close inspection showed that many granules are close to the nucleus while some are somewhat more distributed through the cytoplasm, mimicking the observations in the transfection experiments. Thus in skin type XVII collagen is synthesized at the same subcellular location as in cultured cells. The second striking observation was that the expression along the basement membrane zone (BMZ), where the hemidesmosomes reside, is far from spectacular

and, unlike reported before, only shows very faint linear intensifications. When we repeated the experiment but now added a little Triton, most cytoplasmic expression was lost and a thin line along the BMZ emerged (not shown). This population must represent the hemidesmosomal type XVII collagen fraction. That the expression patterns we describe here have not been reported before has two causes. First, most staining experiments were performed with antibodies that are specific to the extracellular domain. These, however, stain both the full-length and the shedded 120 kDa molecule. The latter molecule is located in a linear fashion just underneath the basal cell. Previous researchers always described their observed staining patterns as typical for type XVII collagen without realizing that their images were in fact contaminated with the shedded molecule. Second, most skin sections had fixations that may remove -as Triton- the cytoplasmic staining.

Our data demonstrate that type XVII collagen synthesis is a very active process in basal keratinocytes and the amount of type XVII collagen not present in hemidesmosomes by far exceeds the population that is hemidesmosome bound.

It was suggested that lateral localized type XVII collagen is stock for the forming of new hemidesmosomes, as these proteins were demonstrated to be already in the trimer form, and since other hemidesmosomal protein components had also been observed laterally[17]. Our data do not contradict this suggestion but on the other hand do demonstrate that identical distributions are seen in suprabasal cells, which cannot be explained as stock for new hemidesmosomes.

At first sight the perinuclear location of the long *COL17A1* mRNA variant seems surprising considering type XVII collagen is a membrane bound protein. Transmembrane proteins, however, are translated on the membrane bound rough endoplasmatic reticulum (ER). The first N-terminal stretch is translated in the cytosol by free ribosomes, after which the ribosomal complex with the protein is directed to the rough ER by a signal recognition particle (SRP) that binds to an N-terminal signal sequence[191,192]. After completing the translation at the rough ER the resulting protein is then targeted to the membrane through either the Golgi apparatus or lysosomes.

A hypothesis to explain the different location of the two 3'UTR forms could be that the long *COL17A1* mRNA transcript translates the membrane bound type XVII collagen destined for hemidesmosomes, whereas the short variant translates type XVII collagen involved in some other processes. In such a hypothesis, the type XVII collagen translated by the short mRNA variant should lack the N-terminal signal sequence, since it seems to be translated on free ribosomes. This suggests variance at the protein level for type XVII collagen. For this no evidence has been found although it should be taken in account that also the heterogeneity in the *COL17A1* 5'UTR is currently unexplained.

Most important however, our data point out an essential role for the *COL17A1* 3'UTR in the perinuclear localization of the transcript. Therefore, we propose an alternative hypothesis for the localization of the *COL17A1* mRNA transcripts: trafficking by *trans*-acting proteins through *cis*-acting sequences in the 3'UTR. Such

mechanism has already been observed for several other genes and here in most cases conserved stem-loops are responsible for the targeting of the mRNA transcripts[193-195]. *Trans*-acting RNA-binding proteins form the link between the mRNA and the cytoskeleton[193,196]. In this way mRNA transcripts may traffic throughout the cell *via* the cytoskeleton. Secondary structures of the *COL17A1* 3'UTR (nucleotides 4591-5546) show the existence of a stem-loop in this long 3'UTR variant that is absent in the short variant (Fig. 10). The sequence (nucleotides 4728-4797) of this stem loop is highly conserved between species (van Zalen *et al,* submitted).
The two other conserved sequences (nucleotides 4946-4986 and 5043-5095) in the unique 610 nucleotides sequence of the long variant have less predicted complex secondary structures. To prove the existence of such a mechanism actual protein binding to the 3'UTR has to be demonstrated.
This perinuclear translation of type XVII collagen fits in a model in which type XVII collagen translated at the endoplasmatic reticulum and is then transported to the cell membrane for incorporation into the hemidesmosomes. The GFP-protein used in our expression study is no membrane-bound protein and lacks such a signal needed for transport to the cell membrane. In type XVII collagen the membrane localisation sequence is found in the N-terminus[53,197,198].
There is some support for our model. Hopkinson and Jones stated that type XVII collagen and BP230, another structural component of the hemidesmosome, may associate in the cytoplasm before incorporation into the hemidesmosome[199]. We hypothesise that this connection is achieved perinuclear, and this is supported by hemidesmosomes-like structures seen at the cell surface in the perinuclear region shortly after plating[200]. BP230 expression has already been observed near the nucleus, and this localisation was directed by a conserved short protein stretch in the plakin domain[201]. Therefore, after translation near the nucleus BP230 may already interact with type XVII collagen before being transported to the cell membrane.

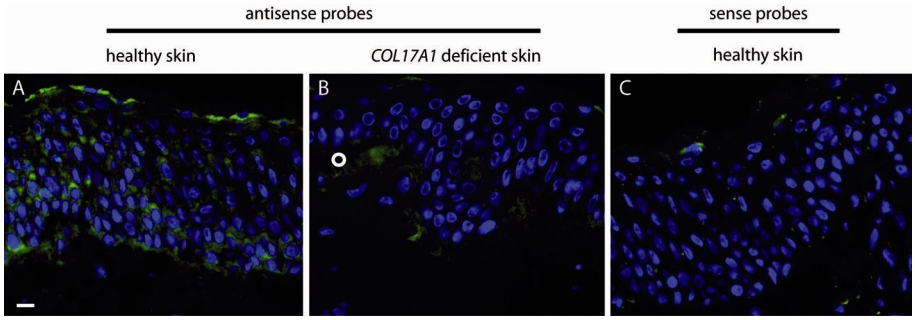

Figure 7. *COL17A1* mRNA expression is observed in both basal and suprabasal keratinocytes.
(A) FISH with antisense probes against the coding region of *COL17A1* mRNA on normal human skin shows expression of *COL17A1* mRNA in both the basal and the suprabasal layer. (**B**) The same experiment on type XVII collagen deficient HEB skin demonstrates the specificity of the signal in **A** as no staining is seen here. The circle (o) indicates a blister cavity. (**C**) Control hybridisation of the corresponding sense probes on normal human skin is also negative. Bar is 10 μm.

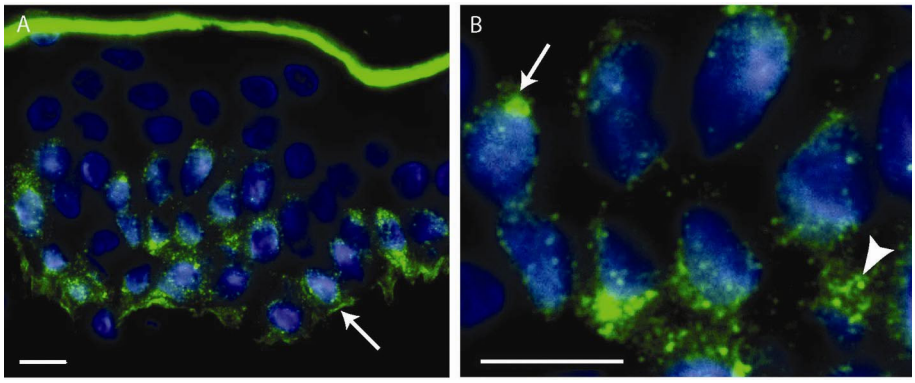

Figure 8. Immunofluorescent localization of full-length type XVII collagen in skin.
Normal human skin was stained with the monoclonal 1A8C that is directed against an intracellular epitope of type XVII collagen. (**A**) Type XVII collagen is, next to basement membrane, also expressed in both basal and suprabasal keratinocytes and it localises in a granular fashion throughout the cytoplasm. In suprabasal keratinocytes the typical linear basement membrane zone staining (arrow) is absent. (**B**) Magnification of suprabasal cells of A. Note the perinuclear expression (arrow) and the more random localisation (arrowhead). Bars are 10 μm.

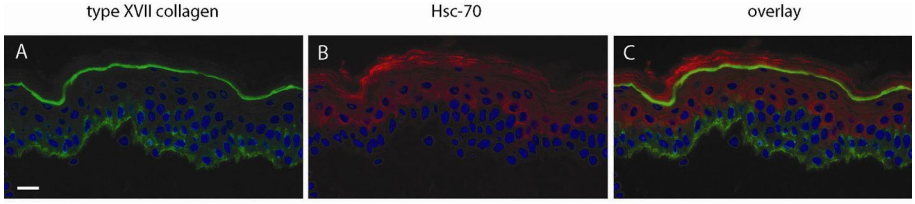

Figure 9. Double immunofluorescence staining shows that type XVII collagen expression is only present in non-differentiated keratinocytes in suprabasal layers. Normal human skin was stained with monoclonal antibody 1A8C against type XVII collagen (in green) and a monoclonal against Hsc-70 (in red), that is specifically expressed in differentiated keratinocytes. (**A**) Strong expression of type XVII collagen is observed similar as in Fig. 9. (**B**). Cytoplasmatic expression of Hsc-70. (**C**). Overlay of **A** and **B** shows that type XVII collagen is not expressed in cells that express Hsc-70 and vice versa. Bar is 10 μm.

The model attributes a crucial role to type XVII collagen in the assembly of hemidesmosomes. Supportive evidence comes from studies on HEB cells in which absence of either full-length or truncated type XVII collagen leads to disturbed expression of hemidesmosomal components[198,202]. For instance, BP230 expression was observed in the cytoplasm rather than at the cell membrane[202] and surface expression of integrin $\alpha_6\beta_4$ in these keratinocytes was half of the expression in healthy keratinocytes[198]. In these cells hemidesmosomes-like structures were formed but only re-expression of type XVII collagen led to incorporation of BP230 into these structures[198]. In line, deletion of the amino-acids 145-230 of type XVII collagen leads to strongly reduced incorporation of BP230 into hemidesmosomes[49]. When the interaction between type XVII collagen and integrin $\alpha_6\beta_4$ was prohibited, then type XVII collagen and BP230 still interacted. No clear hemidesmosome formation was observed, thus demonstrating that nevertheless integrin $\alpha_6\beta_4$ being indispensable for hemidesmosome formation, it is not needed for type XVII collagen-BP230 interaction[52].

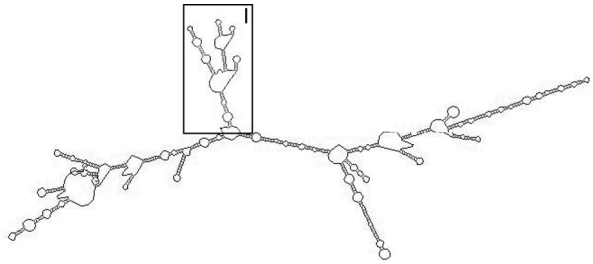

Figure 10. Schematic depiction of possible secondary structure of the long *COL17A1* 3'UTR variant.
Computer modelling was performed with Mfold software[203,204] and the structure with lowest free energy is shown. Box I depicts the conserved region spanning from nucleotides 4728-4797, which is located in the unique insert in the *COL17A1* 3'UTR. Nucleotide numbering is according to Guidice[4].

