CHAPTER 6

SUMMARY, GENERAL DISCUSSION, AND FUTURE PERSPECTIVES

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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 \textit{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. \textit{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 \textit{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 \textit{chapter 2} we demonstrated that the \textit{COL17A1} 3'UTR shows extensive heterogeneity, which may well be important for regulation of type XVII collagen expression. 3'UTRs in general harbour several specialised features that may regulate translation, and these include length, uORF, uAUG, specific cis-acting sequences, and internal ribosome entry sites (IRES)\textsuperscript{133}. The six \textit{COL17A1} 3'UTRs differ in length and the alternative splicing thereby also determines the incidence of the predicted transcription motifs for each particular 3'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 \textit{COL17A1} gene, since the expression pattern of AP-1 proteins changes when keratinocytes differentiate\textsuperscript{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 \textit{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\textsuperscript{151}. Altered incidence of motifs due to altered 3'UTR splicing may then result in a shift of \textit{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\textsuperscript{152-154}. Second, type XVII collagen expression is altered during wound healing and during
development of squamous cell carcinoma. 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 \textit{COL17A1} 5'UTR transcripts in wound healing models, in squamous cell carcinoma, and in other tissues. Also investigating \textit{COL17A1} transcripts in suprabasal keratinocytes may help as we already saw that both \textit{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 \textit{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.

\textbf{Chapters 3 and 4} showed that the alternative splicing of the \textit{COL17A1} 3'UTR has a functional meaning. Translation regulation via mRNA stabilisation and subcellular localisation of mRNA transcripts has already been demonstrated for several other genes and in most cases the 3'UTR was involved. Our findings again confirm the importance of 3'UTRs in regulating gene expression and show that for the \textit{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. 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 \textit{COL17A1} mRNA levels in carcinoma 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.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>All \textit{COL17A1}-variants</th>
<th>Long \textit{COL17A1}-3'UTR variants</th>
<th>Short \textit{COL17A1}-3'UTR variants</th>
<th>Ratio long variant/short variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHKs</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>1.84±0.43</td>
</tr>
<tr>
<td>A431\textsuperscript{a}</td>
<td>27.9</td>
<td>27.0</td>
<td>25.1</td>
<td>2.3</td>
</tr>
<tr>
<td>HaCaT\textsuperscript{b}</td>
<td>37.3</td>
<td>29.6</td>
<td>48.3</td>
<td>1.3</td>
</tr>
<tr>
<td>SCC 22B\textsuperscript{b}</td>
<td>26.9</td>
<td>11.1</td>
<td>56.3</td>
<td>0.4</td>
</tr>
<tr>
<td>WiDr\textsuperscript{c}</td>
<td>29.4</td>
<td>27.3</td>
<td>8.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Glc-a2\textsuperscript{d}</td>
<td>187.7</td>
<td>171.8</td>
<td>222.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Glc-p1\textsuperscript{b}</td>
<td>5.9</td>
<td>2.9</td>
<td>3.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Hep-2\textsuperscript{d}</td>
<td>2.9</td>
<td>4.4</td>
<td>2.4</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Originally derived from: \textsuperscript{a} Epidermoid carcinoma, \textsuperscript{b} Squamous cell carcinoma \textsuperscript{c} Colon carcinoma \textsuperscript{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\textsuperscript{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 \textit{trans}-acting factors to the 3'UTR. The three conserved stretches in the \textit{COL17A1} 3'UTR may narrow the search for binding of \textit{trans}-factors, since conserved regions in 3'UTRs are well-known for their \textit{cis}-activity\textsuperscript{235}. Waggoner and Liebhaber investigated several thousands of mRNAs, including type \textit{COL17A1} mRNA, for binding with the two major \textit{α}CP-isoforms – well-known \textit{trans}-acting factors via a micro-array technique and they did not observe binding of these two to the \textit{COL17A1} mRNA\textsuperscript{236}. This does not totally preclude \textit{COL17A1} mRNA from binding to \textit{α}CP-proteins, since the approach in the study above was very strict and the CP-family contains other members\textsuperscript{237}.

Another candidate for binding to the \textit{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\textsuperscript{193,238}. Furthermore, annexin II expression is observed at the cell periphery in all layers of the epidermis, except for the stratum corneum\textsuperscript{239}. In \textit{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 \textit{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 α₆β₄ is the first component present, which is then followed by the other components. In that model in vitro studies also ascribed a crucial role to plectin, but this could not be confirmed in vivo.

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, 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. 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. 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\textsuperscript{240,241}. This suggest that 3'UTRs in general may influence the NMD response and, therefore, the alternative splicing of the \textit{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\textsuperscript{242}.

The finding that cultured cells of our LABEB patients have less than 10% mRNA of normal is promising for future gene therapy. Dallinger \textit{et al} found 6% effective repair of a mutated \textit{COL17A1} gene by the spliceosome-mediated RNA-trans-splicing (SMaRT\textsuperscript{TM}) technique\textsuperscript{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% \textit{COL17A1} repair would be enough to restore good epidermal adhesion. The first study therefore would be to measure the \textit{in vivo} mRNA level in the skin of our HEB patients to confirm our \textit{in vitro} data.

\section*{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 \textit{COL17A1} 5'UTR and we found a function for the alternative splicing of the \textit{COL17A1} 3'UTR. In addition, we confirmed that NMD is responsible for the \textit{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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