In summary, we demonstrate that the alternative 3'UTR splicing of *COL17A1* is, next to regulation of mRNA stability, also important for the localization of the mRNA. It underlines the importance of 3'UTRs in localization of mRNA transcripts. The observation that the perinuclear localization of type XVII collagen is determined by its long mRNA transcript only may be of help in determining the sequence of incorporation of the individual components into the hemidesmosome. Furthermore, we show that both *COL17A1* mRNA and type XVII collagen are also found in non-differentiated suprabasal keratinocytes. This strengthens the assumption that type XVII collagen has other functions than solely anchoring the basal cells to the basement membrane.

ACKNOWLEDGEMENTS

The antibody 1A8C against an intracellular epitope of type XVII collagen was a generous gift from Dr. K. Owaribe, Nagoya, Japan

CHAPTER 5

EFFECT OF NONSENSE MUTATIONS ON *COL17A1* mRNA TRANSCRIPT LEVELS IN KERATINOCYTES OF HEMIDESMOSOMAL EPIDERMOLYSIS BULLOSA PATIENTS

Sebastiaan van Zalen, Anna M.G. Pasmooij, Miranda Nijenhuis, Marcel F. Jonkman, and Hendri H. Pas

Submitted

ABSTRACT

Hemidesmosomal Epidermolysis Bullosa (HEB) is an inherited bullous skin disorder characterized by diminished epidermal-dermal adhesion. The clinical phenotype includes generalized skin blistering, dental anomalies, universal alopecia, and nail dystrophy but the severity may vary between individual patients. The underlying defect is reduced expression of either type XVII collagen or laminin 5 due to mutations in their coding genes.
Most of the reported mutations in the *COL17A1* gene result in premature termination codons (PTCs). These mutations lead to reductions of the *COL17A1* mRNA transcripts probably due to nonsense mediated mRNA decay (NMD).
In this study we investigated the actual amount of decay of the two major alternative type *COL17A1* mRNA transcripts in keratinocytes of seven HEB patients that had different mutations and compared this to the expression level of type XVII collagen protein and the clinical phenotype. All mutations, irrespective their nature or the presence of in-frame skipping of the mutated exon, caused a severe reduction of mRNA expression that was lower than in general observed in other genetic diseases. No correlation between the phenotype and the amount of decay was detected. Cycloheximide blocking of translation resulted in increased expression of *COL17A1* mRNA transcripts in line with decay due to NMD. Moreover, we found *in vivo* evidence for known *in vitro* NMD mechanisms. Interestingly in two patients that had one PTC containing exon removed through alternative splicing we did not find increased mRNA levels. We conclude that other than NMD mechanisms must also be active in keratinocytes of these patients.

INTRODUCTION

Mutations in the *COL17A1* gene lead to Hemidesmosomal Epidermolysis Bullosa (HEB), a subset of bullous genodermatoses characterized by generalized blistering from birth, alopecia of scalp, absence of body and secondary hair, nail dystrophy, and tooth enamel hypoplasia[32,33,88,97,98,132]. *COL17A1* codes for type XVII collagen: a transmembrane hemidesmosomal adhesion molecule. Hemidesmosomes are essential structures of the basal epithelial cells that anchor these to the underlying basement membrane matrix[129].

The type XVII collagen protein consists of 155 kDa of amino acid sequence, contains extracellular carbohydrate moieties and can be phosphorylated[15]. It has a type II transmembrane orientation, thus with a COOH-terminal ectodomain and an NH_2-terminal cytoplasmic domain. The ectodomain contains 15 collagenous sub-domains (COL1-COL15) by which the molecular tail folds into a triple-helical structure[4]. In addition, a second soluble form of 120 kDa is found, which consists of the cleaved ectodomain[19].

The *COL17A1* gene is located on the long arm of chromosome 10, in band 10q24.3. It spans 52 kb of genomic DNA and contains 56 exons that finally lead to a coding sequence of 4491 nucleotides[2-5]. Differential splicing of exon 56 results in two alternative transcripts that differ 610 nucleotides in length in the 3'Untranslated Region (3'UTR)[12]. At the 5' end six different 5'UTRs are found of which two are major (Van Zalen *et al*, in press).

Most of the reported mutations in *COL17A1* are either nonsense or indel mutations that both lead to premature termination codons (PTCs)[98]. These mutations lead to different phenotypes varying between severe and very mild. The severe phenotype includes generalized blistering with alopecia (generalized atrophic benign epidermolysis bullosa, GABEB), whereas the mild phenotype (localized atrophic benign epidermolysis bullosa, LABEB) is limited to blistering of hands, feet, and face and does not display alopecia (Pasmooij *et al*, submitted). The milder phenotypes (LABEB) are especially seen when PTCs are removed by in-frame skipping of the mutated exon, which then results in the rescue of a shorter, but still partly functional type XVII collagen protein[105,107].

Missense mutations may lead to different degrees of mild phenotypic effects depending on the functional importance of the changed amino acid[108,205]. Examples of less milder phenotypes are amino acid substitutions in the collagenous domain that interfere with correct folding of the triple helix. Incorrect folding of the helix leads to non-specific degradation of the mutant type XVII collagen and abnormal dentition[102-104].

PTCs in *COL17A1* lead to lower levels of *COL17A1* mRNA[205,206]. These lower levels of *COL17A1* mRNA are thought to be the result of nonsense-mediated mRNA decay (NMD)[207]. This is emphasized by the up-regulation expression of NMD involved genes in HEB keratinocytes[101].

However, hardly any data are available on the actual contribution of NMD to the decay of *COL17A1* mRNA in relation to the specific mutation and the phenotype of the patient. NMD is an mRNA-degradation process that specifically allows for the removal of PTC-containing mRNA transcripts. This prevents truncated proteins from being formed, and precludes possible dominant-negative effects of such truncated proteins.
NMD is both a splicing and a translation-dependent event since the NMD pathway is linked to the splicing dependent deposition of a protein complex (exon junction complex; EJC) at 20-24 nucleotides 5' of each exon-exon junction on the coding mRNA[208-210].
This EJC consists of several proteins that are loaded onto the mRNA in the nucleus, and then removed in the cytoplasm by ribosomes during the pioneer round of translating[211]. However, if a PTC is located more than 55 nucleotides upstream of the last EJC this results in the retention of the EJC which then triggers the NMD response[212-214].
In this study we investigated in HEB patients the decay of the *COL17A1* mRNA transcripts by real-time PCR and Northern blotting, and correlated the results with the clinical phenotypes and the expression of the type XVII collagen protein.

MATERIAL AND METHODS

Patients

We used cultured keratinocytes of seven HEB patients that all had mutations leading to PTCs on both alleles (Table 1). For details we refer to the same patient code in Pasmooij *et al* (submitted).
Patient EB 086-01 was an 11-week-old child with LABEB, who was compound heterozygote for the mutations 1877-2A>C and 3432delT[106]. The 1877-2A>C mutated allele was rescued by outsplicing of the whole exon 22 and of part of exon 22 that both restored the reading frame. The resultant proteins were slightly shorter and Western blot demonstrated in cultured cells a protein level of 4-5% compared with normal cells. Electron microscopy showed intra-epidermal split-level very low in the basal keratinocytes.
Patient EB 098-01 was a 36-year-old male with LABEB, who was compound heterozygote for the mutations 2356C>T and 3432delT[105]. The 2356C>T allele was rescued by outsplicing exon 30 resulting in a 1 kDa shorter protein. Protein level in cultured cells was 15% of normal healthy keratinocytes.
Patient EB 035-01 was a 35-year-old male with GABEB, who was compound heterozygote for the mutations 2342delG and 3781>T[165]. The protein level in keratinocytes was below detection limits.

Table 1. Patient mutations

#	*Sex*	*Phenotype*	*Mutation at DNA level*	*Resulting mRNA*	
EB 086-01	F	LABEB*	1877-2A>C/3432delT	in-frame exon skip/PTC	Pasmooij *et al*, 2004 a
EB 098-01	M	LABEB	2356C>T/3432delT	in-frame exon skip/PTC	Pasmooij *et al*, 2004 b
EB 035-01	M	GABEB**	2342delG/3781C>T	PTC/PTC	Scheffer *et al*, 1997
EB 011-01	M	GABEB	2342delG/2342delG	PTC/PTC	Scheffer *et al*, 1997
EB 117-01	F	GABEB	3236delC/3236delC	PTC/PTC	Pasmooij, submitted
EB 093-01A***	M	GABEB	3781C>T/4425insC	PTC/PTC	Pasmooij *et al*, 2005
EB 026-01A	F	GABEB	1706delA/3781C>T	PTC/PTC	Jonkman *et al*, 1997
EB 026-01B	F	revertant	Gene conversion/3781C>T	wild type/PTC	Jonkman *et al*, 1997

* localized atrophic benign epidermolysis bullosa
** generlized atrophic benign epidermolysis bullosa
***no revertant cells were available for cultures.

Patient EB 011-01 was a 40-year-old male with GABEB, who was homozygous for the 2342delG mutation. As patient No. 1 in the original paper on type XVII collagen deficiency he had severely reduced long and short *COL17A1* RNA variants by Northern blot, and no detectable type XVII collagen protein by Western blot in extracts of cultured keratinocytes[132].

Patient EB 117-01 was a 42-year-old female with GABEB, who was homozygous for the 3236delC mutation (Pasmooij *et al*, submitted). She had the most severe phenotype of this series with universal alopecia and cutaneous squamous cell carcinomas in blistering areas.

Patient EB 093-01 was a 75-year-old male and compound heterozygote for 3781C>T and 4425insC. This patient appeared to be mosaic for type XVII collagen expression having the GABEB phenotype with in addition one little patch of sturdy healthy skin on the middle finger. Immunofluorescence demonstrated restored protein expression in several biopsies. The molecular basis for the repair of the different patches in this particular patient has recently been described by Pasmooij *et al*[112]. We obtained keratinocytes cultured from negative skin and these did not produce detectable type XVII collagen.

Patient EB 026-01 was a 27-year-old female with GABEB, and compound heterozygote for the mutations 1707delA and 3781C>T. This patient also was a mosaic patient with larger patches of normal looking skin on the arms and ankle. In such patches the type XVII collagen expression was restored and molecular analysis revealed that different patches had undergone repair by different mechanisms[112]. We have used two types of cultured cells for analysis, the negative cells and the cells that had the 1706delA mutation repaired by gene conversion[110].

Skin analysis by immunofluorescence demonstrated that patients EB 086-01 and EB 098-01 were positive, although reduced, for the intracellular 1A8C epitope, whereas patients EB 035-01, EB 011-01, EB 117-01, EB 093-01, and EB 026-01 were negative, with exception of the revertant patches in patients EB 093-01 and EB 026-01. The extracellular 233 and NCC-Lu-227 epitopes were present in all patients with the exception of patient EB 117-01.

Cell culture

The squamous cell carcinoma line UMSCC-22B, originally derived from a tumour of the hypopharynx, was cultured in DMEM (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (Cambrex, Verviers, Belgium), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) at 37°C with 5% CO_2[134]. Keratinocytes were cultured from punch biopsies from clinically unaffected skin. The epidermis was separated from the dermis after overnight incubation with 0.03% trypsin, 0.02% EDTA at 37°C for 10 minutes. The harvested keratinocytes were cultured under serum-free conditions using Keratinocyte-Serum Free Medium (SFM) (Gibco) according to Mitra and Nickoloff[135]. This medium was supplemented with 25 μg/ml Bovine Pituitary Extract (BPE) and 0.1 ng/ml Recombinant Epidermal Growth Factor (rEGF) (all from Gibco).
For translation inhibition experiments keratinocytes were treated with 10 μg/ml cycloheximide (Sigma, St. Louis, MO) for six hours. Thereafter, cells were harvested by extraction with 1 ml Trizol/25 cm^2 (Invitrogen, Carlsbad, CA).

mRNA isolation

Cells were grown to sub-confluence and then harvested by extraction with 1 ml/25 cm^2 Trizol reagent (Invitrogen). Chloroform was added and after centrifugation the total RNA was precipitated from the aqueous phase with isopropyl alcohol. Then the poly(A^+) mRNA was purified from this with the mRNA isolation kit from Roche (Mannheim, Germany) that works with poly(dT)-conjugated magnetic beads. This last step was performed twice to ensure that the mRNA was extremely pure for use in Northern blotting experiments.

Real time PCR

Two-hundred nanogram total RNA was used for synthesis of first-strand cDNA with SuperScript III RNase H minus reverse transcriptase (Invitrogen) in a 20 μl final volume containing 300 ng of random hexamers (Invitrogen) and 40 units of RNase OUT (Invitrogen).
Combinations of unlabeled PCR primers and Taqman® minor groove binder (MGB) probes (FAM™ dye-labeled) for use in real-time RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands). The targets included the housekeeping gene β-actin (assay ID: Hs9999903), all *COL17A1* transcripts (Hs00166711), the short variant *COL17A1* transcript (Hs00996062), and the long variant *COL17A* transcript. This latter set was specially developed by Assay-on-Design of Applied Biosystems against the unique long variant 3'UTR sequence.
For each gene the final concentration of primers and MGB probes in TaqMan PCR MasterMix (Applied Biosystems) was respectively 900 and 250 nM, and 1 μl of cDNA was

added to the PCR-mix. TaqMan real-time RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Amplification was performed with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15s at 95°C and 60s at 60°C. Triplicate real-time RT-PCR analyses were executed for each sample. Threshold cycle values (C_t) were determined with the help of SDS2.2.2 software and were averaged for each sample. The two *COL17A1* mRNA variant specific primers sets were tested for non-specific annealing and amplifying by real-time RT-PCR analyses of pCR4-vectors (TOPO™ TA cloning kit, Invitrogen) in which either one of the two 3'UTR variants of type XVII collagen was cloned. The primer set specific for the longer 3'UTR variant was not able to detect any plasmid containing the shorter 3'UTR variant and *visa versa*, indicating the specificity of the two primer sets.
Vandenbroecke *et al* proposed to establish standard curves based on plasmids containing appropriate inserts in order to validate the absolute mRNA concentrations when comparing expression splice variants[215]. To establish standard curves for the type XVII specific primer sets we cloned three type XVII collagen PCR-products, that contained the appropriate target sequences, into separate pCR4-vectors. Real-time RT-PCR of ten-fold serial dilutions of these vectors with the respective primer sets resulted in reliable standard curves. With the help of these standard curves we were able to calculate absolute mRNA concentrations in keratinocytes of HEB patients. mRNA concentrations in keratinocytes of three different healthy donors were averaged and used as reference for *COL17A1* mRNA expression in each real-time RT-PCR experiment.
An alternative method to determine relative expression of mRNA transcripts is to use the δC_t:δC_t= C_t(type XVII collagen variant)- C_t(β-actin). Relative expression of each variant is determined by the inverse of the quotation: $E= 2^{-\delta Ct}$. Expression levels calculated in this way did not significantly differ from values interpolated with standard curves (data not shown).

RNA probes

Digoxigenin (DIG)-labeled antisense RNA probes were synthesized using the DIG-Labeling mix (Roche) according to the manufacturer's protocol with some minor modifications as described below. DNA fragments were generated by PCR on SCC cDNA that was produced by transcribing SCC mRNA with Superscript reverse transcriptase (Invitrogen). PCR products were cloned into vector pCR4 (Invitrogen). Plasmids were linearized with restriction enzymes NotI or BcuI (MBI Fermentas, Vilnius, Lithuania), depending on the RNA polymerase, respectively T3 or T7 (Fermentas), that was used in the subsequent transcription reaction. Probes were then generated by *in vitro* run-off transcription on linearized plasmid. For detection of *COL17A1* mRNA two antisense probes were used (location 230x626 and 3679x4131; numbering according to Giudice[4]) and for detection of β-actin one probe was used (location 915-1336; numbering on basis of Genbank accession number NM101001)

Northern blotting

Four-hundred ng samples of mRNA were electrophoresed on 6,6% formaldehyde-1,2% agarose gel and capillary transferred to a positively charged Nylon Membrane membrane

(Amersham Biosciences, Braunschweig, Germany) in 10xSCC (1.5 M NaCl, 150mM NaCitrate, pH 7.0). The mRNA was covalently immobilized to the membrane by 5 minutes 304 nm UV-cross-linking. Next, the membrane was hybridized overnight with the DIG-labeled RNA probes at 68˚C in DIG Easy Hyb buffer. The membrane was washed two times with 2xSCC+0,1% SDS at room temperature followed by two more stringency washes with 0,5xSCC+0,1% at 68˚C. Next the membrane was incubated with anti-DIG-AP F_{ab} fragments 1:20,000 in blocking solution. Chemiluminescence was developed by CPD-star 1:100 in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) and luminescence was detected by exposing the membranes to Lumi-Film Chemiluminescent Detection film. To check for equal loading of mRNA a probe against β-actin was co-hybridized. All reagents used in the incubation and signal development were purchased from Roche.

RESULTS

mRNA expression in HEB keratinocytes

Keratinocytes of all HEB patients had strongly reduced *COL17A1* mRNA levels with the exception of revertant keratinocytes of patient EB 026-01 (Fig. 1A). The reduction was over twenty-fold in HEB keratinocytes compared to wild type *COL17A1* mRNA expression with the exception of patient EB 093-01. Keratinocytes that had nonsense mutations located further upstream of the last exon-border tended to have stronger reductions of mRNA expression (Fig. 2A). However, this correlation was not statistically significant.
Surprisingly, the keratinocytes of patients EB 086-01 and EB 098-01 with LABEB only had slightly higher mRNA expression levels than the type XVII collagen negative cells of the other patients, although still significant ($P<0.05$ for all primer sets). We had expected much higher values considering the in-frame skipping of the mutated exon, and the production of shortened, but partial functional type XVII collagen in cultured cells and in skin in vivo.

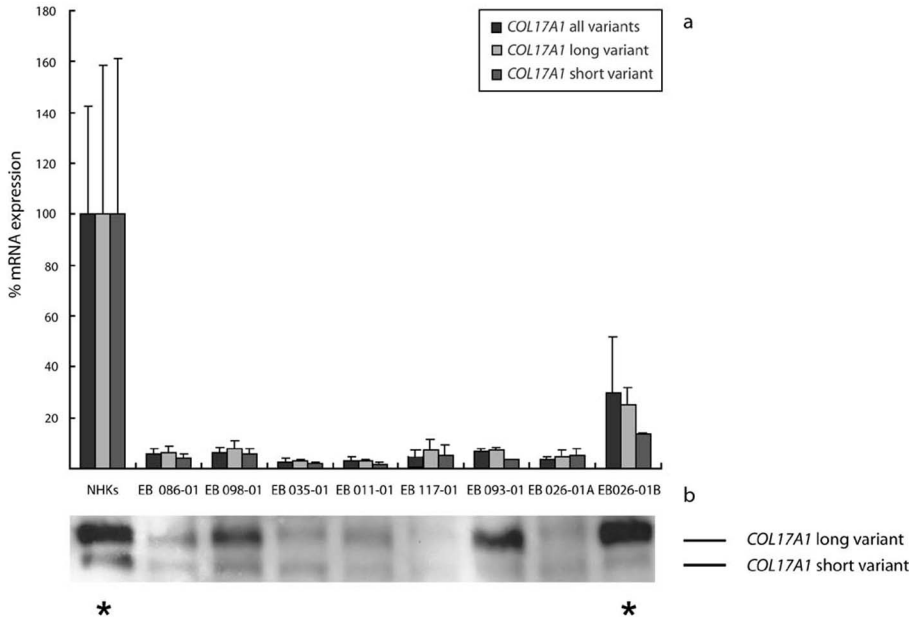

Figure 1. *COL17A* mRNA expression in patients' keratinocytes
(A) mRNA expression of the two major *COL17A* transcripts in HEB cells is severely lower than *COL17A1* mRNA expression in normal keratinocytes. Expression of *COL17A1* mRNA is determined with real time RT-PCR and is corrected for ß-actin mRNA expression and is expressed as percentage compared to normal human keratinocytes for each primer set that is set as 100%. Data are the mean and standard deviation of at least two independent experiments and in each experiment keratinocytes of three healthy donors were used. Real time RT-PCR experiments were performed on total RNA, since control experiments showed no difference in expression when total RNA was used in stead of mRNA.
(B) Northern blotting of *COL17A1* mRNA of HEB keratinocytes confirms the decay observed in the real-time RT-PCR techniques. Anti-sense ß-actin probe was co-hybridized to control for mRNA loading (not shown). *In lanes NHKs and EB 026-01B 100 ng mRNA is loaded while all other lanes contain 400 ng mRNA. One representative experiment out of three is shown and in this experiment keratinocytes of one healthy donor were used.

The type XVII collagen negative cells of patient EB 093-01 showed a relatively higher level mutated *COL17A1* mRNA, although a 14-fold reduction in contrast with NHKs was still observed. As this patient was mosaic we took special care to assure that no contamination with revertant cells had occurred. Neither immunoblot analysis of cells, nor immunofluorescence staining of biopsies, nor sequence analysis of the cDNA batch used in the real time PCR experiments did give any evidence of contamination.

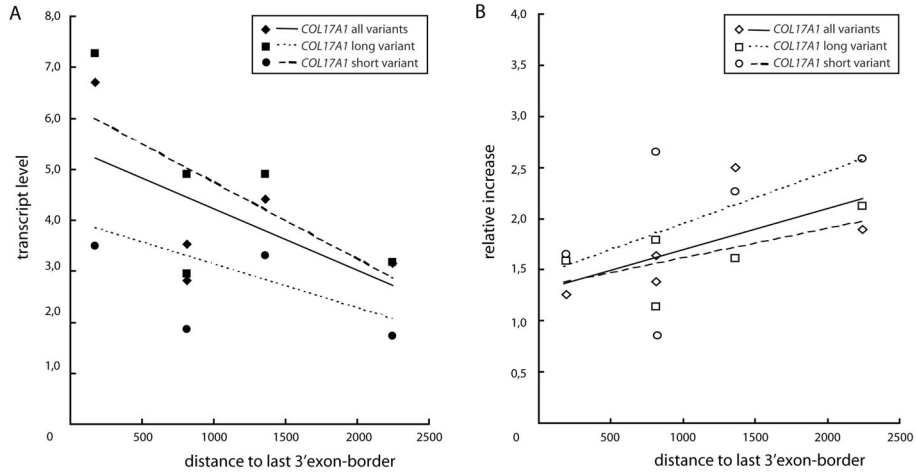

Figure 2. NMD response in GABEB patients cells tend to be stronger as the distance between the PTC and the 3'most exon increases.
(**A**) Patient mRNA levels plotted against the distance to the last exon-border for the most downstream PTC of the respective patient. Diamonds represent expression level of all *COL17A1* variants, squares the long variant level and circles the short variant levels. Lines are trend lines. (**B**) Raise of transcript levels after cycloheximide treatment as function of the distance between the most downstream PTC and the last exon-border. Symbols are similar as in A. Lines are trend lines.

As an independent control on our quantitative PCR data we also analyzed the mRNA level by Northern blotting, which confirmed the relative differences in expression levels between the different patient keratinocytes (Fig. 1B).
The short variant *COL17A1* mRNA transcript was relatively more reduced in patient cells than the long variant mRNA (mean value 3.7 versus 5.4). Student's t-test revealed that this difference was significant ($P<0.01$). This was also demonstrated by the fact that the ratio, between the longer and the smaller variant, was significantly higher in keratinocytes of HEB patients compared to normal keratinocytes (Fig. 3).

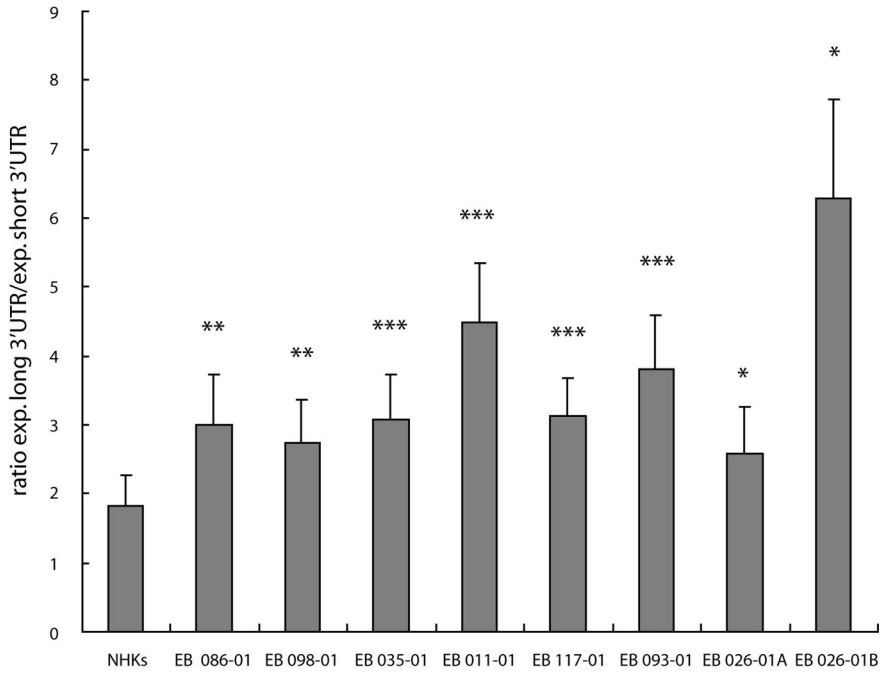

Figure 3. Different decay of the long and short variant *COL17A* mRNA.
Ratio of expression between the larger and the shorter *COL17A1* mRNA transcript is larger in keratinocytes of HEB patients in comparison with NHKs. *P<0.05, **P<0.01, ***P<0.001 compared to NHKs with the help of Student's t-test for independent samples, two-tailed. Expression is determined on basis of real time RT-PCR experiments and is corrected for ß-actin mRNA expression. Data are the mean of at least two independent experiments and in each of these experiments keratinocytes of three healthy donors were used.

A prerequisite for the execution of NMD is that PTCs must be located more then 50 nucleotides upstream of the last exon border[213,216]. In all patients' keratinocytes mutations were located more then fifty nucleotides 5' of the last exon border of the both *COL17A1* mRNA transcripts (Table 2).

COL17A1 mRNA decay is caused by nonsense mediated mRNA decay

To investigate whether mutant *COL17A1* mRNA decay is under NMD control we treated control and HEB keratinocytes with cycloheximide. This inhibits the NMD machinery by blocking general protein translation and thereby it suppresses NMD decay. Consequently those transcripts under NMD control will demonstrate increased levels.

Table 2. Distance of each PTC to last 3'exon-border and *COL17A1* mRNA expression levels.

mRNA concentrations of patients were compared to the expression in normal healthy keratinocytes for both primer sets and expression was presented as a percentage compared to healthy keratinocytes, which was set at 100% for both primer sets. Expression data are the mean of at least two independent experiments.

Pat#	*distance in nucleotides 5' to the last exon-border*	*% relative expression long transcript*	*% relative expression short transcript*	*n*
EB 086-01	1164	6.44	4.29	4
EB 098-01	1164	7.93	5.83	4
EB 035-01	2245/815	2.95	1.87	3
EB 011-01	2245/2245	3.18	1.74	2
EB 117-01	1360	4.90	3.31	3
EB 093-01	815/171	7.26	3.50	2
EB 026-01A	2890/815	4.91	4.98	3
mean ± 2sd	-	5.37 ± 3.86	3.65* ± 3.05	
EB 026-01B	-	24.89	13.42	1
NHK	-	100	100	4

*P< 0.01 compared to the relative expression of the long transcript with the help of Student's t-test for paired samples, two-tailed

Treatment of HEB keratinocytes with cycloheximide indeed led to an increase of *COL17A1* mRNA transcripts in HEB keratinocytes, indicating that *COL17A1* mRNA transcripts containing a nonsense mutation are under control of NMD (Fig. 4). In contrast, *COL17A1* mRNA transcript levels in normal keratinocytes slightly decreased after cycloheximide treatment.

This increase was observed in all HEB keratinocytes and for four patients the increase of expression of the short variant was statistically significant. For two patients we could not determine whether the increase was significant due to shortage of patient material. For no obvious reason, in patient EB 026-01 hardly any increase was seen.

Overall the extent of increase tended to be higher in keratinocytes that had nonsense mutations further upstream of the last exon-border (Fig. 2B). Also this correlation was not statistically significant. When comparing the effect of the cycloheximide on the long and short variant mRNA the average increase of the smaller transcript was significantly (P= 0.05) higher (2.3) than that of the larger transcript (1.7).

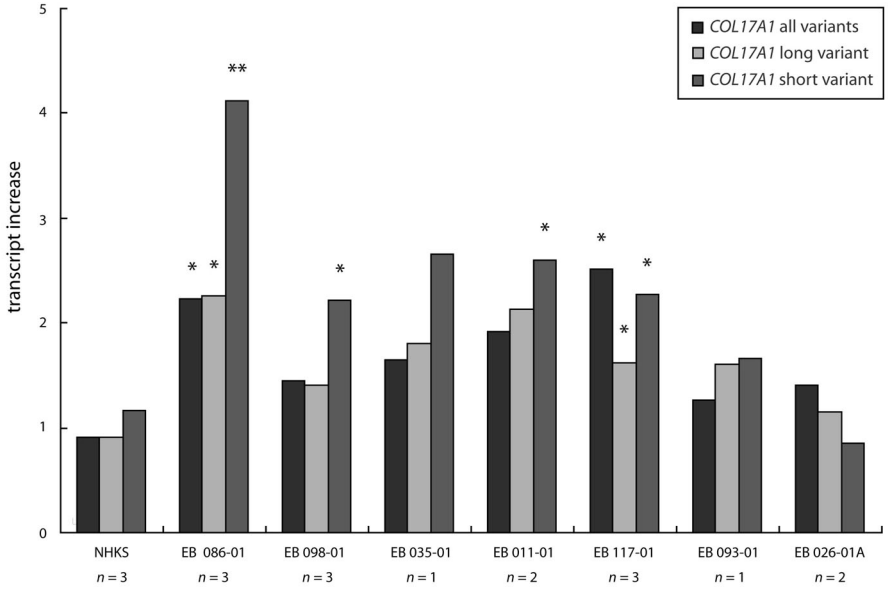

Figure 4. *COL17A1* mRNA levels are elevated after cycloheximide treatment.
Induction of expression is observed in HEB keratinocytes after blocking of protein translation with cycloheximide. Induction is expressed by *COL17A1* mRNA expression after treatment divided by *COL17A1* mRNA expression in keratinocytes of the same patients that were not treated, for each primerset. mRNA expression was determined with real-time PCR experiments on total RNA and is corrected for ß-actin mRNA expression *P<0.05, **P<0.01 compared to untreated keratinocytes with the help of Student's t-test for paired samples, one-tailed.

DISCUSSION

In this study we demonstrate that nonsense mutations in the *COL17A1* gene lead to severely low levels of *COL17A1* mRNA transcripts. Surprisingly, reading frame rescue through outsplicing of the PTC-containing exons does not result in much higher levels.
Most mRNAs bearing PTCs are degraded by nonsense mediated mRNA decay (NMD), although some PTCs can escape NMD[217.] NMD is both a splicing and translation-dependent process, which involves a cascade of proteins[208]. Inhibition of the translation turns of the NMD pathway, which results in an elevated expression of those mRNA transcripts subject to NMD. When we treated cells with cycloheximide we indeed observed in all HEB keratinocytes raised *COL17A1* transcript levels, while transcript levels of normal human keratinocytes slightly decreased.

This latter effect may be due to the fact that cycloheximide is a broad-spectrum inhibitor of protein translation, which not only blocks the NMD machinery, but also may influence the transcription of mRNA or regulation of mRNA stability along treatment. So far, elevated expression of PTC bearing transcripts after treatment with non-specific inhibitors of protein translation have been considered as proof of principle for NMD. However, specific inhibition of the main components of the NMD machinery will provide better insight in the actual contribution of NMD on the decay of PTC bearing transcripts.
The data in this study thus establish that the *COL17A1* transcripts are subject to NMD. The reduction in the mRNA levels is remarkably lower than those described for PTC bearing transcripts of most other proteins[217-219]. So far, only a minority of nonsense mutations with severely low mRNA levels as our patient's *COL17A1* mRNA levels have been reported. These were observed for genes with programmed DNA gene rearrangements, such as TCR-β and immunoglobulin genes and also few mutations in other genes showed comparable severe decay. In the TCR-β gene a uncharacterized down-regulatory-promoting element (DPE) is hypothesized to be responsible for this strong down-regulation of PTC-containing transcripts[220]. PTCs in the *COL10A1* gene cause, as expected, NMD in cartilage tissue. However, in non-cartilage tissue no NMD was observed, indicating that NMD can be under tissue control[221]. The severe decay of PTC-bearing *COL17A1* transcripts may suggest that NMD is especially strongly elicited in skin. However, in contrast, other PTC-containing transcripts of skin expressed proteins showed only moderate mRNA decay denying this suggestion[222,223].
Among the seven HEB patients, two patients with the LABEB phenotype had the reading frame restored through removal of the PTC containing exon in one allele. These patients cells produced type XVII collagen, as witnessed by IF antigen mapping of the skin (Pasmooij *et al,* submitted), and by Western blot, albeit at a considerable lower level than normal. Nevertheless, this was not reflected in much higher mRNA expression levels compared to the GABEB patients since only slightly, although significant, higher values were found. We expected expression levels up to 50%, since NMD should only effect the mutated allele here. Only in the reverted cells of patient EB 026-01 we did observe such a higher level, but this patient had a repaired allele that contained again the wild-type sequence. However, the correction of one allele only restores 25% of mRNA production of two wild type alleles. This meagre result agrees with the observation of Pasmooij *et al*, that an hemizygous *COL17A1* status such as in LABEB patients, heterozygous carriers, and in revertant mosaicism, effects the distribution of type XVII collagen in the skin: the apical-lateral type XVII collagen staining of basal cells is lost (Pasmooij *et al*, submitted).
We do not know what mechanism is responsible for the exon-splicing in patients EB 086-01 and EB 098-01 with LABEB, although we think nonsense associated altered splicing (NAS) is the most probable candidate. The presence of a mutation, which may effect splicing in both patients, is in favor of this hypothesis[105,106]. Similar

to NMD, also NAS is elicited by PTCs. Both mechanisms are supposed to operate independent of each other[224,225]. The low *COL17A1* mRNA expression in these patients is in line with the independence of NAS and NMD with each other, since despite the strong NMD response, expression of type XVII collagen is seen, as shown by expression of the epitopes 1A8C and 233[105,106].

Both alternative 3'UTR transcripts, the long and the short variant, were subject to breakdown, with the short variant decaying to a lower relative level than the long variant. On the average the long variant was present at 5.4% of the normal keratinocytes value while the short variant decayed to an average of 3.7% of normal. In addition, the cycloheximide effect on the *COL17A1* mRNA expression was significantly stronger on the shorter transcript, thus indicating that it is more subject to NMD. The reason for this remains unclear but we suggest that sequences of the *COL17A1* 3'UTR may be involved in the NMD process, since this is the only difference between the long and the short *COL17A1* transcripts.

When we look at GABEB patients' steady state mRNA levels and the increase in level after cycloheximide treatment we see that these levels and the distance of the most downstream PTC to the last exon-border tend to correlate. As the distance upstream to the last exon-border increases the steady state levels decrease while the effect of the cycloheximide blocking increases. A possible cause is a phenomenon known as polar effect: nonsense mutations close to the last 5' exon border cause less decay of the resulting mRNA in comparison with PTC further upstream of this last exon border[226]. In support of a polar effect, in sequence analysis as part of mutation screening in patient EB 093-01 was observed that the mRNA transcript containing the more upstream PTC was more decayed than the other transcript. Thus, a polar effect on NMD decay of mutated *COL17A1* mRNA may be suggested although our patient group is too small to draw a final conclusion.

We did not find a correlation between the location of the mutations and the severity of the symptoms in our patients, since in keratinocytes of patient EB 117-01, who had the most severe symptoms, no lower *COL17A1* mRNA levels were observed. Such a location dependency has been observed in other genetic deficiencies. The location of nonsense mutations in the cystic fibrosis gene, CFTR, influenced the severity of the disease[227,228]. Similar findings were found for the *COL7A1* gene by Tamai *et al* who observed a correlation between mutation location and severity in Japanese Dystrophic Epidermolysis Bullosa (DEB) patients. In the three cases described the symptoms seemed to depend on the distance between the mutation and the last 3'exon border[229]. Other authors suggested that NMD could also be dependent on locations of individual PTCs. Ishiko *et al* described a Japanese milder DEB case that did not fit the suggestion made by Tamai *et al* [230]. And thus is suggested that the sequence surrounding the PTC may also be of importance[219,227,231].

We conclude here that in HEB the best correlation with the clinical phenotype is found with the expression of the type XVII collagen protein as witnessed by immunofluorescence antigen mapping. Our two LABEB cases had a residual staining of the 1A8C epitope, which is proof of presence of the full-length molecule,

since the 1A8C epitope is found on the intracellular domain. In the four patients with the GABEB phenotype the 1A8C epitope could not be demonstrated probably due to the concentrations of the full-length molecule being below the detection limit. They however must have a low synthesis level since in contrast with the 1A8C epitope the 233 epitope was clearly present. The 233 epitope is found on the extracellular domain (amino acids 1118-1143[232]) and thereby reflects the sum of the full-length and the soluble 120 kDa form. In these patients most of the type XVII collagen is apparently present as the soluble 120 kDa molecule. If the synthesis level drops further and becomes that low that also the 233 epitope is lost we find the more severe phenotype as in patient EB 117-01.
In summary, PTCs in *COL17A1* mRNA lead to lower transcript levels than observed in most other genetic diseases. Although we confirmed NMD to be involved in mRNA decay, more important we also demonstrated that other mechanisms must be active. The mRNA level is no indication of actual protein synthesis as no correlation between transcript level and protein level can be demonstrated. These data are of interest for *COL17A1* aimed gene therapy of HEB patients, and for further research on mechanisms of mRNA decay.

CHAPTER 6

SUMMARY, GENERAL DISCUSSION, AND FUTURE PERSPECTIVES

Sebastiaan van Zalen, Marcel F. Jonkman, and Hendri H. Pas

Overview

The aim of this thesis was to expand the basic knowledge on the transcription and translation of type XVII collagen. As has been extensively pointed out in the introduction of this thesis, type XVII collagen is an important molecule in epithelial tissues, and pathogenic processes that effect type XVII collagen function lead to major health problems. Type XVII collagen appears to be heterogeneous at both the mRNA transcript and the protein level. Considering the participation of these alternative molecules in normal homeostasis and in pathogenesis, and also in the light of increasing chances of successful gene therapy, the lack of knowledge on the generation and function of these alternative molecules is surprising.
In this thesis we therefore focused on characterisation of both the 5' and the 3' Untranslated Regions (UTRs) of the *COL17A1* mRNA and their possible involvement in translation. Furthermore we investigated the influence of nonsense mutations on the decay of mRNA and expression of type XVII collagen in Hemidesmosomal Epidermolysis Bullosa (HEB) patients. In this last chapter we will summarise our results and we will discuss the implications of our findings for further studies on type XVII collagen.

Summary

In **chapter 1** we started with making an inventory of type XVII collagen directed research during the last three decades; on its role in normal healthy tissue and even more on its role in the pathogenesis in a range of diseases. We concluded that, although these studies have provided much information about type XVII collagen, certain aspects are still misunderstood. Most studies confirmed the main function of type XVII collagen: anchoring of the basal keratinocytes to the basement membrane. Several observations, however, suggests that type XVII collagen may have other additional functions as well. Also fundamental information on the transcription of the *COL17A1* gene and the translation to type XVII collagen is lacking.
Main enigmas include the unknown function of the shedding of type XVII collagen, the deregulated expression of type XVII collagen in squamous cell carcinogenesis, and the function of the alternative splicing of the *COL17A1* 3'Untranslated Regions (UTR). Considering the established involvement of the untranslated regions of mRNA in various protein expression related mechanisms, we choose to mainly focus on exploring the function the 3'UTR and the primary characterisation of the still unknown 5'UTR. By studying *COL17A1* transcript levels in Hemidesmosomal Epidermolysis Bullosa (HEB) patients with various phenotypes, we tried to find answers on what transcript levels are minimally needed to provide good anchoring of the epidermis and what is the relation of the mRNA level with the HEB skin phenotype. These answers will also be important for studies aimed at gene correction as therapy for Epidermolysis Bullosa.
In **chapter 2** we characterised the *COL17A1* 5'UTR and we demonstrated that the 5'UTR is, like the 3'UTR, also alternatively spliced. RACE and RPA experiments demonstrated the presence of six different 5'UTRs, all with different start points, of which two major transcripts accounted for 75% of the total *COL17A1* expression. The finding of two major transcripts also at the 5' end raised the question if each of the major 5'UTR was connected to a particular 3'UTR. In long template PCR experiments this hypothesis could not be confirmed, so we conclude that both 5' ends are equally shared by the two alternative 3'UTRs. Moreover, motif analysis of the sequence upstream of the translation start site showed the existence of several transcription motifs. These transcription motifs may be important in regulating type XVII collagen expression, not only in normal homeostasis but also under conditions where synthesis has to be shut down. For instance, when keratinocytes differentiate and leave the basal layer, and under conditions where increased expression –often accompanied by a strong cytoplasmic presence- is observed as in wound healing and carcinogenesis.
Chapters 3 and 4 were dedicated to the function of alternative splicing of the *COL17A1* 3'UTR. We investigated whether the alternative splicing is connected with the translation of the ORF, either in a quantitative way and/or in directing its subcellular localisation.

In the first of these two chapters we transfected several cell types with different luciferase-*COL17A1*-3'UTR constructs to investigate translation levels, and in separate experiments we blocked transcription to investigate transcript stability. We showed that when the long variant 3'UTR was cloned behind the coding region of a reporter gene increased reporter gene translation levels were observed in comparison to constructs containing the short variant 3'UTR. The mRNA stability experiments revealed that this is probably caused by decreased stability of the short variant transcript.
In the subsequent chapter we again transfected cells with *COL17A1*-3'UTR constructs, but now we used a GFP-reporter vector that enabled us to localise the subcellular site of translation products. Moreover, we investigated the subcellular location of original *COL17A1* transcripts in cultured keratinocytes and in skin by double fluorescent in situ hybridisation (FISH) experiments.
We demonstrated that GFP-protein translated from transcripts containing the long variant 3'UTR is mainly observed near the nucleus, whereas GFP-protein translated via the short 3'UTR transcript localises more randomly. Through double colour FISH we showed that it is the 3'UTR that is responsible for the targeting of the two major *COL17A1* mRNA transcript to distinct subcellular compartments as the distribution of these transcripts mimics the distribution of the GFP protein. We hypothesise that conserved stretches in the unique insert of the *COL17A1* 3'UTR are responsible for these mechanisms.
The conclusion arising from these experiments is that keratinocytes have the ability to shift type XVII collagen expression, both the level and the site of translation, by differential splicing of the *COL17A1* gene. In this way, cells may be able to alter type XVII collagen translation very specifically and such a mechanism may also be active in the shift in type XVII collagen expression observed in wound healing and carcinogenesis.
That type XVII collagen is translated at more than one location evokes the question whether the resulting populations of protein have different destinations, and what relation exists with the incorporation of the individual hemidesmosome components into the emerging hemidesmosomal complex.
In skin we found by FISH *COL17A1* mRNA expression in basal and first suprabasal layers. Visualisation of type XVII collagen by immunofluorescence confirmed that the protein was indeed found in basal and suprabasal keratinocytes. Besides a linear staining parallel to the basement membrane, we observed a granular pattern similar to that observed in our experimental cell studies. These granules are the first observations of the 'birth' of the type XVII molecule in skin. Due to the absence of hemidesmosomes in suprabasal layers the question is raised what function type XVII collagen has in the suprabasal layer.
In **chapter 5** we investigated the *COL17A1* mRNA levels in a panel of HEB patients whom all had mutations leading to PTC on both alleles. We also investigated if the lower transcript levels were due to nonsense mediated mRNA decay (NMD) by blocking the protein translation and thus the synthesis of NMD

components. *COL17A1* mRNA levels in keratinocytes of HEB patient appeared severely decreased, not only compared to normal keratinocytes, but also with other genetic diseases. Furthermore we observed a possible polar effect in NMD, and this is the first time this was demonstrated in a series of patients.
The most intriguing observation in this study was that keratinocytes of patients with the mild subtype of nH-HEB, localized atrophic benign Epidermolysis bullosa (LABEB), in which the PTC was removed by exon skipping, still had low *COL17A1* mRNA levels. Other mechanisms than NMD must thus be active since NMD is elicited by PTCs. Despite the low mRNA levels these patients had a mild phenotype. We concluded that the best way to predict the phenotype of an HEB patient is to examine the presence of the different epitopes of type XVII collagen. Patients, whom express the full-length molecule at a detectable level in skin, have milder phenotypes than patients with no detectable expression of full-length type XVII collagen. Loss of also the full-length epitopes leads to the more severe HEB subtype generalized atrophic benign epidermolysis bullosa (GABEB) phenotype.

General discussion and future perspectives

In **chapter 2** we demonstrated that the *COL17A1* 5'UTR shows extensive heterogeneity, which may well be important for regulation of type XVII collagen expression. 5'UTRs in general harbour several specialised features that may regulate translation, and these include length, uORG, uAUG, specific cis-acting sequences, and internal ribosome entry sites (IRES)[133]. The six *COL17A1* 5'UTRs differ in length and the alternative splicing thereby also determines the incidence of the predicted transcription motifs for each particular 5'UTR. This may be important for the regulating type XVII collagen translation.
A prominent role in gene expression regulation may be expected for the Sp1 and AP-1 motifs found in the promoter region of the *COL17A1* gene, since the expression pattern of AP-1 proteins changes when keratinocytes differentiate[233]. The c-Fos, Fra-2, and Jun D are found in the basal layer, whereas the other members of these families are absent here. Due to this altered distribution the keratinocytes can shift gene expression. It is not unthinkable that the Sp1 motifs in the *COL17A1* promoter region have a function in shutting down type XVII collagen expression when the keratinocytes leave the basal layer. Furthermore, AP-1 induced change of gene expression is speculated to be especially important when rapid action is required such as in the complex and dynamic process of wound healing[151]. Altered incidence of motifs due to altered 5'UTR splicing may then result in a shift of *COL17A1* gene expression. Although completely hypothetical, several observations support such an idea. First, increased expression of AP-1 proteins is seen in wound healing models as is upregulation of c-Fos in renal epithelium after damage[152-154]. Second, type XVII collagen expression is altered during wound healing and during

development of squamous cell carcinoma[124]. The coincidence of these observations needs further attention.
A first step to explore this possible regulation in more detail is to investigate the distribution of the *COL17A1* 5'UTR transcripts in wound healing models, in squamous cell carcinoma, and in other tissues. Also investigating *COL17A1* transcripts in suprabasal keratinocytes may help as we already saw that both *COL17A1* mRNA and type XVII collagen expression is changing during the differentiation process.
We already made a first attempt by performing quantitative PCR on both normal human keratinocytes and the UM-SCC22B cell line, and this taught us that the ratio of the two major 5'UTRs did not significantly differ as we also saw in the RPA experiments.
Moreover, the functional importance of the different lengths of the six *COL17A1* 5'UTRs and the putative transcription motifs should be investigated in reporter gene expression assays. However, this will not be easy since a major problem of such assays is the low transfection efficiency of primary keratinocytes, leading to probably small expression differences that are hard to interpret. Other cells like CHO-cells, which are much easier to transfect, may provide a reliable alternative.
Chapters 3 and 4 showed that the alternative splicing of the *COL17A1* 3'UTR has a functional meaning. Translation regulation via mRNA stabilization and subcellular localisation of mRNA transcripts has already been demonstrated for several other genes and in most cases the 3'UTR was involved[169-175,193,194,234]. Our findings again confirm the importance of 3'UTRs in regulating gene expression and show that for the *COL17A1* gene the 3'UTR is functionally an important region. As discussed before for the 5'UTR, the 3'UTR alternative splicing may also be involved in conditions of aberrant type XVII collagen expression. Here we suggest that attention in particular should be given to the observed shifts from membrane to cytoplasmic expression[57,120,123]. The finding that alternative splicing of the 3'UTR effects both the expression level and protein localisation easily leads to the idea that splicing controls expression level and localisation, and therefore the expression ratio of long variant and short variant could be investigated in carcinoma cells and in models of wound healing.
Preliminary investigations of these levels in a panel of carcinoma cell lines however demonstrated that in most carcinoma cells the ratio of the long versus short variant remains in favour of the long variant and in some cells even a strong increase of the long variant is observed (Table 1)

Table 1. Preliminary results on *COL17A1* mRNA levels in carcimoma cell lines.
Expression levels are expressed as percentage compared to expression of each transcript variant in normal healthy keratinocytes (NHKs). A total of 14 different cell lines was investigated by real time PCR (for experimental details, see chapter 5). Eight cell lines showed expression levels below 1% compared to NHKs and these were considered as negative.

Cell line	*All COL17A1-variants*	*Long COL17A1-3'UTR variants*	*Short COL17A-3'UTR variants*	*Ratio long variant/short variant*
NHKs	100	100	100	1. 84±0.43
A431[a]	27.9	27.0	25.1	2.3
HaCaT[b]	37.3	29.6	48.3	1.3
SCC 22B[b]	26.9	11.1	56.3	0.4
WiDr[c]	29.4	27.3	8.2	7.2
Glc-a2[d]	187.7	171.8	222.7	1.7
Glc-p1[b]	5.9	2.9	3.2	2.0
Hep-2[d]	2.9	4.4	2.4	4.1

Originally derived from: [a]Epidermoid carcinoma,[b]Squamous cell carcinoma [c]Colon carcinoma [d]Adenocarcinoma

Thus reciprocal ratios in comparison with NHKs are not an intrinsic characteristic of carcinoma cells and the shift observed in the UMSCC22B cell line remains a unique feature of this particular line. As Parikka *et al* described that the type XVII collagen expression fluctuates with the stage of the tumour, first decreasing in dysplasia and then strongly increasing with maturation[70], it may be more informative to study the transcript ratios by FISH on carcinoma tissue sections and subcellular type XVII collagen expression by immunofluorescence.

Further research concerning the mechanism of the 3'UTR regulation should include actual protein binding studies of *trans*-acting factors to the 3'UTR. The three conserved stretches in the *COL17A1* 3'UTR may narrow the search for binding of *trans*-factors, since conserved regions in 3'UTRs are well-known for their *cis*-activity[235]. Waggoner and Liebhaber investigated several thousands of mRNAs, including type *COL17A1* mRNA, for binding with the two major αCP-isoforms – well-known *trans*-acting factors- via a micro-array technique and they did not observe binding of these two to the *COL17A1* mRNA[236]. This does not totally preclude *COL17A1* mRNA from binding to αCP-proteins, since the approach in the study above was very strict and the CP-family contains other members[237].

Another candidate for binding to the *COL17A1* 3'UTR is annexin II that has been shown capable of binding mRNA via sequences in the 3'UTR and is required for cytoskeleton mediated transport in epithelial cells[193,238]. Furthermore, annexin II expression is observed at the cell periphery in all layers of the epidermis, except for the stratum corneum[239]. In *in vitro* cells some cytoplasmic expression is also seen. However, when annexin II will direct subcellular localisation of type XVII collagen, it should bind both *COL17A1* mRNA and type XVII collagen, since we propose that membrane bound type XVII collagen is translated perinuclear.

The data in **chapter 4**, in which we visualised perinuclear synthesis of type XVII collagen, may be used for further study of how individual components are incorporated into maturing hemidesmosomes. In fact our data show that it should be feasible to study this in skin sections instead of in cultured cell models, using specific antibodies to the separate proteins.
We also suggested a more important role for type XVII collagen in this process than originally thought. The current model assumes that integrin $\alpha_6\beta_4$ is the first component present, which is then followed by the other components[49]. In that model *in vitro* studies also ascribed a crucial role to plectin, but this could not be confirmed *in vivo*[60].
Our hypothesis could be tested by confocal microscopy in order to reveal the 3-dimensional location of the products of GFP-*COL17A1*-constructs. Additional studies should then include cloning *COL17A1* 5' terminal sequences, suggested to be interacting with BP230[53,197,198], before the coding region of GFP and then follow the fate of the produced GFP, preferably in a time frame.
From **chapter 5** it emerged that not only the NMD process is responsible for decay of mutated transcripts. Our data revealed that also non-PTC containing transcripts are subject to NMD. However, some type XVII collagen is translated from these transcripts, thus other mechanism must be present. Focusing on what mechanisms other than NMD are active in these two LABEB patients will broaden the understanding of mRNA decay.
The eventual polar effect we observed in our panel of seven patients should be confirmed in a larger series of patients in order to get actual proof of such a mechanism. The quantitative PCR method we used seems an appropriate technique to do so. In addition, in mutation analysis of cDNA of keratinocytes of heterozygous patients the distribution of both transcripts can be determined. This approach was already used in keratinocytes of patient EB 035-01 and the data here denied the existence of a polar effect; the transcript containing the more downstream PTC was subject to more breakdown than the other. Noteworthy, the PTCs in patient EB 035-01 are further upstream than in the first description of the polar effect in which only PTCs within 400 nucleotides of the last exon-border were introduced[226]. Therefore, the polar effect may only be effective on short distances to the last exon-border.
Moreover, we noticed that NHKs demonstrated intrinsic variation, even between keratinocytes of the same donor. This variation may be caused by the dynamic assembly and breakdown of the hemidesmosome[71]. Keratinocytes are able to incorporate type XVII collagen into the hemidesmosome in less than half an hour after breakdown of hemidesmosomes. Therefore, rapid upregulation of type XVII collagen might be necessary and this may be the cause of the intrinsic variation in NHKs.
We also saw that the short variant decayed to lower levels than the long variant. So far, we have no explanation for this discrepancy, so we can only suggest that the *COL17A1* 3'UTR effects the NMD mechanism. In yeast an element 3' downstream

of the PTC is required for execution of NMD, and NMD was triggered when a ribosome failed to terminate adjacent to a properly configured 3'UTR[240,241]. This suggest that 3'UTRs in general may influence the NMD response and, therefore, the alternative splicing of the *COL17A1* 3'UTR may be involved in regulation of the NMD response, although up to now no actual proof that human 3' sequences are involved in the NMD response has been presented.
Originally, it was thought that NMD was simple a way to remove mRNA transcripts that contained a PTC and our findings are another confirmation of this simple decay mechanism. However, our findings are also in line with recent observations that already showed that NMD is more than a simple destroyer, and that it is far more complex than was originally thought and it suggested that NMD is another form of post-transcriptional regulation[242].
The finding that cultured cells of our LABEB patients have less than 10% mRNA of normal is promising for future gene therapy. Dallinger *et al* found 6% effective repair of a mutated *COL17A1* gene by the spliceosome-mediated RNA-trans-splicing (SMaRT™) technique[113]. They were able to repair the mutation in 25% of the cells, so they stated that the actual repair efficiency was 24%. Considering our data, 24% *COL17A1* repair would be enough to restore good epidermal adhesion. The first study therefore would be to measure the *in vivo* mRNA level in the skin of our HEB patients to confirm our *in vitro* data.

Conclusion

The initial goal of this thesis was to obtain more basic information on type XVII collagen in order to better understand its functioning in health and disease. In this thesis we resolved the entire sequence of the *COL17A1* 5'UTR and we found a function for the alternative splicing of the *COL17A1* 3'UTR. In addition, we confirmed that NMD is responsible for the *COL17A1* mRNA decay in HEB patients. The decrease was larger than generally observed in genetic deficiencies.
Thus, our research led to unexpected discoveries on the mRNA and its translation to type XVII collagen, of which some were really surprising. This raises a number of new questions that no doubt will be the basis for further studies

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CHAPTER 7

NEDERLANDSE SAMENVATING VOOR DE NIET-INGEWIJDE

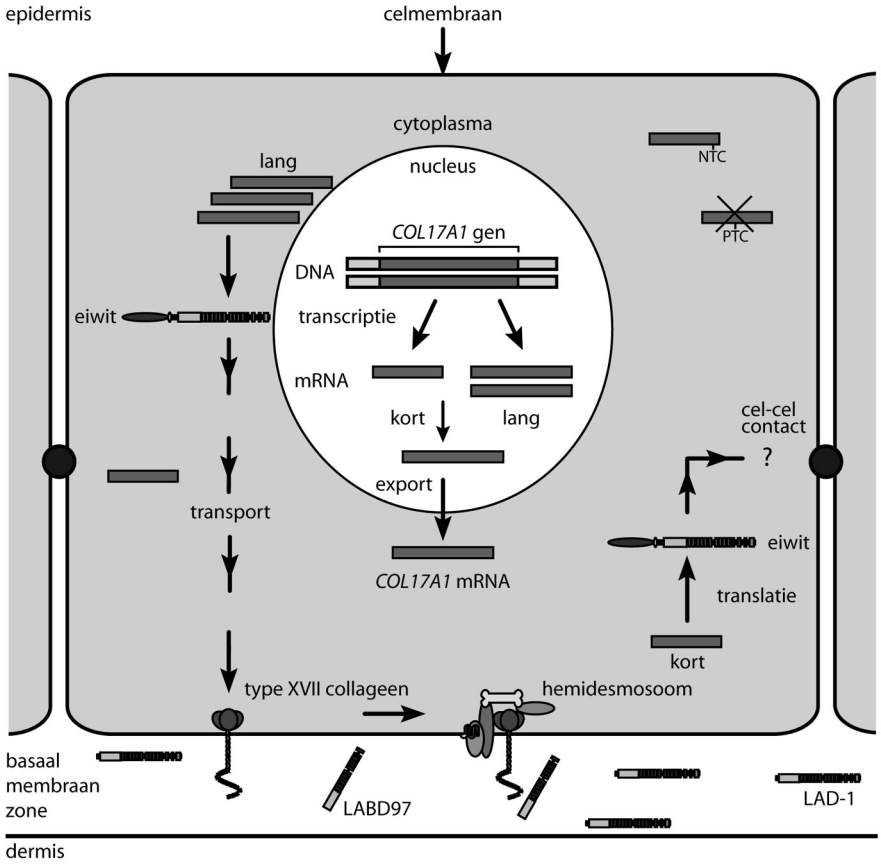
Sebastiaan van Zalen, Marcel F. Jonkman, en Hendri H. Pas

In de eerste stap van de productie van een eiwit wordt van het DNA, waarop het gen ligt, een afdruk gemaakt, het messengerRNA (mRNA). Deze stap wordt de transcriptie genoemd. Het mRNA bevat nu de informatie waarmee de aminozuren, waaruit een eiwit opgebouwd is, in een bepaalde volgorde aan elkaar gekoppeld kunnen worden. De eiwitsynthese is de tweede stap en wordt de translatie genoemd. Het eiwit is de functionele eenheid van een gen in de cel.
Dit proefschrift beschrijft en onderzoekt verschillende aspecten van de transcriptie van het *COL17A1* mRNA vanaf het *COL17A1* gen en de translatie van dit *COL17A1* mRNA in type XVII collageen eiwit (Fig. 1). De kennis die we hiermee verwerven helpt ons om de regulatie van de translatie van type XVII collageen eiwit beter te begrijpen.
We hebben ons gericht op de alternatieve splicing van *COL17A1* welke plaatsvindt in de onvertaalde gedeeltes van het mRNA. Wat is de invloed van het onvertaalde gedeelte op de eiwitsynthese? Is het van invloed op het syntheseniveau? Kan het zijn dat, gezien het feit dat de basale keratinocyt een gepolariseerde cel is, ook van invloed zijn op de lokalisatie van de transcripten? Wat is de relatie met het type XVII collageen? Leidt de alternatieve splicing van het gen tot eiwitten met verschillende functies? Welk effect hebben mutaties op het mRNA niveau en hoe beïnvloedt dit de eiwitsynthese?

Figuur 1
Schematische en vereenvoudigde weergave van de transcriptie en translatie van *COL17A1* gen in type XVII collageen eiwit in de basale keratinocyt.
De transcriptie van het *COL17A1* mRNA vindt plaats in de kern (nucleus). Door differentiële transcriptie worden zowel een lange als een korte *COL17A1* mRNA variant afgeschreven (alternatieve splicing). Deze worden naar het cytoplasma geëxporteerd waar de translatie tot het type XVII collageen plaatsvindt.
Uit ons onderzoek blijkt de lange variant na het transport naar het cytoplasma zich dichtbij de kern bevindt (peri-nucleair; linksboven) terwijl de kleine meer random in het cytoplasma lokaliseert (o.a. rechtsonder).
Onze momentele hypothese is dat het type XVII collageen dat door de afzonderlijke transcriptvormen wordt getransleerd naar verschillende locaties in de cel wordt getransporteerd. Een populatie wordt naar het celmembraan getransporteerd en ingebouwd in het hemidesmosoom (linksonder). Hier kan shedding optreden wat resulteert in de twee andere vormen van type XVII collageen, LAD-1 en LABD97, welke we onder de celmembraan vinden. Een andere populatie zou bedoeld kunnen zijn voor het laterale celmembraan waar het een functie vervult op de plaats van cel-cel contacten (rechtsmidden).
Rechtsboven is weergegeven wat er gebeurt als een mRNA transcript een prematuur terminatie codon (PTC) bevat: deze wordt afgebroken. Het transcript met een normaal terminatie codon (NTC) blijft aanwezig en wordt vertaald tot type XVII collageen.

Inleiding

Type XVII collageen wordt vooral in de huid waargenomen en is daarin een onderdeel van het hemidesmosoom (Hfd 1, Fig. 2, blz. 14). De functie van het hemidesmoom en dus ook van type XVII collageen is verankering van de basale huidcellen (keratinocyten) in de onderste cellaag van de opperhuid (epidermis) aan het ondergelegen weefsel, om precies te zijn aan het basale membraan, net boven de dermis (Fig. 1).
Het hemidesmosoom bestaat uit zes eiwitten waarvan twee alleen in het binnenste van de cel, het cytoplasma, voorkomen, terwijl de 4 andere de wand van de cel, het celmembraan, doorkruizen (transmembraan). Door de onderlinge bindingen tussen deze eiwitten ontstaat een zeer sterke structuur (Fig. 1).



Figuur 1.

Type XVII collageen is één van de zogenoemde transmembraan eiwitten, met de NH_2-terminale kop in de cel en de COOH-terminale staart buiten de cel (Hfd 1, Fig 1, blz. 12). De COOH-terminale staart kan buiten de cel afgeknipt worden, zodat een tweede oplosbare vorm van het eiwit ontstaat, LAD-1. Deze vorm kan verder geprocessed worden tot een nog iets kleiner molecuul, LABD97 en hierdoor bestaan er in de huid dus 3 type XVII collageen vormen (Fig. 1). Het afknippen gebeurt waarschijnlijk door metalloproteases, ook wel sheddases genoemd (proteases zijn enzymen die meer of minder specifiek eiwitten in stukken knippen).
Naast deze heterogeniteit op eiwitniveau bestaan er op mRNA niveau twee verschillende transcripten (Hfd 1, Fig 1, blz. 12). Deze verschillen in de staart van het mRNA transcript, de 3'Untranslated Region (UTR) en het mechanisme wat dit veroorzaakt wordt alternatieve splicing genoemd. Zoals de naam UTR al suggereert zit het verschil dus in een niet-translerend gedeelte van het mRNA en de informatie in dit gedeelte is dan ook niet in het eiwit terug te vinden. Wel is bekend dat 3'UTRs een grote rol kunnen spelen bij de regulatie van translatie door bijvoorbeeld het beïnvloeden van onder andere de stabiliteit of de subcellulaire lokalisatie van mRNA transcripten. Door de alternatieve splicing mist de ene vorm 610 nucleotiden (bouwstenen van het mRNA) in de 3'UTR (vanaf hier de korte variant genoemd), terwijl de andere wel de volledige sequentie bevat (lange variant). De sequentie van de kop van het mRNA, de 5'UTR, is nog volledig onbekend (Hfd 1, Fig 1, blz. 12).

Onbekende aspecten in transcriptie en translatie

In **hoofdstuk 1** zijn, naast de bovengenoemde informatie, de vele andere bevindingen van veertig jaar onderzoek aan type XVII collageen op een rij gezet. Het onderzoek heeft zich gericht op zowel de functie van type XVII collageen in de gezonde situatie als de bepalende rol van type XVII collageen in het ontstaan van verschillende ziektebeelden. In Hemidesmosomale Epidermolysis Bullosa (HEB) kan een mutatie in het *COL17A1* gen leiden tot de totale afwezigheid van type XVII collageen. En, in bullous pemphigoid kunnen auto-antistoffen tegen type XVII collageen leiden tot immuundeposities in de huid wat een ontstekingsreactie tot gevolg heeft. Beide ziektebeelden leiden tot blaarvorming: het loslaten van de epidermis van de dermis. Dit is een bevestiging van de belangrijke rol die type XVII collageen speelt bij de verankering van de epidermis aan de ondergelegen dermis.
Een ander ziektebeeld waarin type XVII collageen mogelijk een rol speelt is het plaveiselcelcarcinoom. Analyse van carcinoom cellen laat namelijk zien dat type XVII collageen opgereguleerd wordt en dat er mogelijk een verschuiving plaatsvindt in de plaats waar het eiwit gevonden wordt: van het membraan naar het cytoplasma (Hfd 1, Fig 4, blz. 25). Soortgelijke observaties zijn gedaan in het wondgenezingsproces.

Onderzoek van de gezonde situatie heeft zich vooral gericht op de expressie in andere weefsels, op de functie van de al genoemde shedding en de volgorde van opbouw (incorporatie) van de verschillende componenten in het hemidesmosoom. Uit deze onderzoeken bleek dat expressie werd gezien in tal van andere weefsels, dat de functie van shedding vooralsnog nog niet duidelijk is en dat bij de incorporatie type XVII collageen slechts de andere hemidesmosomale eiwitten lijkt te volgen en geen bepalende rol speelt.
Wat verder zeer interessant is dat andere onderzoeken de suggestie wekken dat type XVII collageen ook andere functies kan hebben naast het ankeren van de basale keratinocyt aan de dermis.

Doel van het proefschrift

Uit hoofdstuk 1 blijkt vóóral dat ondanks de vele data die het onderzoek aan type XVII collageen opgeleverd hebben, er nog veel aspecten van de transcriptie en translatie onopgehelderd zijn. Het doel van dit proefschrift is dan ook om de basiskennis van deze fundamentele processen te vergroten. Dit hebben we gedaan door de sequentie van de 5'UTR in kaart te brengen en de functie van de alternatieve splicing van de 3'UTR te onderzoeken. Daarnaast hebben we de afbraak van gemuteerd mRNA bestudeerd in keratinocyten van HEB patiënten.

Sequentie van de *COL17A1* 5'UTR

De nucleotiden volgorde (sequentie) van de 5'UTR werd in **hoofdstuk 2** opgehelderd: ook deze bleek alternatief gespliced te zijn en uit 6 transcripten van verschillende lengte te bestaan (Hfd 2, Fig 2, blz. 38). Twee van deze uiteinden met lengtes van 102 en 220 nucleotiden zijn verantwoordelijk voor 75% van het totale *COL17A1* mRNA. De aanwezigheid van deze twee dominante transcripten aan het 5' uiteinde van het mRNA wekt de suggestie dat elk van deze uiteindes afzonderlijk gekoppeld is aan een specifiek 3' uiteinde. Long template PCR experimenten konden dit vermoeden echter niet bevestigen en dus lijken de twee meest abundante 5' uiteinden gelijkelijk over de twee alternatieve 3'UTR uiteinden verdeeld.
In **hoofdstuk 2** werd ook de DNA-sequentie vóór de het begin van de *COL17A1* mRNA sequentie onderzocht en deze sequentie bleek verschillende transcriptiemotieven te bevatten die mogelijk eiwitten van de AP-1 en Sp1 familie kunnen binden. Dergelijke eiwitten kunnen de transcriptie van genen aan- of uitzetten door te binden aan transcriptiemotieven in de DNA sequentie. Daarmee kan de mate van transcriptie van type XVII collageen gereguleerd worden. Verandering in expressie kan van belang zijn, bijvoorbeeld in het geval dat

keratinocyten differentiëren en de basale laag verlaten of op het moment dat snel wondgenezing plaats moet vinden.

Functie van de alternatieve splicing van de 3'UTR

De functie van de alternatieve splicing van de *COL17A1* 3'UTRs is onderzocht in de **hoofdstukken 3** en **4**. In het eerste van deze twee hoofdstukken werd bepaald wat voor invloed de verschillende 3'UTRs hebben op de mate van translatie van het eiwit. Hiervoor werden beide 3'UTRs afzonderlijk achter een luciferase reporter-gen geplaatst (*COL17A1*-3'UTR-constructen; Hfd 3, Fig 1, blz. 47). Na het in het cytoplasma brengen (transfectie) van de constructen in verschillende soorten cellen werd de hoeveelheid licht die de cellen uitstraalden bepaald en daarmee de hoeveelheid getransleerd construct. Hieruit bleek dat cellen waarin het construct met de lange 3'UTR gebracht was, meer licht uitstraalden in vergelijking tot cellen waarin de korte variant gebracht was. (Hfd 3, Fig 2, blz. 50). Dit verschil lijkt veroorzaakt te worden door een hogere stabiliteit van het langere mRNA transcript (Hfd 3, Fig 3, blz. 52). Een stabieler transcript zorgt voor meer type XVII collageen eiwit.
In het volgende hoofdstuk werden de beide 3'UTRs achter een GFP-reporter gen geplaatst (Hfd 4, Fig 1, blz. 61). Het translatie product van dit gen fluoresceert groen en kan daardoor waargenomen worden door een fluorescentie microscoop. Dit geeft de mogelijkheid om de subcellulaire lokalisatie van de translatie producten te onderzoeken. Naast deze transfectie experimenten werd ook de lokalisatie van de oorspronkelijke alternatieve *COL17A1* mRNA transcripten in gekweekte keratinocyten onderzocht. Hiertoe werd een dubbele fluorescente *in situ* hybridisatie (visualiseren van mRNA) opgezet die het mogelijk maakte om de locatie van beide alternatieve mRNA transcripten afzonderlijk zichtbaar te maken. Het lange transcript werd specifiek groen aangekleurd, terwijl alle transcripten samen (lang+kort) een rode kleur kregen. Dit maakte het mogelijk om de locatie van het kleine transcript te deduceren.
Wij vonden dat GFP-eiwit met de lange 3'UTR achter de coderende sequentie, voornamelijk vlakbij de kern, peri-nucleair, gelokaliseerd wordt, terwijl GFP met als staart de kleine 3'UTR door het hele cytoplasma (random) gezien wordt (Hfd 4, Fig 2, blz. 66). Deze lokalisatie is in overeenstemming met de locatie van de *COL17A1* mRNA transcripten. Het lange transcript wordt namelijk ook vooral peri-nucleair gezien, terwijl het kleine transcript door het hele cytoplasma te zien is (Hfd 4, Fig 5, blz. 70). Locatie van transcripten en eiwit synthese komen dus overeen. Wij poneren de hypothese dat kleine gedeeltes in de 3'UTR, die geconserveerd bleven tijdens de evolutie, verantwoordelijk zijn voor deze mechanismen.
De experimenten in deze twee hoofdstukken tonen aan dat keratinocyten door differentiële splicing van het *COL17A1* gen de mogelijkheid hebben om de translatie van type XVII collageen te beïnvloeden, ofwel in hoeveelheid ofwel in plaats van

synthese, ofwel een combinatie van beide. Deze specifieke veranderingen kunnen het mechanisme zijn bij de veranderingen in type XVII collageen translatie die gezien worden bij wondheling en in het ontstaan van plaveiselcelcarcinomen.
Doordat type XVII collageen op verschillende plaatsen in de cel aangemaakt kan worden rijst de vraag of de resulterende eiwitproducten misschien verschillende bestemmingen hebben in de cel (Fig. 1). Bekend is dat naast de incorporatie in het hemidesmosoom type XVII collageen ook affiniteit heeft voor actinine-4 en P120 catenine. Actinine-4 en P120 catenine worden gevonden in plaatsen van cel-cel contact tussen keratinocyten en dubbelkleuringen hebben aangetoond dat type XVII collageen hier ook wordt aangetroffen.
Een extra en verrassende observatie tijdens de mRNA *in situ* hybridisatie proeven was dat er ook *COL17A1* mRNA te zien was in de hogere lagen van de epidermis, de suprabasale lagen (Hfd 4, Fig 7, blz 74). Ook type XVII collageen eiwit werd in deze suprabasale lagen waargenomen. In de basale laag werd naast de in de literatuur beschreven lineaire expressie ook gespikkeld (granulaire) expressie gezien in het cytoplasma (Hfd 4, Fig 8, blz. 74). Deze granulaire expressie kwam overeen met de expressie van GFP-translatie na transfectie in keratinocyten.
Door de afwezigheid van hemidesmosomen in de suprabasale lagen was deze observatie nogal onverwacht en dus is de hamvraag: wat is de functie van type XVII collageen in de suprabasale lagen? Zoals hierboven al gezegd is, zijn er aanwijzingen dat type XVII collageen betrokken is bij cel-cel contacten en dit zou de meest waarschijnlijke bestemming van het cytoplasmatische type XVII collageen kunnen zijn (Fig. 1).

COL17A1 mRNA niveaus in HEB patiënten

In **hoofdstuk 5** onderzochten we de *COL17A1* mRNA transcript niveaus in keratinocyten in een panel van HEB patiënten. Deze patiënten missen type XVII collageen door een mutatie in het *COL17A1* gen. Alle patiënten die we onderzochten hadden zogenoemde premature terminatie codons (PTCs) op beide allelen. Een PTC ligt op het mRNA voor het normale terminatie codon (NTC) waar normaal gesproken de eiwitsynthese stopt. Door een PTC stopt de translatie echter voortijdig waardoor een onvolledig eiwit zou kunnen ontstaan. De cel heeft echter een speciaal mechanisme -non-sense mediated mRNA decay (NMD)- dat specifiek mRNA transcripten met een PTC herkent en afbreekt (Fig. 1). Deze afbraak is noodzakelijk omdat onvolledige eiwitten mogelijk schade aan de cel kunnen toebrengen.
Wij vonden dat de *COL17A1* mRNA transcripten in cellen van HEB patiënten zeer sterk afgebroken zijn met een restniveau van minder dan 10% in vergelijking tot gezonde keratinocyten (Hfd 5, Fig 1, blz. 85). Deze afname was groter dan gevonden is bij ziekten ten gevolge van andere genetische deficiënties. Het

blokkeren van het NMD mechanisme zorgde voor een verhoging van het *COL17A1* mRNA niveau. Dit toont aan dat NMD zeer waarschijnlijk verantwoordelijk is voor de sterke afbraak van *COL17A1* mRNA.
Het meest intrigerende was echter dat in twee patiënten, waar van één allel het PTC door een reddingsmechanisme was verwijderd, nog steeds vergelijkbaar lage *COL17A1* mRNA transcript niveaus gevonden werden. Wij hadden hogere mRNA niveaus, tot aan 50%, verwacht omdat het transcript zonder PTC niet door het NMD zou moeten worden afgebroken.
In deze twee patiënten werd wel een redelijke -5 tot 15%- hoeveelheid type XVII collageen eiwit geproduceerd. De verhoogde eiwitsynthese in combinatie met het lage mRNA niveau geeft aan dat er naast NMD nog andere afbraak mechanismen werkzaam zijn; welke dat zijn is voor ons op dit moment nog onbekend.
Beide patiënten hadden milde symptomen (fenotype) van HEB. Wij concluderen dan ook dat de beste manier om het fenotype te voorspellen, gebaseerd moet zijn op de mate van eiwitsynthese, aangezien in patiënten met een ernstiger fenotype nauwelijks type XVII collageen eiwit detecteerbaar was.
Dat er maar een minimale hoeveelheid *COL17A1* mRNA aanwezig hoeft te zijn om de belangrijkste functie van type XVII collageen uit te voeren is ook van belang voor genreparatie studies. Tot nu toe werd gedacht dat de mRNA expressie in gerepareerde cellen minimaal 50% zou moeten zijn voor het goed functioneren van de huid. Onze data geven echter aan dat dit ook bij lagere niveaus mogelijk is.

Conclusie

Centraal in dit proefschrift stond de karakterisatie van het *COL17A1* mRNA en de functie van de alternatieve splicing. Kennis van het mRNA zal leiden tot meer kennis over type XVII collageen zelf. Het onderzoek leidde tot onverwachte ontdekkingen met betrekking tot transcriptie en translatie van *COL17A1* mRNA. Als elk proefschrift heeft ook dit proefschrift een hoop nieuwe vragen opgeroepen die ongetwijfeld de basis vormen voor verder onderzoek aan type XVII collageen.

LIST OF ABBREVIATIONS

αCP: α-complex
ADAM: a disintegrin and metalloproteinase
AP-1: activating protein-1
ARE: adenylate/uridylate (AU)-rich element
AUG: start codon
BCC: basal cell carcinomas
BMZ: basement membrane zone
BP: bullous pemphigoid
bps: basepairs
CD: cluster of determination
cDNA: copy DNA
CHO: Chinese hamster ovary
COL: collagenous
DEB: Dystrophic Epidermolysis Bullosa
DIG: digoxigenin
DPE: down-regulatory promoting element
EJC: exon-junction complex
ER: endoplasmatic reticulum
EGF-R: epidermal growth factor receptor
FISH: fluorescent *in situ* hybridisation
GABEB: generalized atrophic benign epidermolysis bullosa
GFP: green fluorescent protein
hnRNP: heterogeneous nuclear ribonucleoproteins
IF: immunofluorescence
IRES: internal ribosome entry sites
kb: kilobases
kDa: kiloDalton
LABEB: localized atrophic benign epidermolysis bullosa
LABD97: 97 kDa linear IgA bullous dermatosis antigen
LAD-1: 120 kDa linear IgA dermatosis antigen
MMP: mucous membrane pemphigoid
MRF4: muscle regulatory factor
NAS: nonsense associated altered splicing
NC: non-colleganous
NHKs: normal human keratinocytes
HEB: Hemidesmosomal Epidermolysis Bullosa
NMD: nonsense mediated mRNA decay
nt: nucleotide
ORF: open reading frame
PCBP: poly(C)-binding protein
PKC: protein kinase C
PLC: Phospholipase C
PTC: premature termination codon

RACE: 5'rapid amplification of cDNA ends
RPA: ribonuclease protection assays
RT-PCR: reverse transcriptase- polymerase chain reaction
SCC: squamous cell carcinomas
SRP: signal recognition particle
TACE: TNF-alpha converting enzyme
TBP: TATA-binding protein
TPA: 12-*O*-tetradecanoylphorbol-13-acetate
uAUG: upstream start codon
uORF: upstream open reading frame
UTR: Untranslated Region

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